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Online ozonolysis methods for the determination of double bond position in unsaturated lipids

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Abstract
Modern lipidomics relies heavily on mass spectrometry for the structural characterization and quantification of lipids of biological origins. Structural information is gained by tandem mass spectrometry (MS/MS) whereby lipid ions are fragmented to elucidate lipid class, fatty acid chain length, and degree of unsaturation. Unfortunately, however, in most cases double bond position cannot be assigned based on MS/MS data alone and thus significant structural diversity is hidden from such analyses. For this reason, we have developed two online methods for determining double bond position within unsaturated lipids; ozone electrospray ionization mass spectrometry (OzESI-MS) and ozone-induced dissociation (OzID). Both techniques utilize ozone to cleave C-C double bonds that result in chemically induced fragment ions that locate the position(s) of unsaturation.

Keywords
unsaturated, lipids, position, online, bond, double, determination, methods, ozonolysis, CMMB

Chapter 21

OnLine Ozonolysis Methods for the Determination of Double Bond Position in Unsaturated Lipids

Michael C. Thomas, Todd W. Mitchell, and Stephen J. Blanksby

Summary

Modern lipidomics relies heavily on mass spectrometry for the structural characterization and quantification of lipids of biological origins. Structural information is gained by tandem mass spectrometry (MS/MS) whereby lipid ions are fragmented to elucidate lipid class, fatty acid chain length, and degree of unsaturation. Unfortunately, however, in most cases double bond position cannot be assigned based on MS/MS data alone and thus significant structural diversity is hidden from such analyses. For this reason, we have developed two online methods for determining double bond position within unsaturated lipids; ozone electrospray ionization mass spectrometry (OzESI–MS) and ozone-induced dissociation (OzID). Both techniques utilize ozone to cleave C–C double bonds that result in chemically induced fragment ions that locate the position(s) of unsaturation.

Key words: Ozonolysis, Double bond position, Structural characterization, Unsaturated lipids, Mass spectrometry, OzESI–MS, OzID

1. Introduction

Modern, high throughput lipid analysis techniques are increasingly reliant on mass spectrometry for the structural identification and quantification of individual lipids within complex mixtures *(1)*. With this approach, structural characterization is based solely on the collision induced dissociation (CID) behavior of the lipid ions. This is particularly effective in phospholipid analysis, where CID spectra are easily interpreted to yield information on lipid class and the total number of carbons and double bonds of any esterified fatty acids *(2)*. As powerful as CID may be, significant limitations remain in assigning fatty acid sn-position – especially in mixtures of regioisomers – and determining the position

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and geometry of C–C double bonds. While difficulties remain in characterizing the relative percentages of two regioisomers differing only in fatty acid sn-position, the most abundant isomer can usually be identified from CID spectra *(2)*. Conversely, low energy negative ion CID spectra, as acquired from triple quadrupole, QToF, or ion trap instruments, do not form ions informative of double bond position. Furthermore, the relative ratios of fragment ions in the CID spectra of the $[M - H]^-$ phospholipid regioisomers differing only in double bond position are indistinguishable *(3)*. Since no information is gained on double position, most analyses assign the position of unsaturation purely on the basis of fatty acid natural abundances as determined from gas chromatography (GC). For example, the *m/z* 281 18:1 carboxylate ion commonly observed in CID spectra of phospholipid anions would usually be assumed to be 9Z-18:1 (oleic acid). While oleic acid is a highly abundant fatty acid, other regioisomers such as 6Z-18:1 (petroselinic acid) and 11Z-18:1 (vaccenic acid) are also found in biological systems *(4)*. Traditional lipid analysis has revealed twelve commonly occurring isomeric fatty acids (**Table 1**) *(5)*. The failure of CID to identify double bond position is thus a significant short-coming since isomeric lipids differing only in double bond position can have distinct biochemical and biophysical properties *(6)*.

Several methods are currently available to identify double bond position, either within fatty acid methyl esters or intact lipids. Traditional gas-chromatography based methods are the most commonly applied and can distinguish regioisomeric fatty acids *(7)*. This approach, however, requires that all fatty acid ester bonds are hydrolyzed and converted to their methyl esters. As a consequence, the assignment of each fatty acid identified to its parent lipid(s) within a complex mixture is impossible without

Table 1
A list of common isomeric fatty acids differing only in double bond position

Fatty acid (no. carbons: no. double bonds)	Fatty acid class
16:1	n-7, n-9
18:1	n-7, n-9, n-12
18:3	n-3, n-6
20:3	n-3, n-6, n-9
22:5	n-3, n-6

Other naturally occurring isomeric fatty acids are also known.

extensive, multidimensional fractionation. To gain greater structural detail it is therefore advantageous to be able to determine double bond position within intact lipids. Several mass spectrometric methods have been developed that enable this to be done. Conceptually, the simplest of these methods is the CID of the carboxylate anions formed upon fragmentation of the parent phospholipid anion in an MS^3 experiment. Comparison of the resultant MS^3 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 *(8)*. To promote fragmentation at the site of unsaturation, chemical derivatization of the double bond prior to ESI–MS/MS analysis has also been demonstrated to be useful in identifying double bond position. In two notable examples, (a) Moe et al. pretreated phospholipids and free fatty acids with osmium tetroxide prior to ESI–MS/MS analysis *(3, 9, 10)*. 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 *(3, 9, 10)*. (b) 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 ozonides 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 *(11)*. While chemical derivatization of lipids can be used for locating double bond position, it has the undesirable requirement of additional sample preparation prior to analysis. Ideally, online methods for double bond position elucidation are preferred. Such a method has been developed by Brenna and co-workers and has been applied to fatty acid methyl esters *(12, 13)* and triacylglycerols *(14)*. 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, complementary methods that are applicable to more complex lipids, such as phospholipids, are needed.

Our efforts towards determining double bond position have centred on the use of ozone chemistry in conjunction with mass spectrometry. We have developed two techniques whereby ozonolysis is either performed in the ion-source or the ion-trapping region of a mass spectrometer. The simplest of these techniques is ozone electrospray ionization–mass spectrometry (OzESI–MS). In this technique, ozonolysis is initiated in the electrospray ionization source of a commercial mass spectrometer and provides two chemically induced fragment ions for each double bond that can readily identify double bond position *(15, 16)*. This technique is quite similar to using ozonolysis in

conjunction with field-induced droplet ionization but can be achieved without specialized instrumentation *(17)*. OzESI–MS is easy to use and highly effective for individual lipids or simple lipid mixtures, however, interpretation of OzESI–MS spectra of complex mixtures is exceedingly difficult. To overcome the limitations of OzESI–MS we developed OzID where mass selected lipid ions are isolated and trapped in the presence of ozone vapor within an ion trap mass spectrometer *(18)*. In this chapter, the materials, modifications, and methods to utilize these two techniques are discussed.

2. Materials

2.1. Equipment

2.1.1. OzESI–MS (In Situ Corona Discharge)

1. ThermoFinnigan LTQ® ion-trap mass spectrometer (San Jose, CA, USA) fitted with an Ionmax® source.
2. 1/4-in. teflon tubing (approximately 2-m length).

2.1.2. OzESI–MS (External Ozone Generation)

1. Micromass QuattroMicro (Manchester, UK) triple quadrupole mass spectrometer fitted with a z-spray® ion source.
2. Ozone generator capable of generating ozone at 0.3% (v/v) in oxygen (in our experiments we have used an ozone generator from a NO_x analyzer, Model 8840, Monitor Labs, Englewood, CO, USA). Ozone generators use either UV photolysis or corona discharge for ozone production. Corona discharge is preferred since the maximum ozone concentration that can be produced from UV photolysis ozone generators is lower (maximum of 0.2% by weight).
3. 1/4-in. teflon tubing (approximately 2-m length).
4. Tubing is required to connect the ozone generator to the ESI source. Silicone tubing is recommended and rubber tubing should not be used. There are a range of websites that provide useful information on ozone compatibility *(19, 20)*.
5. Soft plastic adaptor to join the teflon tubing and silicone tubing together.
6. Flow-metering valve.
7. Flow meter (the NO_x analyzer used in our experiments contained an in-built flow meter).
8. Solvex unlined nitrile, 0.28-mm thick, 33-cm length, slip on gloves, 37–145 (Ansell, Richmond, Vic, Australia).

2.1.3. OzID

1. ThermoFinnigan LTQ ion-trap® mass spectrometer (San Jose, CA, USA) fitted with an Ionmax source®.
2. HC-30 ozone generator (Ozone Solutions, Sioux Center, IA, USA).

3. Pump 11 Pico Plus syringe pump (Harvard Apparatus, Holliston, MA, USA).
4. Model 203 variable leak valve (Granville-Phillips, Boulder, CO, USA).
5. Stainless steel 1-piece 40G series 3-way ball valve (Swagelok, Salon, OH, USA).
6. Stainless steel 1-piece 40G series ball valve (Swagelok, Salon, OH, USA).
7. Stainless steel union tee (Swagelok, Salon, OH, USA).
8. 1/8-in. stainless steel tubing.
9. PEEKsil tubing restrictor (100-mm L × 1/16-in. OD × 0.025-mm ID, SGE Analytical Science, Ringwood, Vic, Australia).
10. Appropriate Swagelok fittings for connections (Swagelok, Salon, OH, USA)
11. 10-mL Norm-ject luer slip syringe (Henke Sass Wolf, Tuttlingen, Germany).
12. Boston PVC 6-mm ID tubing (Fix-a-tap, Asquith, NSW, Australia), soft plasting adaptor and plastic 7-mm OD T connector (U 099223162, Livingstone, Rosebery, NSW, Australia) for ozone collection.
13. Teflon tap.
14. Glass Dreschel bottle (or "bubbler", 1 L).
15. ADM2000 Intelligent flowmeter (J & W Scientific, Folsom, CA, USA).
16. Solvex unlined nitrile, 0.28-mm thick, 33-cm length, slip on gloves, 37–145 (Ansell, Richmond, Vic, Australia).

