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Separation and identification of phosphatidylcholine regioisomers by combining liquid chromatography with a fusion of collision- and ozone-induced dissociation

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The differentiation of closely related lipid isomers is increasingly important to our evolving understanding of lipid biochemistry but it is equally challenging to contemporary chromatographic and mass spectral analyses. Recently, we described a novel ion-activation approach based on combining collision- with ozone-induced dissociation (CID/OzID) for the identification of the relative acyl chain substitution positions in glycerophospholipids. Here we demonstrate, for the first time, that CID/OzID can be effectively combined with reversed-phase chromatography to enable the separation and unambiguous identification of regioisomeric pairs of phosphatidylcholines that differ only in the arrangement of acyl chains on the glycerol backbone.

Keywords: mass spectrometry, ozone-induced dissociation, liquid chromatography, lipids, isomers, phosphatidylcholines

Introduction

The composition of the lipidome (i.e., the structure and abundance of different lipids within a biological system) can vary significantly between different organisms, different organs or even within different regions of the same tissue.[1] While enzymatic regulation, lipid synthesis, recycling pathways and transport all play an important role in dictating the molecular structure and abundance of the lipids present, factors such as diet, exercise, infection or other insult can also have a profound influence.[2] In order to unravel these complex biochemical mechanisms and to understand the role(s) of lipids in health and disease it is essential to have access to sensitive and highly specific methods for the identification and quantification of individual lipid molecular species.

Glycerophospholipids are among the most abundant classes of lipids in nature and significant attention has been focused on developing analytical methods to elucidate the molecular structure of these species and to quantify them within complex lipid extracts.[3] Contemporary tandem mass spectrometry (MS/MS) is an essential and powerful tool for such analyses and can provide a rapid identification of the class

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of the lipid [e.g., phosphatidylcholine (PC)] and the number of carbons and degree of unsaturation in each of the acyl chains [e.g., PC 16:0_20:4 where the presence of a saturated 16-carbon chain (16:0) and 20-carbon polyunsaturated chain (20:4) are indicated].[4] A significant limitation of commonly deployed mass spectrometric assays, however, is the inability to differentiate closely related isomeric lipids. For example, the collision-induced dissociation (CID) mass spectrum of PC 16:0/20:4 (where the 20:4 chain is esterified to the *sn*-2 position on the glycerol backbone) is very similar to that obtained from the positional isomer PC 20:4/16:0 (where the 20:4 is at the *sn*-1 position). This challenge is further compounded by the fact that both isomers are likely present in many complex extracts.[5,6] While these structures may appear quite similar, the biochemistry of the isomers can be profoundly different, indicating that in order to answer certain biological questions the isomeric pairs need to be resolved and separately characterized.

Liquid chromatography (LC) has been extensively used in conjunction with mass spectrometry for dramatically reducing the complexity of lipid extracts prior to mass spectral analysis.[7–14] There are, however, only a limited number of examples in which LC has successfully been combined with mass spectrometry for the separation and identification of phospholipid isomers.[15–19] Of these, only one previous study has demonstrated the successful separation of pairs of PC *sn*-positional isomers using reversed-phase ultra-high performance liquid chromatography.[16] In the same study, MS/MS—specifically MS^3 fragmentation—was used to assign the molecular structure to each of the separately eluting isomers. As noted above, however, such product ions are usually not unique to a particular isomer and rather the relative abundance of the spectral peaks is used to infer the acyl chain substitution positions.[5,20] Uncertainty in such structural assignments is further compounded by the often low abundance of the mass spectral peaks. Recently, our group developed a novel ion-activation method that combines collision- and ozone-induced dissociation (CID/OzID) in sequence to yield abundant product ions that are diagnostic of the *sn*-position and can thus be deployed to identify and differentiate regioisomeric lipids.[21] Here we describe, for the first time, the combination of LC and CID/OzID into a single workflow to enable the separation and identification of PC *sn*-positional isomers.

Materials

All the solvents used, including water, were Optima LCMS grade and purchased from Thermo Fisher Scientific (Scoresby, Victoria). Analytical-grade butylated hydroxytoluene (BHT) was purchased from Sigma-Aldrich (St. Louis, Missouri). Analytical-grade sodium acetate was purchased from Ajax Chemicals (Auburn, New South Wales, Australia). Industrial-grade compressed oxygen was obtained from BOC (Cringila, New South Wales, Australia). The following synthetic phospholipid standards were purchased from Avanti Polar Lipids, Inc. (Alabaster, Alabama): PC 18:1/16:0 [1-(9*Z*-octadecenoyl)-2-hexadecanoyl-*sn*-glycero-3-phosphocholine], PC 16:0/18:1 [1-hexadecanoyl-2-(9*Z*-octadecenoyl)-*sn*-glycero-3-phosphocholine], PC 16:0/20:4 [1-hexadecanoyl-2-(5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenoyl)-*sn*-glycero-3-phosphocholine], and PC 16:0/22:6 [1-hexadecanoyl-2-(4*Z*,7*Z*,10*Z*,13*Z*,16*Z*,19*Z*-docosahexaenoyl)-*sn*-glycero-3-phosphocholine].

