Supporting Information

Determining Double Bond Position in Lipids Using Online Ozonolysis Coupled to Liquid Chromatography and Ion Mobility-Mass Spectrometry

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Supplemental Figures and Tables

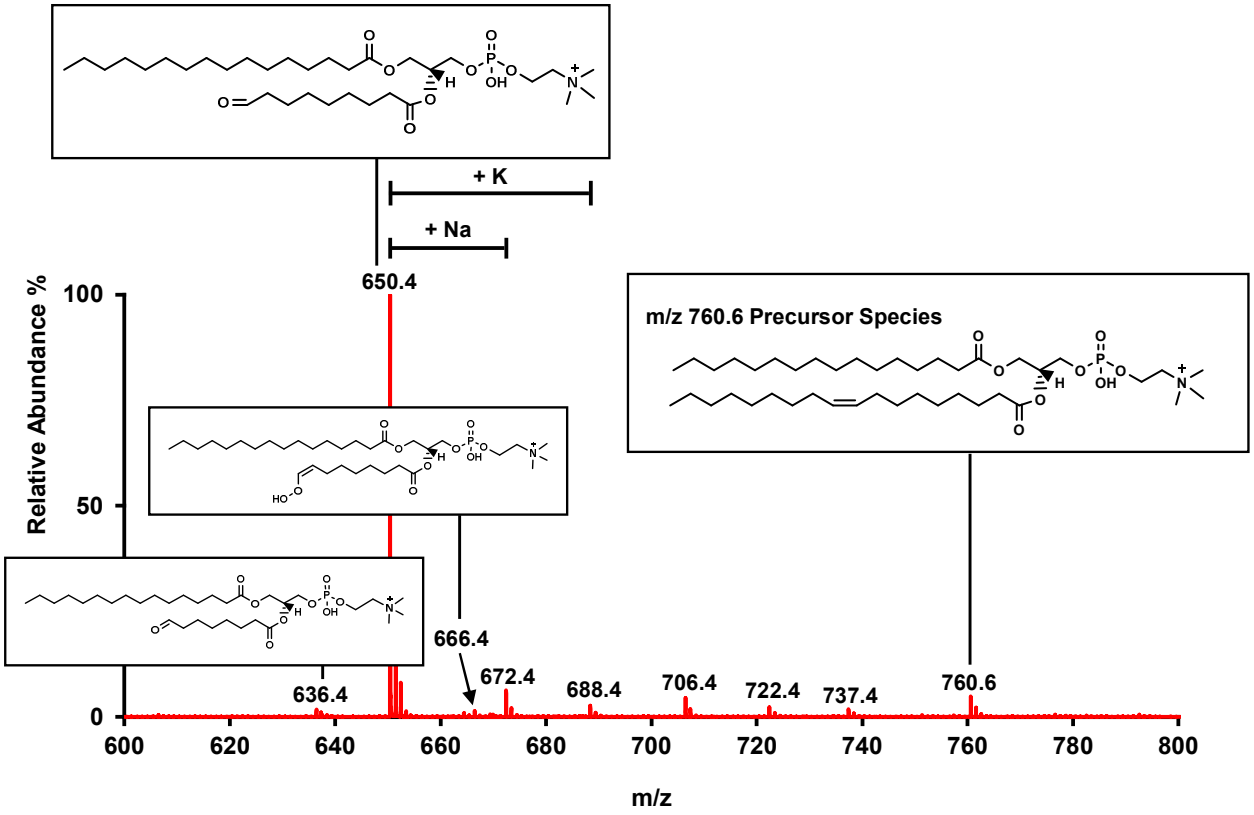


Figure S1. An expanded version of the PC 16:0/18:1 spectra following irradiation with the ozonolysis device at 10 µL/min. Observed ions are labeled with their corresponding mass-to-charge, and structures are proposed for several species based on the exact mass measurement (<10 ppm). It should be noted that the ion at *m/z* 737.4 is not likely an ozonolysis product due to its odd-numbered mass and the fact that it does not appear on the same mobility trend line as the other ozonolysis product ions. The diagnostic aldehyde product ion (*m/z* 650.4) indicative of double bond cleavage at the 9th carbon atom in one of the alkyl chains of the precursor (*m/z* 760.6) is the dominant species in the spectrum. Product ions corresponding to sodiated and potassiated forms of the aldehyde product are also observed at *m/z* 672.4 and *m/z* 688.4, respectively. Additionally, the product ion at *m/z* 666.4 is likely a vinyl hydroperoxide or the corresponding carboxylic acid, resulting from rearrangement of the Criegee intermediate.[1,2] Further oxidation of this species at *m/z* 666.4 is thought to result in the formation of the species at *m/z* 636.4 by loss of formaldehyde.[3] Finally, the presence of the ion at *m/z* 706.4 can likely be attributed to solvent adduction of acetonitrile to one of the Criegee ions at *m/z* 666.4. Solvent adduction has previously been reported for solution phase ozonolysis, especially when methanol is used as a solvent.[4]

[1] Santrock, J.; Gorski, R. A; O'Gara, J. F. Products and Mechanism of the Reaction of Ozone with Phospholipids in Unilamellar Phospholipid Vesicles. *Chem. Res. Toxicol.* **1992**, *5* (1), 134–141.

[2] Brown, S. H. J.; Mitchell, T. W.; Blanksby, S. J. Analysis of Unsaturated Lipids by Ozone-Induced Dissociation. *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids* **2011**, *1811* (11), 807–817.

[3] Vu, N.; Brown, J.; Giles, K.; Zhang, Q. Ozone-Induced Dissociation on a Traveling Wave High-Resolution Mass Spectrometer for Determination of Double-Bond Position in Lipids. *Rapid Commun. Mass Spectrom.* **2017**, *31* (17), 1415–1423.

[4] Thomas, M. C.; Mitchell, T. W.; Harman, D. G.; Deeley, J. M.; Murphy, R. C.; Blanksby, S. J. Elucidation of Double Bond Position in Unsaturated Lipids by Ozone Electrospray Ionization Mass Spectrometry. *Anal. Chem.* **2007**, *79* (13), 5013–5022.