2.2. Reagents and Supplies

1. HPLC grade methanol and AR grade chloroform (APS Chemicals, Sydney, NSW, Australia) for sample preparation for all methods. AR grade sodium acetate (APS Chemicals, Sydney, NSW, Australia) may be used to form sodium adducts for OzESI–MS (external ozone generation) and OzID analysis.
2. Sodium thiosulfate, potassium iodide and Vitex indicator are required to titrate ozone. Sodium thiosulfate and potassium iodide are also required if high concentration ozone is not destroyed using a catalytic ozone destruct unit during production for OzID.
3. Ultra high purity helium (99.999%) (BOC gases, Cringila, NSW, Australia) for operation of the LTQ ion trap mass spectrometer.
4. Nitrogen (BOC gases, Cringila, NSW, Australia) for operation of the LTQ ion trap mass spectrometer when performing OzID experiments.

5. Industrial grade compressed oxygen (purity 99.5%) (BOC gases, Cringila, NSW, Australia) for ozone production in all methods. An oxygen regulator must be used when working with oxygen.

3. Methods

3.1. OzESI–MS (In Situ Corona Discharge)

1. Close nitrogen supply and release the pressurized nitrogen in the nitrogen line using the sheath and auxiliary gas controls within the instrument control software. Remove the tubing from the nitrogen inlet push fitting located on the back panel of the LTQ ion-trap mass spectrometer (*see* **Fig. 1**).
2. Attach the oxygen cylinder to the nitrogen inlet via 1/4-in. tubing and set the pressure to 100 ± 20 psi using the oxygen compatible regulator.
3. Prepare lipid sample in methanol with a concentration of 1–10 μM for an isolated lipid. Use higher concentrations (~40 μM) for a lipid mixture.
4. Infuse the lipid sample into the ESI source with a flow rate of 3–10 μL/min. Using higher flow rates may reduce the relative yields of ozonolysis products. Furthermore, using higher flow rates may result in the ignition of methanol in the ion source (see safety precautions, **Subheading 3.6**).
5. Set the ionization polarity to negative.
6. Tune to maximize the abundance of the lipid ion of interest using a source voltage of –3 to –4 kV. Tuning can be done by using the automatic tune function to optimize the ion optics. The gas flow rates can be optimized using the semiautomatic

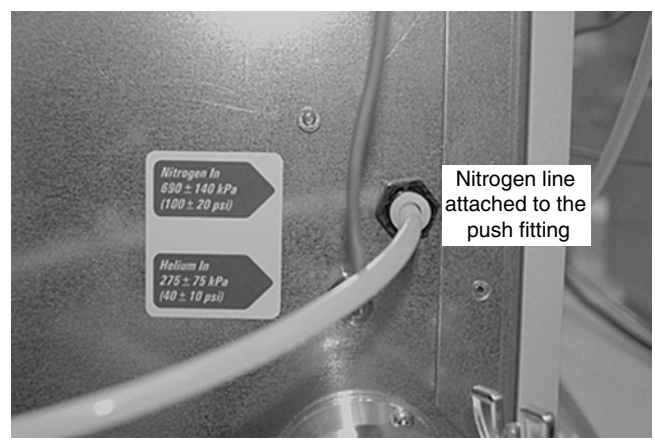

Fig. 1. The nitrogen input push fitting. For in situ corona discharge OzESI–MS the nitrogen line is replaced by connecting an oxygen cylinder via 1/4-in. teflon tubing.

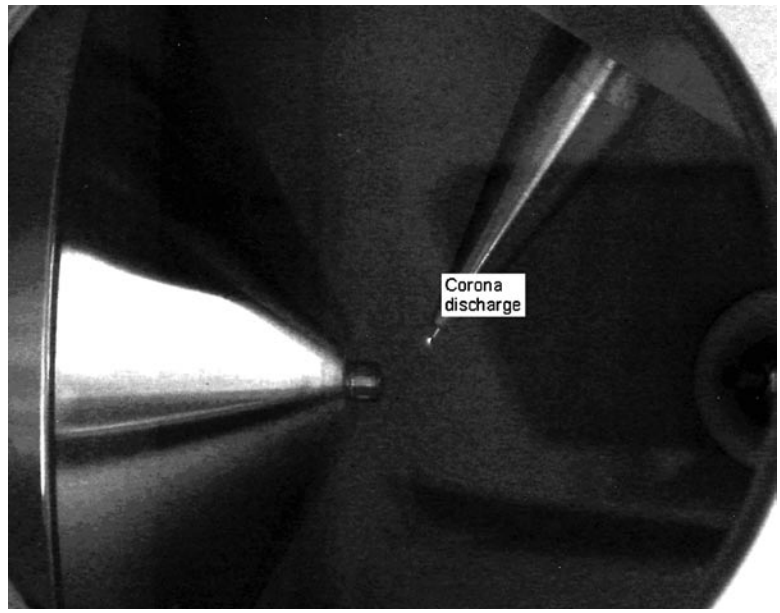

Fig. 2. A photograph of a corona discharge on the ESI capillary caused by increasing the source voltage in negative ion mode with oxygen (instead of nitrogen) as the desolvation gas.

tune function. The sheath and auxiliary gases should not be set greater than 15 (arbitrary units) as this may be detrimental to the concentration of ozone produced.

7. Acquire a conventional ESI–MS spectrum using these optimized conditions.
8. Increase the source voltage until an ozone producing corona discharge is observed (typically –4.5 to –6 kV). A corona discharge is observed as a blue glow at the tip of the ESI capillary (*see* **Fig. 2**). If necessary the lights in the laboratory can be turned off to allow easier visualization of the corona discharge.
9. Acquire the resulting OzESI–MS (in situ corona discharge) spectrum.

3.2. OzESI-Ms (External Ozone Gernation) - Ozone Generator Selection and Measuring Output Ozone Concentration

An ozone generator capable of producing ozone to approximately 0.3% (v/v) in oxygen is required for OzESI–MS. The use of higher ozone concentrations may be a significant hazard and therefore the output ozone concentration should be measured prior to connecting to the mass spectrometer. Ozone concentration can be measured either by titration or using a specialized ozone analyzer. To measure ozone concentration by titration, a setup designed to collect ozone in a plastic syringe needs to be made (refer to **Subheading 3.5**). For this setup, however, a flow-metering valve is required upstream of the ozone generator

and the teflon tap is not needed. After the ozone collection setup is prepared with the ozone generator and oxygen supply attached, perform a titration as follows:

1. Open the oxygen cylinder tap and set the pressure to 5 psi.
2. Using the flow-metering valve set the oxygen flow rate and turn on the ozone generator.
3. Wait for 5 min for ozone production to stabilize.
4. Collect a known volume of the ozone generator output in a gas tight plastic syringe.
5. Bubble collected ozone through acidified aqueous potassium iodide.
6. Back titrate against sodium thiosulfate in the presence of Vitex indicator up to the end-point (color disappears as iodine is reduced to iodide).

Specialized ozone analyzers are commercially available and would also be suitable for this application.

3.3. OzESI-MS (External Ozone Generation) – Modification of Ion Source

For a schematic of the instrument modification for OzESI–MS *see* **Fig. 3**.

1. Connect the oxygen cylinder to a metering-flow valve with 1/4-in. teflon tubing.
2. Connect the metering-flow valve to the ozone generator with an appropriate length of tubing.
3. Connect an appropriate length of 1/4-in. teflon tubing to the ozone generator and using a soft plastic fitting connect a small length of silicone tubing.
4. Ensure that the desolvation gas supply is turned off on the computer interface. Detach the desolvation gas line from the press fitting at front of the QuattroMicro triple quadrupole mass spectrometer.

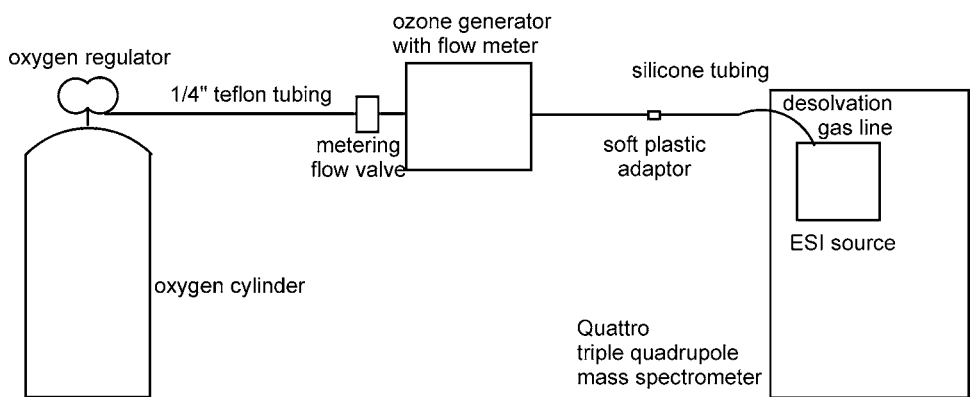

Fig. 3. A schematic representation of the modifications to the Quattro triple quadrupole mass spectrometer for OzESI–MS.