Methods

Stock solutions of all PC standards were prepared in acetonitrile/2-propanol (50/50, v/v) to a final lipid concentration of ca. 300–400 µM with 1% BHT (w/w) and stored at −80°C until analysis. Using the corresponding stock solution, PC standards, PC 16:0/20:4 and PC 16:0/22:6 were each separately diluted using water/acetonitrile/2-propanol (50 : 25 : 25, v/v) to a final concentration of 40 µM. The phospholipid standards, PC 16:0/18:1 and PC 18:1/16:0, were mixed at a 1 : 1 molar ratio in water/acetonitrile/2-propanol (50 : 25 : 25, v/v) to a final total lipid concentration of 80 µM each (i.e., total lipid concentration of 160 µM).

Chromatography was performed using a Surveyor high-performance liquid chromatograph system coupled to an LTQ linear ion trap mass spectrometer (Thermo Fisher Scientific, San Jose, California). Injections of 2.5–10 µL of each PC standard sample were loaded onto an Acquity C_{18} BEH column (1.7 µM, 150 mm × 1 mm internal diameter, Waters, Milford, Massachusetts). For all analyses, the column was maintained at 60–65°C and samples held at 5°C. For analysis of PC standards, PC 16:0/20:4 and PC 16:0/22:6, mobile phases were prepared as follows: A, water, sodium acetate (50 µM), and B, acetonitrile/2-propanol (75/25, v/v), sodium acetate (50 µM). The gradient elution was run at a flow rate of 60 µL min^{-1} as follows: 0–10 min, 70% B; 10–90 min, 75% B; 90–95 min, 99% B; 95–110 min, 70% B. For the 1 : 1 mixture of PC 16:0/18:1(9Z) and PC 18:1(9Z)/16:0, the mobile phases were prepared as follows: A, water, sodium acetate (70 µM), and B, acetonitrile/2-propanol (66 : 34, v/v), sodium acetate (70 µM). The gradient elution was run at a flow rate of 60 µL min^{-1} as follows: 0–60 min, 50–100% B; 60–63 min, 100% B; 63–75 min, 50% B.

The mass spectrometer was modified to enable the introduction of ozone into the ion trap as previously described.[22–24] All analyses were undertaken in positive ion mode and representative source conditions included: capillary voltage 45 V, ionization spray voltage 4 kV; capillary temperature 200°C; tube lens voltage 180 V. Nitrogen gas served as the sheath gas, auxiliary gas and sweep gas and helium as the bath gas with the addition of a small bleed from a continuous flow of externally generated ozone (produced at 170 g m^{-3} by a Titan 100 generator, Absolute Ozone, Edmonton, Canada). These conditions provide a constant level of ozone within the ion trap throughout the experiments. The XCalibur instrument control software (Thermo Fisher Scientific, San Jose, California) was used to trigger alternating full-scan MS and a targeted

CID/OzID scan with most data acquired in the centroid mode. The CID/OzID sequence is formally an MS^3 experiment in which intact $[PC + Na]^+$ ions are mass selected (using an isolation width of 5 Th) and subjected to CID (using a normalized collision energy of 40). The [PC + Na – 183] CID product ion is then re-isolated (using an isolation width of 3 Th) and trapped for 200–1000 ms, with no collision energy, in the presence of ozone. The ion injection time was 100 ms for all CID/OzID scans.