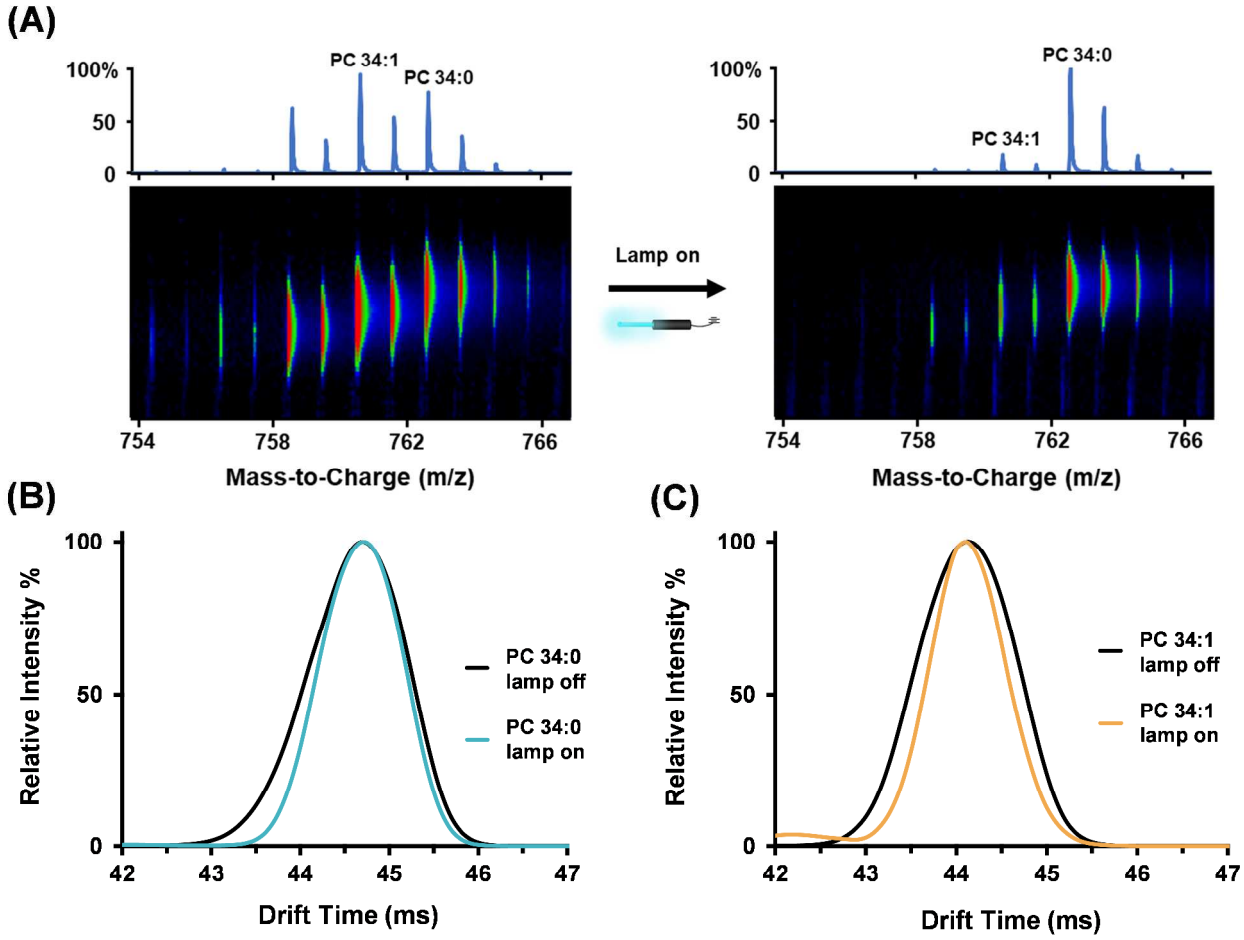