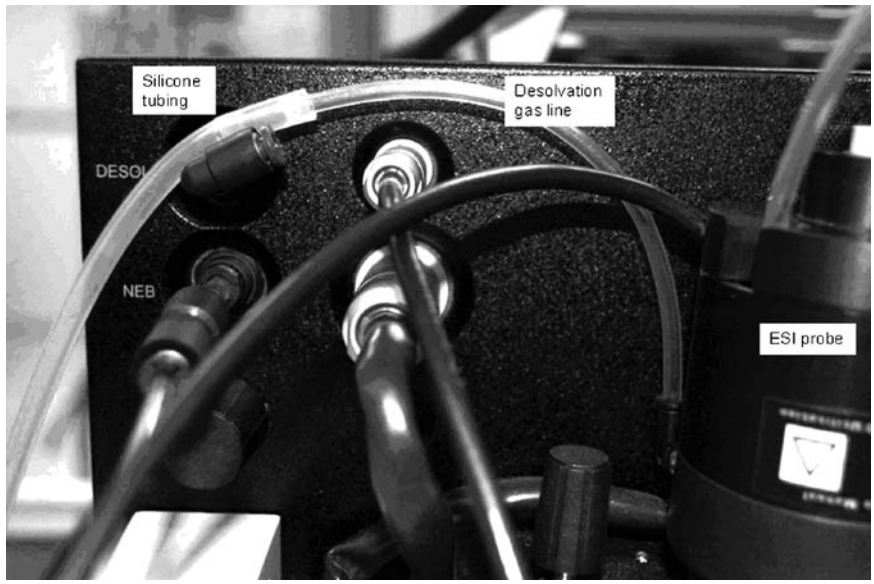

Fig. 4. A photograph of the desolvation gas line connected to silicone tubing. This involved detaching the desolvation gas line from the push fitting.

5. Connect the silicone tubing to the desolvation gas line. Since silicone tubing is soft, this can be done by inserting the desolvation gas line into the silicone tubing (*see* **Fig. 4** for a photograph of the ESI source modified for OzESI–MS). Check that the ion source is sealed and ensure it is vented external to the laboratory. A strip of filter paper soaked with a concentrated aqueous solution of potassium iodide is useful for the detection of leaks. The appearance of a brown coloration indicates ozone is present.

3.4. Performing OzESI-MS (External Ozone Gernation

1. Open the tap of the oxygen cylinder and set the pressure to approximately 5 psi using the oxygen regulator.
2. Using the flow-metering valve, set the oxygen flow rate to the desired flow rate (160 mL/min was used for the ozone generator from the NO_x analyzer).
3. Infuse the sample (10–40 μM in methanol) into the ESI source at a flow rate of 3–10 μL/min. Enter the required voltages for maximum ion abundance. Typical settings for negative ion mode are; capillary voltage of 3.5 kV, cone voltage 50 V with a source temperature of 80°C. For positive ion mode similar settings are used, however, a lower cone voltage is applied, typically either 30 or 40 V.
4. Acquire a conventional ESI–MS spectrum using oxygen as the desolvation gas. Ensure that the desolvation gas is not turned on in the instrument interface to avoid wasting nitrogen.

5. Turn on the ozone generator and wait for several minutes for the ozone concentration to stabilize.
6. Acquire the OzESI–MS spectrum.
7. Turn off the ozone generator and purge the ozone generator and ESI source with oxygen to remove the ozone before disassembling the unit.

In a typical MS spectrum there are often ions resulting from impurities in the sample, furthermore, lipid mixtures can generate a large number of lipid ions. Both impurities and other lipid ions may interfere with identification of ozonolysis product ions in the OzESI–MS spectrum. This is why both conventional ESI–MS and OzESI–MS spectra are acquired. Comparison of the spectra should allow the assignment of ozonolysis products in the OzESI–MS spectrum.

Since the Quattro is a triple quadruple instrument it is also possible to use neutral loss and precursor ion scans when performing ozonolysis. These scans can be extremely useful in simplifying OzESI spectra. In lipid extracts, these scan functions allow the OzESI spectrum to be simplified by only detecting a particular class of lipid. For example, all protonated choline-containing phospholipids and their aldehyde and α -methoxyhydroperoxide ozonolysis product ions fragment to form an ion of m/z 184.1 *(16)*. Therefore, when analyzing phosphatidylcholines or sphingomyelins by OzESI, using a m/z 184.1 precursor ion scan to detect their protonated ions is recommended. To do this select the precursor ion scan function and enter the product mass as m/z 184.1. Turn the collision gas on and set the collision gas pressure to approximately 3×10^{-3} Torr and use a collision energy of 35 eV. All other steps remain the same.

3.5. OzID

With the modified LTQ we set the helium pressure to 5 psi using the regulator attached to the helium cylinder. The flow rate of helium into the mass spectrometer is controlled by the variable leak valve. The Granville-Phillip model 203 variable leak valve used in our modified instrument is generally set to around 10 (arbitrary units) to achieve an ion gauge pressure of approximately 0.8 mTorr. For OzID, high concentration ozone needs to be produced (see safety precautions, **Subheading 3.6**) and collected in the fumehood before being introduced into the mass spectrometer. A setup for ozone production and collection therefore needs to be prepared as shown in **Fig. 5** and outlined below:

1. Connect the oxygen cylinder to the ozone generator inlet using 1/4-in. teflon tubing with Swagelok fittings.
2. Connect an appropriate length of teflon tubing to the ozone generator output port.

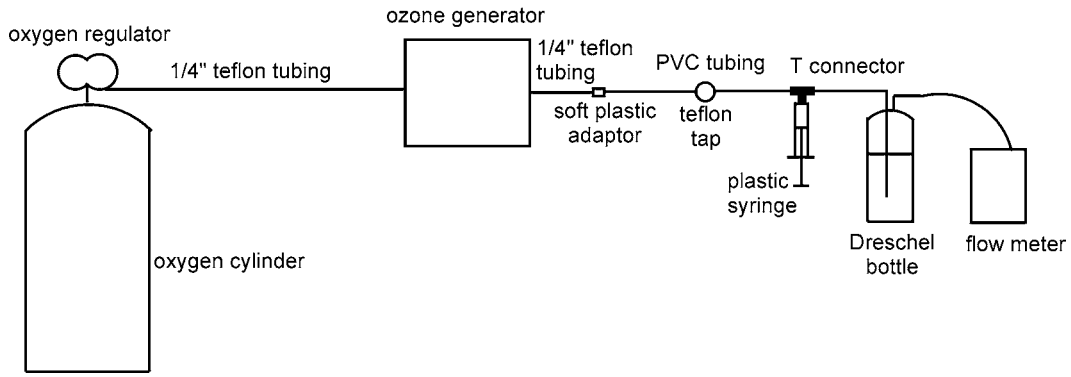

Fig. 5. A schematic diagram of the setup used to collect high concentration ozone. The Dreschel bottle ("bubbler") contains a solution of sodium thiosulfate and potassium iodide and is used to destroy ozone before being released into a fumehood. High concentration ozone is collected by attaching a syringe to the T connector.

3. Connect a piece of PVC tubing (6-mm I.D. Boston) to the teflon tubing using a soft plastic adaptor.
4. With plastic tubing connect a teflon tap and plastic T connector in series (*see* **Note 1**).
5. On the plastic T-connector connect a small piece of PVC tubing and insert a syringe tip into the PVC tubing to act as a connector for the syringe.
6. Connect the PVC tubing to a Dreschel bottle containing a 600 mL of aqueous solution of sodium thiosulfate (70 g) and potassium iodide (5 g). This acts to destroy ozone as it leaves the system. The solution will turn brown when the thiosulfate is totally consumed. This acts as a warning that ozone is not being destroyed and that a fresh solution needs to be prepared.
7. On the outlet of the Dreschel bottle connect an appropriate adaptor so that a flow meter may be attached.

To generate high concentration ozone:

1. Close the teflon tap and set the oxygen pressure to 20 psi using the oxygen regulator.
2. Connect the flow meter to the outlet of the Dreschel bottle and set the oxygen flow rate to 400–500 mL/min using the teflon tap. Once the flow rate is set, remove the flow meter.
3. Turn the ozone generator on and set power output to 68 (arbitrary units).
4. After 20–30 min, attach the flow meter to the Dreschel bottle outlet and reduce the oxygen flow rate to 30–40 mL/min.
5. Wait approximately 5 min before collecting ozone in a 10-mL disposable plastic syringe. Quickly attach a square tip needle to the syringe. Wear gloves when collecting ozone, however, be aware that gloves only provide limited protection from ozone as high concentration ozone can create cracks in the gloves.

6. Turn off the ozone generator, remove the flow meter and leave the flow rate set at 30–40 mL/min to purge ozone from the system for 10 min.
7. Close the oxygen cylinder tap and depressurize the system by opening the teflon tap to increase the oxygen flow rate. Ensure that the pressure does not increase high above 50 psi (the recommended maximum pressure of the HC-30 ozone generator).
8. Close the teflon tap so that water vapor cannot diffuse back to the ozone generator.

Introducing ozone into the ion trap and setting up for OzID:

1. Connect the syringe containing ozone to the PEEKsil restriction (*see* **Fig. 6** for a photograph of an ozone containing syringe attached to a PEEKsil restriction).
2. Place the syringe in the syringe pump and set it to 20–25 μL/min.
3. Infuse the lipid sample into the ESI source with a flow rate of 3–10 μL/min (higher flow rates may be used if needed). Typically lipid samples are prepared in either methanol or methanol:chloroform (2:1) with a concentration of 1–10 μM

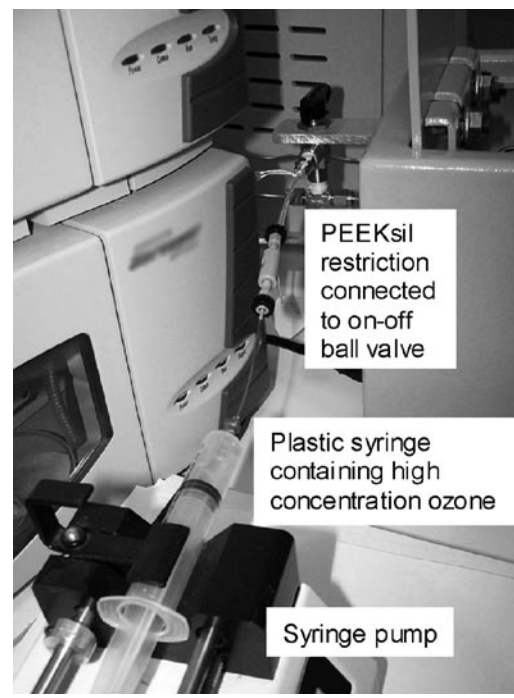

Fig. 6. A photograph of an ozone containing syringe attached to the PEEKsil restriction. Through the PEEKsil restriction the ozone is introduced into a flow of ultrahigh purity helium. The flow of helium is controlled using a variable valve which on our instrument is inside the steel box on the right hand side of the image.

for an isolated lipid and approximately 40 μM for a lipid mixture. Ensure that the sample volume is sufficient for at least 10–15 min of spectral accumulation time.

4. Set the ion gauge pressure to 0.80–0.85 mTorr by adjusting the helium flow with the variable leak valve if necessary (this may not be an accurate pressure reading since the ion gauge is calibrated for helium). Using the correct pressure is critical for acquiring OzID spectra. At this pressure the resolution will be poor and the m/z of the lipid ions in the MS spectrum will shift to approximately 0.5 Da lower. This is due to the presence of oxygen and ozone molecules in the ion trap. Going to higher pressures will result in potentially large mass shifts and a reduction in resolution. Conversely, going to lower pressures will cause problems with ion isolation. The gas pressure can be optimized empirically by observing the effect of helium flow on peak width and mass accuracy.
5. Using the "Define Scan" control panel, isolate the ion of interest using an isolation width of 3 Th. To isolate the correct ion a value up to 1 Da higher than the actual m/z may be required. It is important to examine the peak shape of the isolated ion, check for shoulders on either side of the peak. Adjust the entered m/z and isolation width as required. If the lipid is part of a complex mixture then an isolation width of 2 Th may be needed so that neighboring lipid ions are not also isolated. Using an isolation width of 1 Th is not possible due to the presence of oxygen and ozone in the ion trap.
6. Using the "Define Scan" control panel, set the activation energy to 0, set the number of microscans to 1 and change the activation time to 10,000 ms. In OzID the activation time is more appropriately referred to as a trapping time since no activation energy is used.
7. Acquire OzID spectra. Typically 50 scans are collected for abundant ions. For ions of low abundance it may be necessary to collect more than 100 scans to obtain a satisfactory signal-to-noise ratio.
8. When finished, remove the syringe and dispose of the ozone into a fumehood. It is advisable to pull the plunger on the syringe back to ensure that there is no back pressure in the syringe. This minimizes the chance of ozone being released into the laboratory.

Ozone decomposes with time (*see* **Note 2**) and therefore ozone should be prepared directly before use. When conducting continuous OzID experiments, high concentration ozone should be prepared approximately every 2 h. Ozone concentration is especially critical when performing OzID on ions of low abundance, prepare fresh ozone if required.

Collision-induced fragmentation of the parent ion during isolation can occur due to the presence of heavy molecules in the ion trap. This is observed with the sodium adducts of phosphatidylcholines and sphingomyelins where the neutral loss of trimethylamine (59 Da) is observed. CID fragment ions formed during isolation can be removed by adding a second isolation step. To do this:

1. Enter in the mass of the lipid ion and isolate the lipid ion with an isolation width of 3 Th (or less if needed) and leave the activation time set to 30 ms.
2. Enter the mass of the lipid ion again (as for an MS^3 experiment) and isolate the ion using an isolation width of 10 Th. Set the activation time to 10,000 ms and ensure the number of microscans is set to 1.
3. Acquire the OzID spectrum.

It is also possible to perform CID prior to ozonolysis. An example of where this is potentially useful is phospholipids with two unsaturated fatty acids of different chain lengths but the same number of double bonds. In such cases it may be difficult to assign the position of the double bonds to a particular fatty acid (see CID/OzID results, **Subheading 3.7.7**). It is possible, however, to remove a fatty acid by CID and perform OzID on the resulting fragment ion. This leaves no ambiguity as to the positions of the double bonds on each fatty acid. To perform CID/OzID on a fragment ion:

1. Enter in the mass of the lipid ion and isolate it with an isolation width of 3 Th (or less if needed) and leave the activation time set to 30 ms. Enter in a collision energy that gives a maximum abundance of the fragment ion of interest.
2. Enter the mass of the fragment ion (as in an MS^3 experiment) and isolate the ion using an isolation width of 3–5 Th. Set the activation time to 10,000 ms and ensure the number of microscans is set to 1.
3. Acquire the OzID spectrum.

3.5.1. Instrument Modification for OzID

Modification of the LTQ ion-trap mass spectrometer involves 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. A PEEKsil tubing restrictor (100-mm L × 1/16-in. OD × 0.025-mm ID, SGE) is connected to the helium supply line via a shut-off ball valve and T-junction downstream of the metering flow valve (*see* **Fig. 7**). Full details of this modification have been provided by Harman and Blanksby *(21)*.

3.6. Safety Precautions When Using Ozone

1. There is a potential explosion risk when using methanol and oxygen with the presence of a corona discharge as an ignition

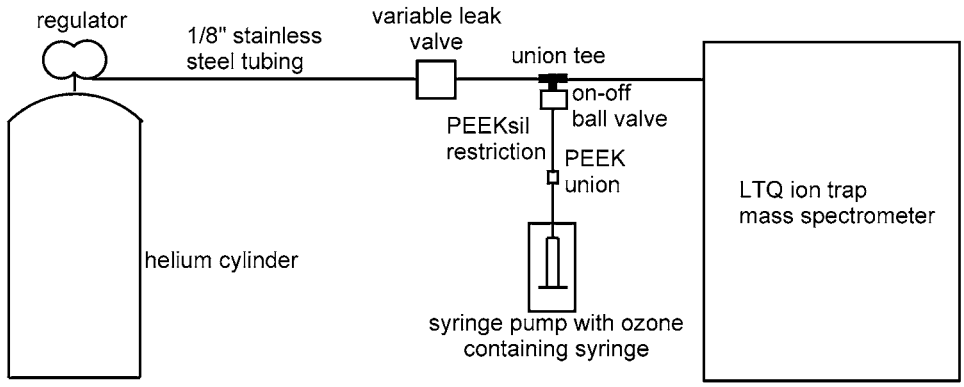

Fig. 7. A schematic diagram of the modification to an LTQ ion trap mass spectrometer for OzID.

source. Under normal operation with sample flow rates of 3–10 µL/min the fuel:oxygen ratio is too low for ignition to occur. With increased sample flow rates there may be a danger of ignitions since the fuel:oxygen ratio is increased. Particular care needs to be taken when washing out a sample with a high flow rate of methanol since methanol vapor may build up in the ESI source.

2. Ozone is a powerful oxidizing agent and care must be taken when using ozone, especially at high concentrations. Ozone is capable of reacting explosively with certain compounds. Furthermore, ozonides and hydroperoxides can be produced from the reaction of ozone with unsaturated organic compounds. Ozonides and hydroperoxides formed from ozonolysis can decompose explosively. The inhalation of ozone causes irritation and possible damage to the respiratory tract. Ozone also causes irritation to the eyes. The odor of ozone is distinctive and detectable at low concentrations (0.02–0.05 ppm). The permissible exposure limit for ozone is 0.1 ppm *(22)*. The Immediately Dangerous to Life and Health (IDLH) level is 5 ppm *(23)*.
3. It is recommended that an ozone monitor is acquired if working with ozone to measure the ambient concentration of ozone. There are many suitable ozone monitors on the market including the EZ-1X EcoZone ozone monitor (Eco Sensors, Santa Fe, USA) and Series-200 ozone monitor (Aeroqual Limited, Auckland, New Zealand). A cheap method for leak detection is using a strip of filter paper wet with a solution of potassium iodide. The presence of ozone will result in a brown discoloration of the filter paper.
4. When producing large quantities of ozone it is also suggested that the ozone is destroyed before being released to the atmosphere. There is a wide range of commercially available catalytic

ozone destruct unit such as the ODS-1P ozone destruct unit (Ozone Solutions, Sioux Center, Iowa, USA). Ozone can also be destroyed using a less elegant method of bubbling ozone though an aqueous solution of sodium thiosulfate and potassium iodide (dissolve 70 g of sodium thiosulfate and 5 g of potassium iodide in approximately 600 mL of water). Ozone is destroyed by reacting with thiosulfate and when the thiosulfate is totally consumed iodide is oxidized to iodine and the solution turns a dark brown/purple. When this occurs, turn off the ozone generator immediately and purge the system with oxygen before preparing a fresh solution of sodium thiosulfate with sodium iodide.