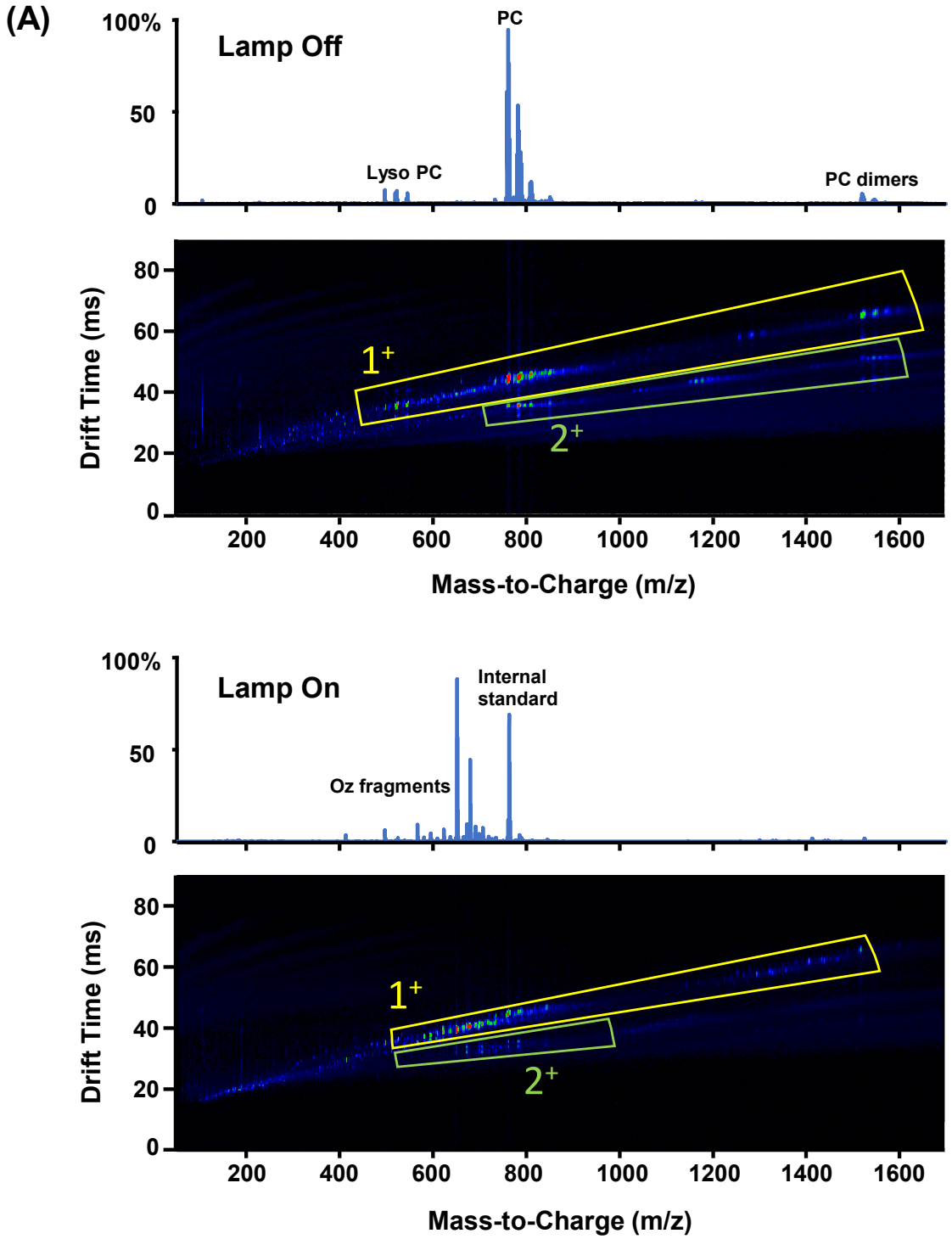