5. It is advisable to read the MSDS for ozone before working with this gas *(24)*.

3.7. Results

This section describes how to interpret OzESI–MS and OzID spectra using the look-up tables provided and demonstrates the approach using a series of worked examples.

3.7.1. OzESI–MS of a GPA Standard (In Situ Corona Discharge)

Ozonolysis may be initiated by corona discharge in the ESI source of a mass spectrometer when using oxygen as the drying gas (*see* **Subheading 3.1**) *(15)*. Shown in **Fig. 8a** is the negative ion

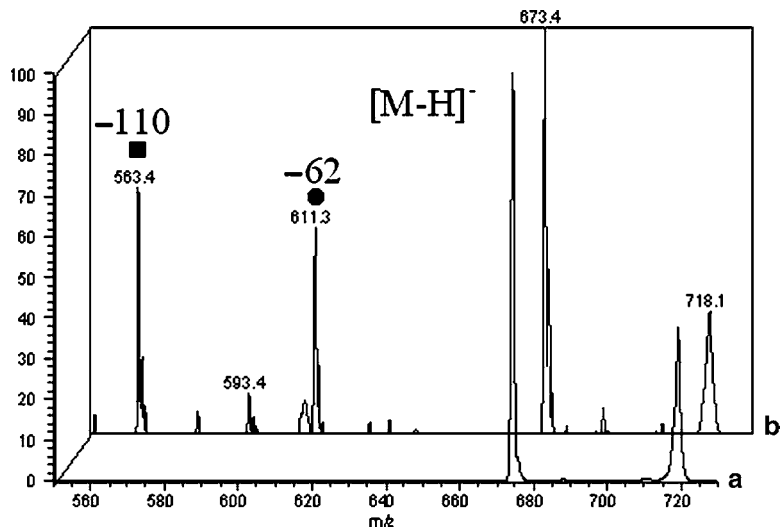

Fig. 8. (**a**) The ESI–MS spectrum of GPA(16:0/9*Z*-18:1) acquired in negative ion mode with a source voltage of −3.5 kV using oxygen instead of nitrogen as the drying gas. (**b**) The OzESI–MS spectrum of the sample acquired using a source voltage of −4.7 kV with oxygen as the drying gas. In this spectrum ozonolysis products are observed at *m*/z 563 and 611 (labeled *filled square* and *filled circle*) representing the neutral losses of 110 and 62 Da. The ion at *m*/*z* 593 results from the loss of water from the *m*/*z* 611 ion. The ion at *m*/*z* 718 is a formate adduct formed during ionization and is observed in both spectra.

ESI–MS spectrum of a methanolic solution of a monounsaturated phosphatidic acid, GPA(16:0/9*Z*-18:1), acquired using oxygen as the drying gas. This spectrum was acquired with a source voltage of –3.5 kV and a corona discharge was not observed at the ESI capillary. In this spectrum the $[GPA(16:0/9Z\text{-}18:1) - H]^-$ anion is observed at *m/z* 673. Shown in **Fig. 8b** is the ESI–MS spectrum when the source voltage was increased to –4.7 kV. Under these conditions a corona discharge was observed at the ESI capillary. In this spectrum, ozonolysis product ions at *m/z* 563, 593, and 611 are observed. The *m/z* 563 and 611 ions are consistent with aldehyde and α-methoxyhydroperoxide product ions from the ozonolysis of the n-9 double bond (*i.e.*, the 9th carbon–carbon bond from the methyl end of the fatty acyl chain). The *m/z* 593 ion is believed to result from the neutral loss of water from the *m/z* 611 ion. Interestingly, the α-methoxyhydroperoxide ozonolysis product results from a reaction of an ozonolysis intermediate with methanol, which was used as the electrospray solvent in this experiment. The participation of the ESI solvent in the ozonolysis reaction has been demonstrated using D_4-methanol and ethanol as the ESI solvent where the *m/z* 611 ion shifted by 4 and 14 Da, respectively *(15)*.

3.7.2. OzESI–MS (External Ozone Generation)

In our experiments it was found that the total ion current (TIC) was adversely affected when using the high voltages needed to initiate a corona discharge. Furthermore, an ozone producing corona discharge could only be established in negative ion mode. Conversely, when using an ozone generator TIC did not decrease under OzESI–MS conditions and OzESI–MS could be preformed in both negative and positive ion mode *(16)*. OzESI–MS experiments were performed on a triple quadrupole mass spectrometer. With this configuration it was found that *m/z* 184 precursor ion scanning was very useful in detecting choline containing, protonated phospholipids and their ozonolysis products and therefore was used routinely for the analysis of phosphatidylcholines (GPCho) and sphingomyelins (SM).

3.7.3. OzESI–MS of GPCho Isomeric Standards (External Ozone Generation)

The OzESI–MS spectra of the regioisomers GPCho(9*Z*-18:1/9*Z*-18:1) and GPCho(6*Z*-18:1/6*Z*-18:1) acquired using a *m/z* 184 precursor ion scan are shown in **Fig. 9**. For the n-9 lipid GPCho(9Z-18:1/9Z-18:1), two ozonolysis product ions were observed at *m/z* 676 and 724. These ions are 110 and 62 Da lower than the GPCho(9*Z*-18:1/9*Z*-18:1) $[M + H]^+$ ion and correspond to the aldehyde and α-methoxyhydroperoxide product ions, respectively. The neutral losses of 110 and 62 Da were also observed in the OzESI–MS spectrum of GPA(16:0/9*Z*-18:1) **(Fig. 8)** and are indicative of an n-9 double bond *(16)*. In the OzESI–MS spectrum of GPCho(6*Z*-18:1/6*Z*-18:1) ozonolysis products were observed *m/z* 634 and 682 corresponding to the

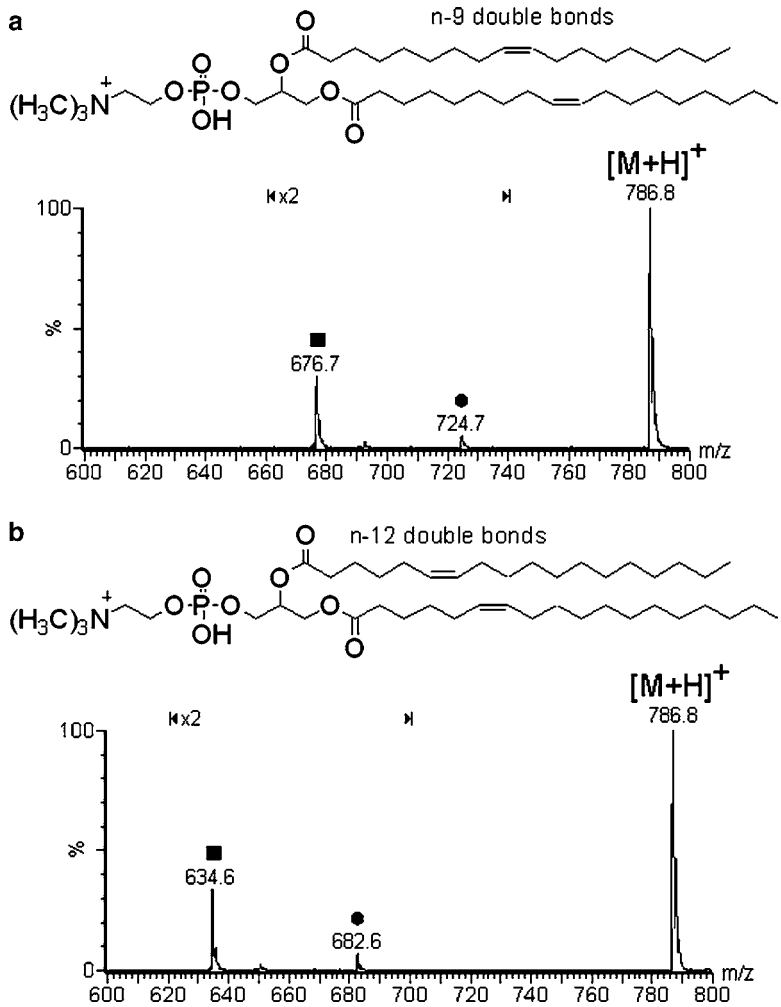

Fig. 9. The OzESI–MS spectrum of a 1 μM of methanolic solution of (**a**) GPCho (9*Z*-18:1/9*Z*-18:1) and (**b**) GPCho(6*Z*-18:1/6*Z*-18:1). Both spectra are recorded as *m/z* 184 precursor ion scans and thus show only the $[M + H]^+$ molecular ion and corresponding chemically induced fragment ions. The symbols *filled square* and *filled circle* identify the ozonolysis product ions as aldehydes and α-methoxyhydroperoxides, respectively. The mass difference between the ozonolysis products and the precursor ion is also given.

neutral losses of 152 and 104 Da. These ozonolysis products are shifted down 42 Da from the ozonolysis products of GPCho (9*Z*-18:1/9*Z*-18:1). This 42-Da mass shift corresponds to three methylene groups and thus demonstrate that GPCho(6*Z*-18:1/6*Z*-18:1) is an n-12 lipid. Clearly, using the mass difference between the parent ion and the aldehyde and α-methoxyhydroperoxide product ions can be used to determine the double bond position. **Table 2** provides a list of expected neutral losses from the OzESI of a range of monounsaturated fatty acids.

Table 2
A list of expected neutral losses or gains for the aldehyde and α-methoxyhydroperoxide product ions from OzESI–MS of lipids containing monounsaturated fatty acids with double bond (DB) positions of n-1 to n-15

DB-position	Neutral loss	
n –	Aldehyde	Methoxyhydroperoxide
1	–2	–50
2	12	–36
3	26	–22
4	40	–8
5	54	6
6	68	20
7	82	34
8	96	48
9	110	62
10	124	76
11	138	90
12	152	104
13	166	118
14	180	132
15	194	146

3.7.4. OzESI–MS of a Human Lens Extract (External Ozone Generation)

OzESI–MS may also be applied to simple lipid mixtures to determine double bond position within abundant unsaturated lipids. The positive ion *m/z* 184 precursor ion scan of a lipid extract of a 50-year old, cataractous human lens is shown in **Fig. 10a**. This spectrum shows the protonated ions of the choline-containing phospholipids, sphingomyelin, dihydrosphingomyelin, and phosphatidylcholine. The two most abundant lipid ions at *m/z* 705 and *m/z* 815 correspond to the saturated and monounsaturated dihydrosphingomyelins, SM(d18:0/16:0) and SM(d18:0/24:1), respectively. Other lipids are of relatively low abundance. The OzESI–MS spectrum of the same sample also acquired as an *m/z* 184 precursor ion scan is shown in **Fig. 10b**. In the spectrum, ions of *m/z* 753 and 781 are observed, which are not present in the standard ESI spectrum **(Fig. 10a)**. Performing CID on the *m/z* 753 and 781 ions (data not shown) revealed

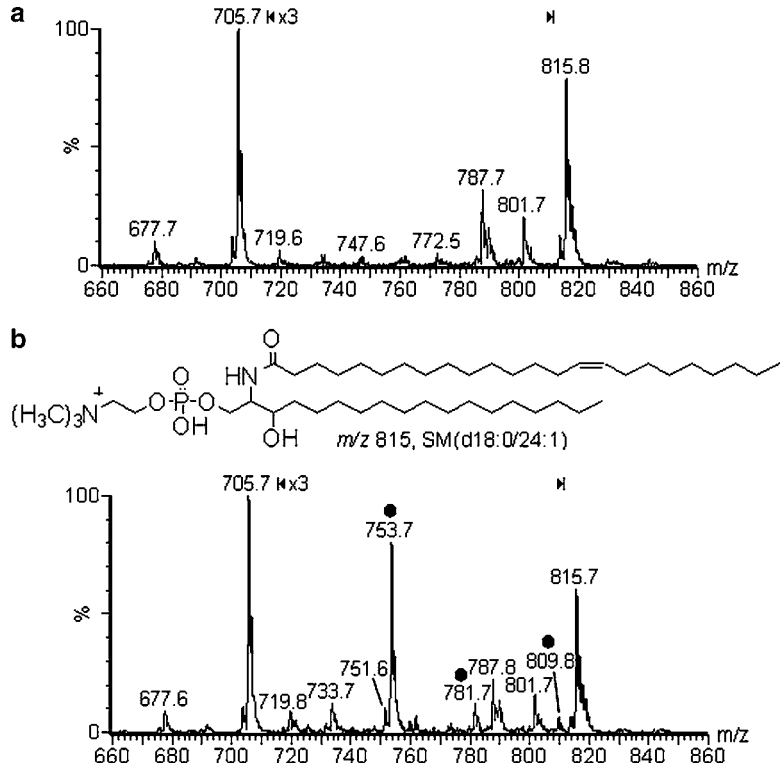

Fig. 10. (**a**) The positive ion ESI–MS spectrum of a lipid extract obtained from a 50-year-old, cataractous human lens recorded as a *m/z* 184 precursor ion scan and thus showing only the $[M + H]^+$ molecular ions of sphingomyelin, dihydrosphingomyelin, and phosphatidylcholine lipids present within 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. The symbol *filled circle* indicates the α-methoxyhydroperoxide ozonolysis product ions arising from the ozonolysis of SM(d18:0/24:1). These ozonolysis products correspond to neutral losses of 62, 34, and 6 Da indicating the possible presence of three isomeric forms of this SM(d18:0/24:1) with double bonds in the n-9, n-7, and n-5 positions, respectively.

the neutral losses of 34 and 49 Da from the precursor ions, which are characteristic of protonated α-methoxyhydroperoxides *(16)*. The α-methoxyhydroperoxide ions of *m/z* 753 and 781 correspond to neutral losses of 62 and 34 Da. Using the list of expected neutral losses for α-methoxyhydroperoxide ions shown in **Table 2** it can be seen that the neutral losses of 62 and 34 Da are indicative of n-9 and n-7 double bond positions, respectively. Since there is only one site of unsaturation, SM(d18:0/24:1) was assigned as two isomers, SM(d18:0/15*Z*-24:1) and SM(d18:0/17*Z*-24:1), where the stereochemistry about the double bond is assumed to be *Z*. Unfortunately, the expected aldehyde product ions from the OzESI–MS of SM(d18:0/15*Z*-24:1)

and SM(d18:0/17*Z*-24:1) are predicted at *m/z* 705 and 733 and are thus isobaric with phospholipids already present in the extract. Interestingly, another ozonolysis product ion at *m/z* 809 was observed corresponding to the α-methoxyhydroperoxide ion of an n-5 double bond. This suggests that there may be a minor abundance of SM(d18:0/19Z-24:1). The abundance of the *m/z* 809 ion, however, was too low to allow confirmation of the α-methoxyhydroperoxide moiety by MS/MS and thus the presence of an n-5 isomer remains uncertain.".

3.7.5. OzID of GPCho Isomeric Standards

OzESI–MS is a useful technique for double bond identification within isolated standards and simple lipid mixtures as demonstrated with the human lens extract **(Fig. 10)**. There are however, several problems that can occur when performing OzESI–MS on lipid mixtures. These include (a) ozonolysis products having the same *m/z* as other lipids ions in the OzESI–MS spectrum and (b) different molecular lipids giving ozonolysis products of the same *m/z*. To overcome these problems we developed OzID whereby ions are mass selected and trapped in the presence of ozone before spectrum acquisition. This allows even lipids present within complex lipid mixtures to be analyzed without the need for chromatographic separation.

The OzID spectra of the $[M + Na]^+$ ions of GPCho(9*Z*-18:1/9*Z*-18:1) and GPCho(6*Z*-18:1/6*Z*-18:1) are shown in **Fig. 11**. Both phospholipid standards were made to a concentration of 1 μM in methanol with 200- μM sodium acetate to aid sodium adduct formation during ESI. Sodium adducts were found to ionize easily and react rapidly with ozone to yield OzID spectra with a good signal-to-noise ratio. To acquire these OzID spectra two isolation steps were performed. The first isolation was made at an isolation width of 3 Th to ensure no neighboring ions were also selected. This isolation step resulted in minor fragmentation of the precursor ion and therefore a second isolation step using an isolation width of 10 Th was used to remove the CID fragments from the OzID spectra. After the second isolation step, ions were trapped for 10 s to react with ozone. For the OzID spectrum of the GPCho(9*Z*-18:1/9*Z*-18:1) sodium adduct, two abundant ozonolysis products were observed at *m/z* 698 and 714. These correspond to the neutral losses of 110 and 94 Da. The neutral loss of 110 Da was previously observed in the OzESI–MS spectrum of protonated GPCho(9*Z*-18:1/9*Z*-18:1) sodium adduct, ozonolysis products were observed at *m/z* 656 and 672 corresponding the neutral losses of 152 and 136 Da. As in OzESI–MS, these ozonolysis product ions are shifted 42 Da and indicate the n-12 double bond position. From this data it is can be seen that aldehyde and Criegee ions are the two main products from OzID. **Table 3** shows the expected neutral losses/gains for the OzID of a range of monounsaturated-fatty-acid-containing lipids.