Figure S2. Comparison of drift time profiles for two lipid species from a PC lipid extract with the ozonolysis device off (black) and on (teal **(B)** or mustard **(C)**). Because lipid species differing in mass by the addition of one double bond to an alkyl chain are spaced 2 Da apart in mass and < 1 ms apart in drift time, the second isotope peak of one species often overlaps with the primary peak of the species containing one fewer double bond **(A)**. As discussed in the primary text, the overlapping of these isotopic envelopes can result in broadened drift profiles and shifts in a given drift profile's measured centroid, both of which can lead to errors in a species' calculated CCS. In the figure above, comparison of two PC lipids with the ozonolysis device turned off and then on reveals the extent of drift profile broadening due to isotopic overlap. Once the lamp is turned on, lipid species containing double bonds are significantly diminished through reaction with ozone so that isotopic overlap is reduced **(A)**. For PC 34:0, the resolving power of the drift peak increased from 35.6 with the lamp off to 41.5 with the lamp on, and PC 34:1 saw a similar increase from a drift peak resolving power of 34.8 to 44.5. Note, however, that neither centroid of the two species were observed to significantly shift upon isotopic subtraction. Nonetheless, the ozonolysis device could aid in direct measurement of the accurate drift profiles of closely related lipid species without need for mathematical corrections due to isotopic overlap, illustrating another potential advantage of this technique.