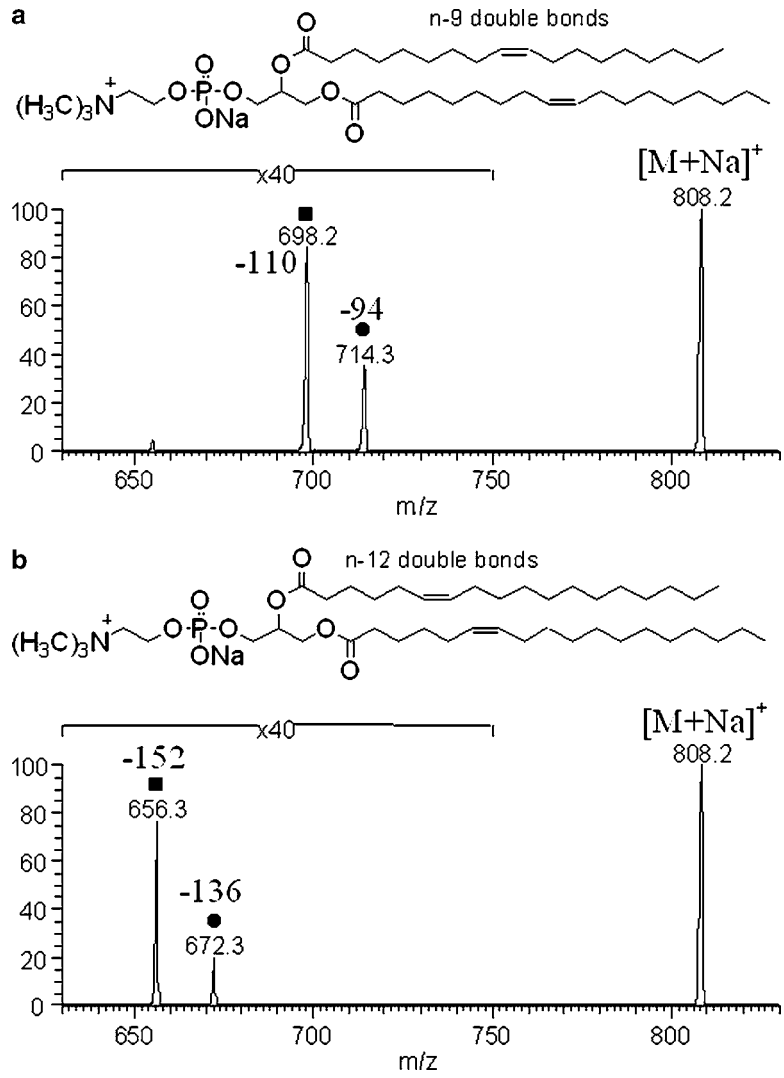

Fig. 11. The OzID spectra of the $[M + Na]^+$ ions of (**a**) GPCho(9*Z*-18:1/9*Z*-18:1) and (**b**) GPCho(6*Z*-18:1/6*Z*-18:1). Both phospholipids were made to a concentration of 1 μM in methanol with 200 μ M of sodium acetate to aid sodium adduct formation during ESI. The pair of ions resulting from ozonolysis of the double bond are labeled with the neutral loss they represent and the *filled square* and *filled circle* symbols indicating aldehyde and Criegee ions, respectively.

3.7.6. OzID of Cow Kidney Phospholipids: Positive and Negative Ion Examples

The negative ion ESI–MS spectrum of a cow kidney lipid extract is shown in **Fig. 12a**. This lipid extract is significantly more difficult to analyze than the human cataractous lens extract due to the larger number of lipids and the increased levels of unsaturation. The most abundant ion within this spectrum is the *m/z* 885 ion which was identified by CID to be GPIns(18:0/20:4) *(18)*. An ion 2 Da greater in mass was observed at *m/z* 887 and was identified as GPIns(18:0/20:3) by CID. Significantly, three isomers of 20:3 have been well documented (**Table 1**), these are skip-conjugated n-9, n-6, and n-3. From the CID spectrum alone,

Table 3
A list of expected neutral losses or gains for the aldehyde and Criegee product ions from OzID of lipids containing monounsaturated fatty acids with double bond (DB) positions of n-1 to n-15

DB-position	Neutral loss	
n–	Aldehyde	Criegee
1	–2	–18
2	12	–4
3	26	10
4	40	24
5	54	38
6	68	52
7	82	66
8	96	80
9	110	94
10	124	108
11	138	122
12	152	136
13	166	150
14	180	164
15	194	178

the isomer(s) that were present in cow kidney GPIns(18:0/20:3) could not be ascertained. The OzID spectrum of the *m/z* 887 ion is shown in **Fig. 12b**. In this spectrum, three pairs of ozonolysis products were observed from the cleavage of each of the double bonds of the 20:3 fatty acid. **(Fig. 12b)**. It is most important to determine the position of the double bond closest to the methyl end since this determines the fatty acid class. In the OzID spectrum of cow kidney GPIns(18:0/20:3) the terminal neutral losses of 68 and 52 Da were observed indicative of an n-6 fatty acid. The three pairs of ozonolysis product ions are also separated by 40 Da indicative of skip-conjugated double bonds **(Fig. 12b)**. No ozonolysis products of either n-9 or n-3 20:3 were observed. Therefore, using a combination of CID and OzID the structure of this phospholipid can be assigned as GPIns(18:0/8*Z*,11*Z*, 14*Z*-20:3), where the stereochemistry of the double bonds is assumed to be *Z*. From a knowledge of the OzID behavior of

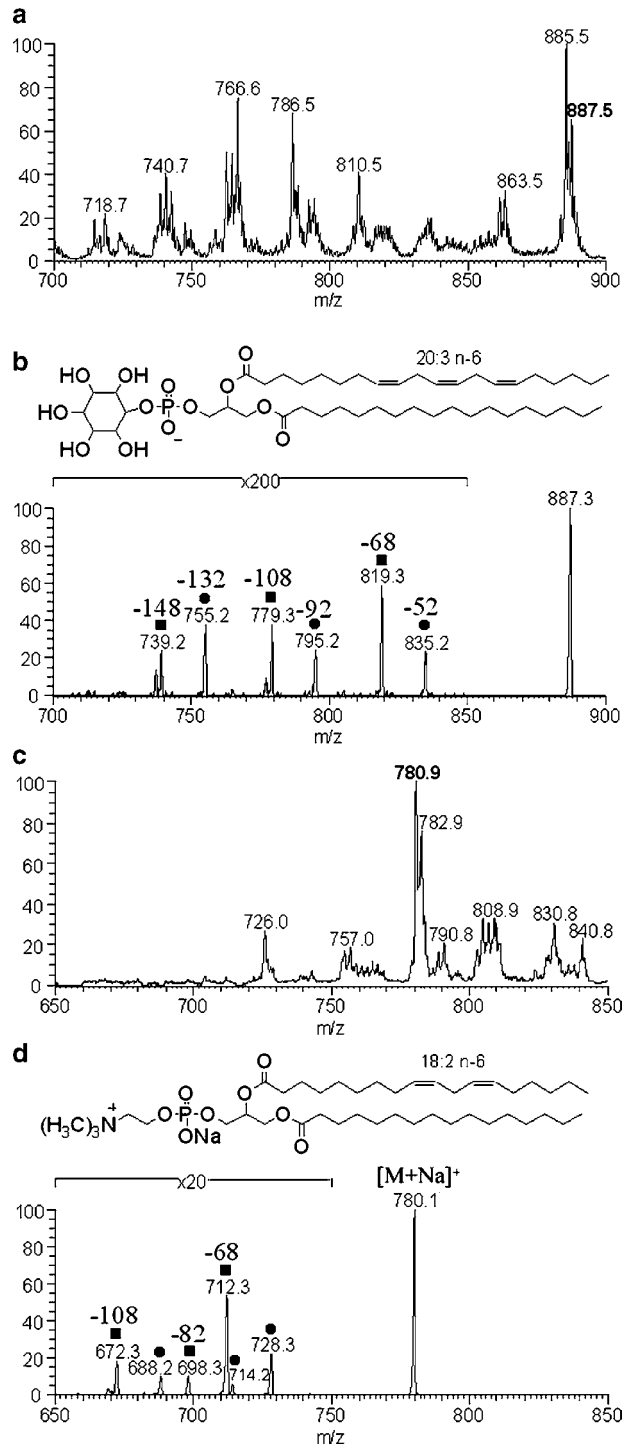

Fig. 12. (**a**) The negative ion ESI–MS spectrum of a cow kidney lipid extract (*ca.* 40 μM in 2:1 methanol–chloroform). The label is bold is the *m*/*z* of the ion selected in the OzID experiment. (**b**) The OzID spectra of phospholipid anion identified by CID as GPIns(18:0/20:3). (**c**) The positive ion ESI–MS spectrum of the same cow kidney extract with 200 μM of NaOAc to produce sodium adducts of the GPCho lipids. The label in bold is the *m*/*z* of the ion selected in the OzID experiment. (**d**) The OzID spectrum of the *m*/*z* 780 ion from a cow kidney lipid extract. This has been identified by MS/MS to be the sodium adduct of GPCho(16:0/18:2) with a minor abundance of GPCho(16:1/18:1). In both OzID spectra the pairs of ions resulting from ozonolysis of each double bond are labeled with *filled square* and *filled circle* indicating aldehyde and Criegee ions, respectively. The neutral losses for both the aldehyde and Criegee ions are indicated in spectrum (**b**), however only the neural losses of the aldehyde ions are indicated in spectrum **d**.

lipids containing polyunsaturated fatty acids, a list of expected neutral losses from OzID was produced and is shown in **Table 4**.

The positive ion ESI–MS spectrum of a cow kidney lipid extract is shown in **Fig. 12c** where 200 μM sodium acetate was added to aid the formation of sodium adducts. The most abundant ion in this ESI–MS spectrum was observed at *m/z* 780 and was identified by CID as predominately the sodium adduct of GPCho(16:0/18:2) with a small contribution from sodiated GPCho(16:1/18:1). The OzID spectrum of the *m/z* 780 ion is shown in **Fig. 12d**. Interestingly, in this spectrum three sets of ions are observed despite the fact that the 18:2 fatty acid has only two double bonds. The first pair of ozonolysis products is observed at 68 and 52 Da below the precursor ion indicative of an n-6 double bond **(Table 4)**. Another pair of ozonolysis products is observed with 40 Da lower indicative of a double bond in the 9th position from the methyl terminus within a skip-conjugated polyunsaturated fatty acid. This information allows GPCho(16:0/18:2) to be assigned as GPCho(16:0/9*Z*,12*Z*-18:2) where the double bond geometry is assumed to be *Z*. Interestingly, a pair of ozonolysis product ions are observed at *m/z* 698 and 714 corresponding to the neutral losses of 82 and 66 Da. By consulting **Table 3** it can be

Table 4
A list of expected neutral losses for the aldehyde and Criegee product ions from OzID of lipids containing n-3, n-6, and n-9 skip-conjugated polyunsaturated fatty acids

		Neutral loss	
Class	**DB-position**	**Aldehyde**	**Criegee**
n-3	3	26	10
	6	66	50
	9	106	90
	12	146	130
	15	186	170
	18	226	210
n-6	6	68	52
	9	108	92
	12	148	132
	15	188	172
	18	228	212
n-9	9	110	94
	12	150	134
	15	190	174

The fatty acid class indicates the position of the first double bond counted from the methyl-end of the acyl chain.

seen that these neutral losses are indicative of n-7 monounsaturated fatty acids. These ozonolysis products therefore are likely arising from the isobaric lipid, GPCho(16:1/18:1).

3.7.7. CID/OzID: Anionic Ether Phospholipid from Human Lens

The more challenging lipids for structural assignment by OzID are lipids containing two or more unsaturated fatty acids. In such cases it is possible that a particular double bond position may not be able to be assigned to an individual fatty acid. Examples of such lipids have previously been found when analyzing human lens lipids by mass spectrometry within our laboratory. The structural characterization of a GPEtn ether lipid by OzID and CID/OzID has previously been published *(18)*. In this section the double bond assignment within another abundant human lens phospholipid will be demonstrated. This phospholipid was observed at m/z 772 in the negative ion MS spectrum **(Fig. 13a)**. The CID fragmentation of this lipid is consistent with a phosphatidylserine (GPSer) with an 18:1 fatty acid and a monounsaturated ether chain. The OzID spectrum of the m/z 772 ion is shown in **Fig. 13b**. In this spectrum the neutral losses of 110, 94, 82, and 66 are observed indicating the presence of both n-9 and n-7 double bonds. From these data alone, however, it is impossible to assign the n-9 double bond position to either the 18:1 fatty acid or the ether chain. To gain this information, the m/z 772 ion was mass selected and subjected to CID and the m/z 403 fragment ion then isolated and trapped in a CID/OzID experiment **(Fig. 13c)**. The m/z 403 ion results from the neutral losses of the GPSer headgroup and 18:1 fatty acid and therefore contains only the unsaturated ether chain. In this CID/OzID spectrum of the unsaturated ether containing fragment ion, ozonolysis products indicative of both n-9 and n-7 are observed **(Fig. 13c)**. Interestingly, however, within the CID/OzID spectrum the abundance of the n-7 ozonolysis products is greater than the n-9 ozonolysis products unlike in the standard OzID spectrum (**Fig. 13b**). This suggests that the n-7 double bond may be predominately located on the ether chain. Therefore, the OzID and CID/OzID data seem to suggest that the m/z 728 GPSer ether maybe a mixture of four isomers; GPSer(11Z-18:1e/9Z-18:1), GPSer(9Z-18:1e/11Z-18:1), GPSer(9Z-18:1e/9Z-18:1), and GPSer(11Z-18:1e/11Z-18:1) where the GPSer(11Z-18:1e/9Z-18:1) isomer is likely to be the most abundant. Double bond geometry was assumed to be *Z*.

3.8. Conclusion

For both OzESI–MS and OzID, two main product ions are observed from double bond cleavage by ozone. Using the mass difference between the precursor lipid ion and the ozonolysis product ion, double bond position can, in most instances, be unambiguously assigned. Of these two techniques, OzID is the most promising tool for further probing molecular structure by mass spectrometry. The significant advantage being that chromatographic separation of complex lipid mixtures is not required. The technique

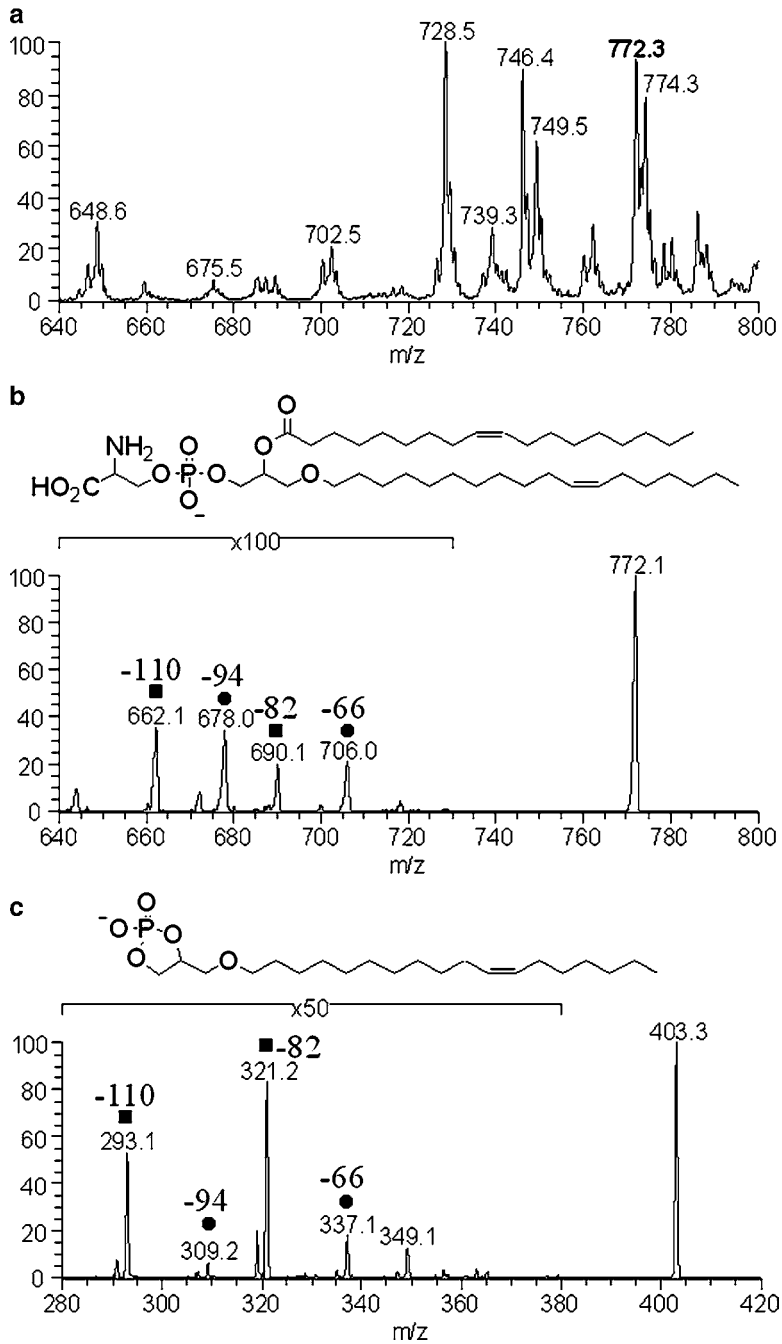

Fig. 13. (**a**) The ESI–MS spectrum of a human lens extract acquired on the LTQ. (**b**) The OzID spectrum of a GPSer ether anion of *m/z* 772 acquired using a trapping time of 10 s. (**c**) The CID/OzID of the *m/z* 403 fragment ion. In this experiment *m/z* 772 is mass isolated and subjected CID, *m/z* 403 is then mass-selected and allowed to react with ozone. The *m/z* 403 ion results from the neutral losses of the GPSer headgroup and 18:1 fatty acid. The pairs of ions resulting from ozonolysis of double bonds are labeled with *filled square* and *filled circle* indicating aldehyde and Criegee ions, respectively. The neutral losses of the ozonolysis products represent are also labeled.

does, however, require a major instrument modification. Conversely, OzESI–MS only requires a minimal instrument modification and is suitable for isolated lipids or simple lipid mixtures.

4. Notes

1. When connecting PVC tubing to parts such as the Dreschel bottle of plastic T-connector first heat the tubing with a heat gun. This softens the tubing making it easier to work with. *Do not use lubricants* as these may explosively react with oxygen and/or ozone.
2. In a study performed in 2002, the half-life of ozone gas in a 1 m^3 PVC reaction chamber at 21°C with 73% relative humidity was found to be 1 h *(25)*. In our experience, after 2 h of use the yield of ozonolysis products decreases significantly. The authors also acknowledge Ms Jane Deeley for providing the human lens spectra and Miss Jessica Nealon for the preparation of the cow kidney sample.

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