(A)
Lamp Off
100%
50
0
PC
Lyso PC
PC dimers
Drift Time (ms)
80
60
40
20
0
1+
2+
200
400
600
800
1000
1200
1400
1600
Mass-to-Charge (m/z)
Lamp On
Internal standard
Oz fragments

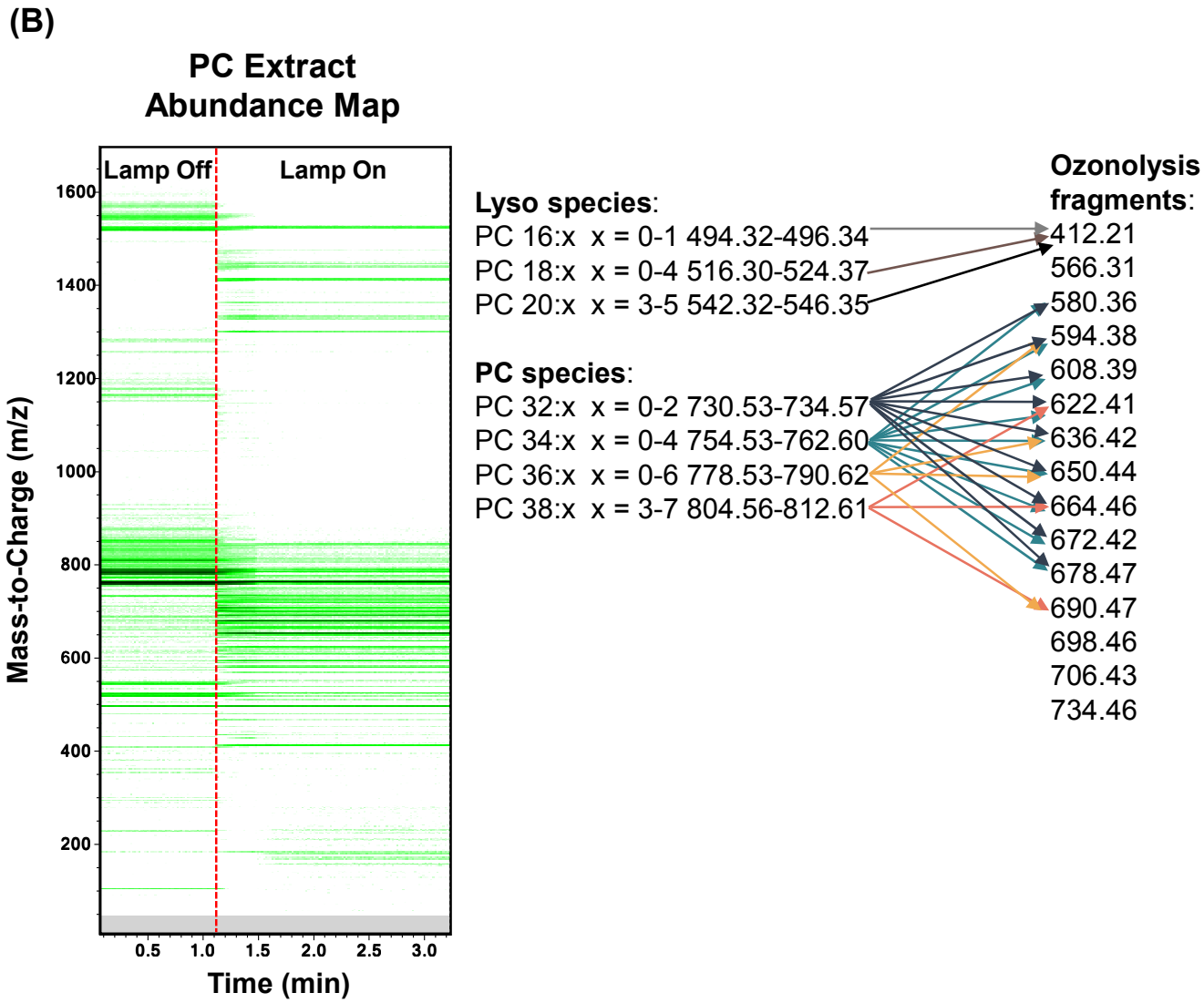


Figure S3. (A, previous page) Full IM-MS spectra of the PC lipid extract with the ozonolysis device off (top) and on (bottom). In both spectra, 1+ and 2+ trendline regions are highlighted and relevant features are labeled on the accompanying mass spectra. This work focused solely on the 1+ lipid species. Prior to onset of ozonolysis three main lipid-rich regions of mass-mobility space are observed. These features correspond to lyso PC species (ca. *m/z* 450 - 600) containing only a single fatty acid tail esterified to the glycerol backbone, PC species containing two fatty acid tails esterified to the glycerol backbone (ca. *m/z* 700-850), and singly charged dimer species formed from the association of two PC monomers (ca. *m/z* 1450-1600). Following ozonolysis in the device, any species containing a double bond is significantly depleted such that the lipid-rich regions previously observed are no longer detected, and ozonolysis product ions are observed in the region of *m/z* 400-750. It can be noted that several species were not observed to deplete upon ozonolysis, most notably *m/z* 762.6, which corresponds to the fully saturated PC 16:0/18:0 lipid standard that was spiked in as an internal standard, and the 1+ dimer of PC 16:0/18:0 at *m/z* 1524.2. An additional fully saturated lipid species at m/z 496.3 corresponding to PC 16:0 endogenous to the sample was also detected and not found to diminish in signal upon ozonolysis. **(B)** The abundance map shown above is another way of visualizing the PC lipid extract data in **(A)**. The intensities for all

mass-to-charge signals are plotted as a function of time, clearly delineating how the observed peaks in the spectrum change as a result of ozonolysis. However, as explained in the text, because the ozonolysis reaction occurs prior to source ionization, there is no way to directly correlate observed ozonolysis product ions with their precursors. Possible ozonolysis products were predicted for several lipid species observed in the sample based upon possible precursor structures generated using LIPID MAPS. It was observed that the detected ozonolysis product ions could have been generated by multiple precursors, and that depending on the precursor structure analyzed, multiple possible ozonolysis product ions could also be generated. Thus, the deconvolution the origins of ozonolysis product ions in complex samples is very challenging. However, it was noted that the majority of product ions detected contained no sites of unsaturation in their fatty acid alkyl chains which could indicate that the reaction proceeded to completion even for species containing multiple sites of unsaturation. The peak at *m/z* 690.47 was one of the few exceptions observed, which is thought to contain one unsaturation in its fatty acid chains. This result stands in contrast to those of the fatty acid containing six double bonds that was analyzed (Figure 4 in the text), in which the preferred ozonolysis product occurred as a result of cleavage at the last double bond on the fatty acid tail, which left five unsaturations remaining on the acyl chain. The source of this discrepancy, namely that ozonolysis of a fatty acid standard produced multiply unsaturated product ions while a PC extract produced mainly fully saturated product ions, is unclear at this time. One possible explanation is that the lipid extract predominantly contained lipids with only a single unsaturation in their acyl chains, resulting in the majority of fully saturated ozonolysis product ions. Separation of lipid precursors via chromatography, as suggested in the text, could help to answer this question by correlating lipid precursors directly to their ozonolysis products.

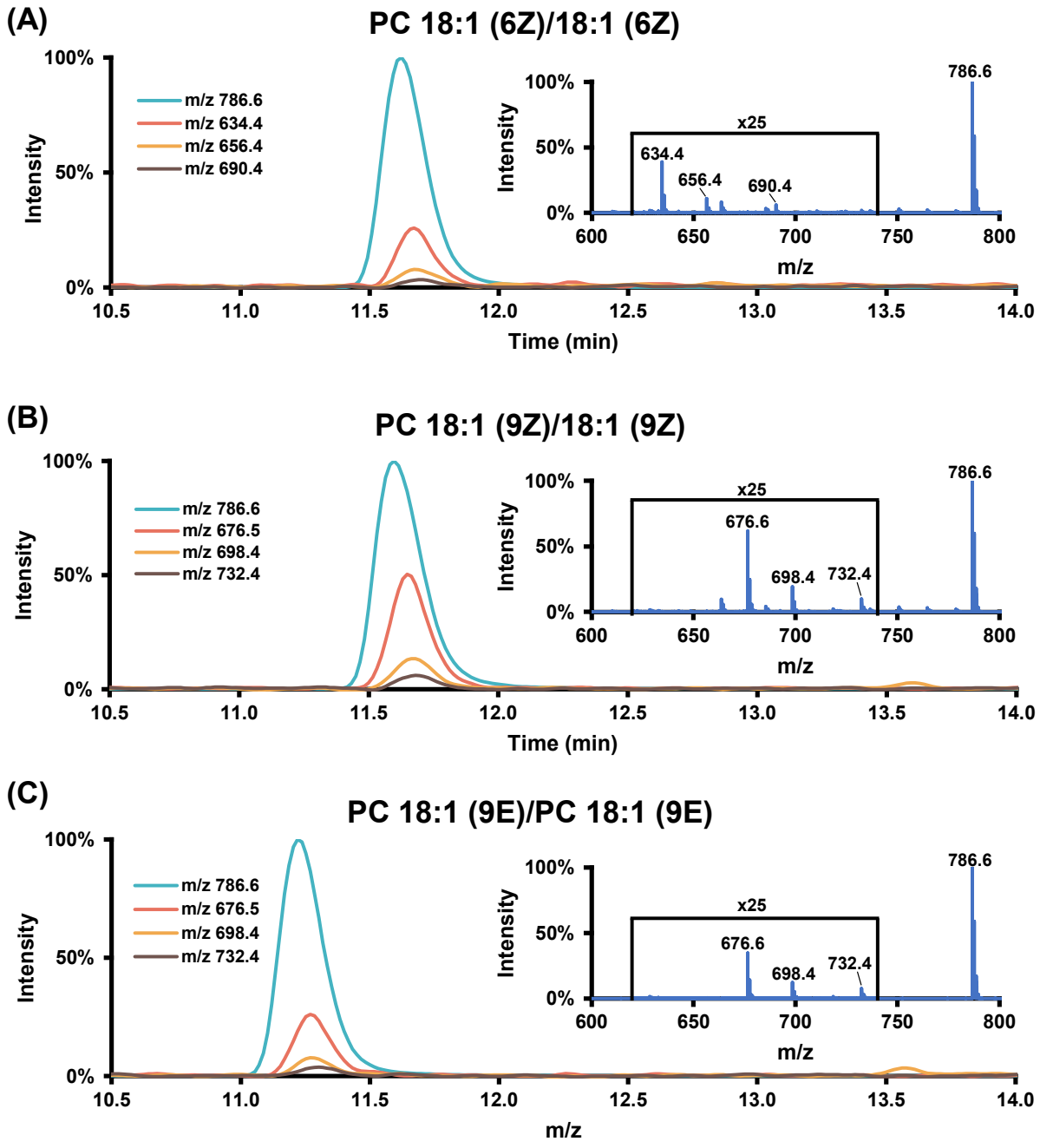


Figure S4. Comparison of extracted ion chromatograms (EICs) for three PC standards ran separately using the LC-Oz-IM-MS technique, with the ozonolysis device turned on. Analysis of the retention time of the precursor at *m/z* 786 reveals that the *trans* species would elute first in a mixture with a retention time of about 11.24 min, while the two *cis* double bond position isomers would likely elute together with a retention time of about 11.63 min. This elution order is in agreement with the data presented in Figure 6 of the manuscript, in which the three species were combined and run as a mixture. Background subtracted mass spectra taken from each of the peaks give ozonolysis fragments consistent with cleavage at the 9th carbon atom along the acyl chain for both **(B)** and **(C)**, and fragments consistent with cleavage at the 6th carbon atom in the acyl chain for **(A).** The types ozonolysis fragments detected are similar to those observed in **Figure S1**, and include the diagnostic aldehyde species, a sodiated form of the aldehyde

species, and an acetonitrile solvent adducted species like that seen in **Figure S1** located at a mass approximately 40 Da above the predicted mass of the Criegee ion (*m/z* 692.5).