Results and discussion

Separation and identification of isomeric PCs

The results of LC-MS analysis of a commercially available, synthetic standard provided as 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (annotated here as PC 16:0/20:4) are shown in Figure 1. The total ion chromatogram for ions at *m/z* 804 [Figure 1(a)], corresponding to the sodium cation of PC 36:4, reveals two peaks with maxima at 44.8 min and 46.4 min. The presence of two chromatographic features with a common *m/z* suggests the presence of isomeric phospholipids within the sample. Previous chromatographic investigations using an identical reversed-phase column showed isomer separation could be observed for synthetic PCs with a PC 36:4 sum composition. In that study, relative peak abundances in tandem mass spectra suggested that the major and minor chromatographic peaks corresponded to the *sn*-positional isomers PC 16:0/20:4 and PC 20:4/16:0, respectively.[16] With these putative peak assignments in mind, CID/OzID mass spectra were obtained across the chromatographic peaks at 44.8 min and 46.4 min and the results are shown in Figure 1(b) and (c), respectively. These spectra are obtained by first mass selecting the $[M + Na]^+$ precursor ion and subjecting it to CID. Ozone is present in the ion trap throughout the experiment, but at sufficiently low concentrations that it does not impact on the conventional functions of the instrument. For example, the CID spectra obtained under these conditions are not significantly affected by the presence of the reagent gas in the helium. In a formal MS^3 experiment, the product ion at $[M + Na - 183]^+$ was then mass selected and stored in the ion trap for 1000 ms in the presence of ozone. The carbon–carbon double bond linking the *sn*-2 acyl chain to the glycerol backbone in the $[M + Na - 183]^+$ ion is chemically cleaved by the ozone, resulting in a neutral loss characteristic of the acyl chain at this position. The origins of these ions have previously been described and the process is briefly summarized in Scheme 1.[21] The CID/OzID spectrum of the first eluting peak [Figure 1(b)] shows abundant product ions at *m/z* 427 and *m/z* 443, corresponding to neutral losses of 194 and 178, respectively. Importantly, however, the observed neutral losses in the CID/OzID spectrum are consistent with predictions for a 16:0 acyl chain occupying the *sn*-2 position of the glycerol backbone (Table 1) and thus support the assignment of the chromatographic peak to the PC 20:4/16:0 isomer. The CID/OzID spectrum of the late-eluting peak is shown in Figure 1(c) and

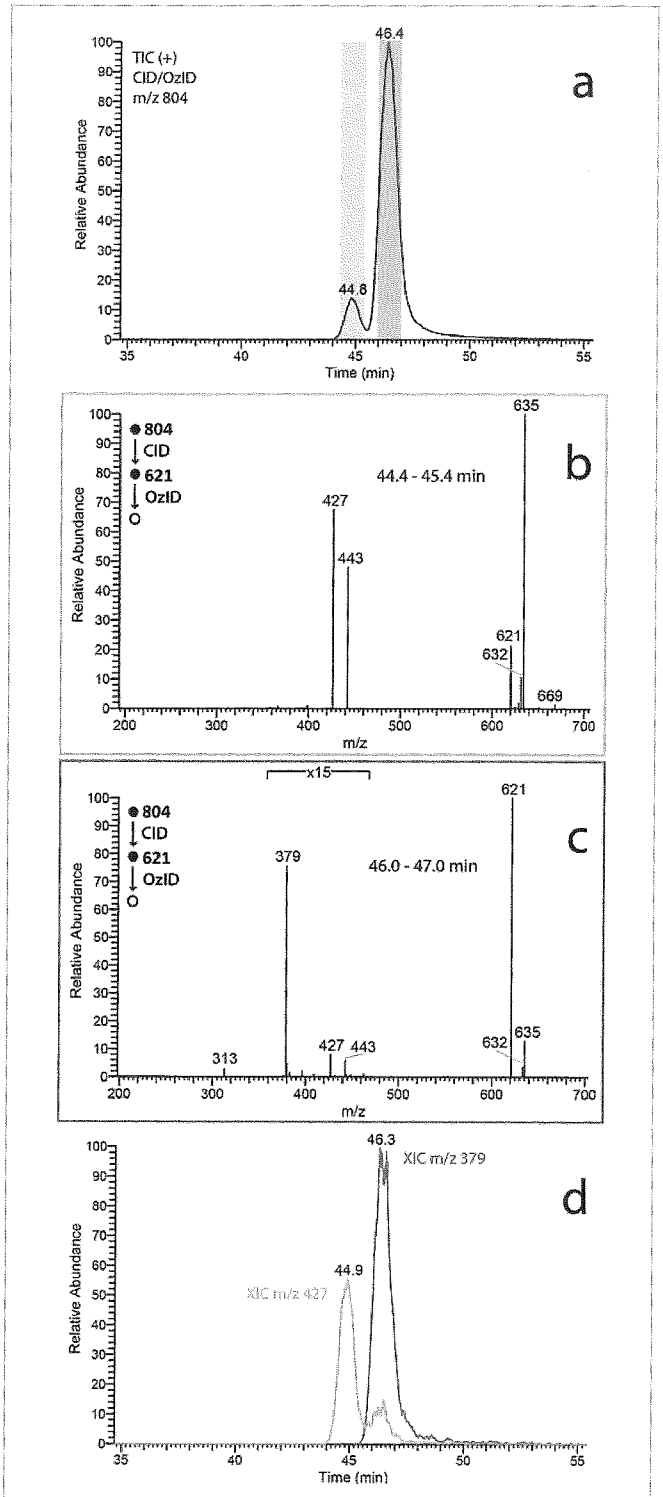
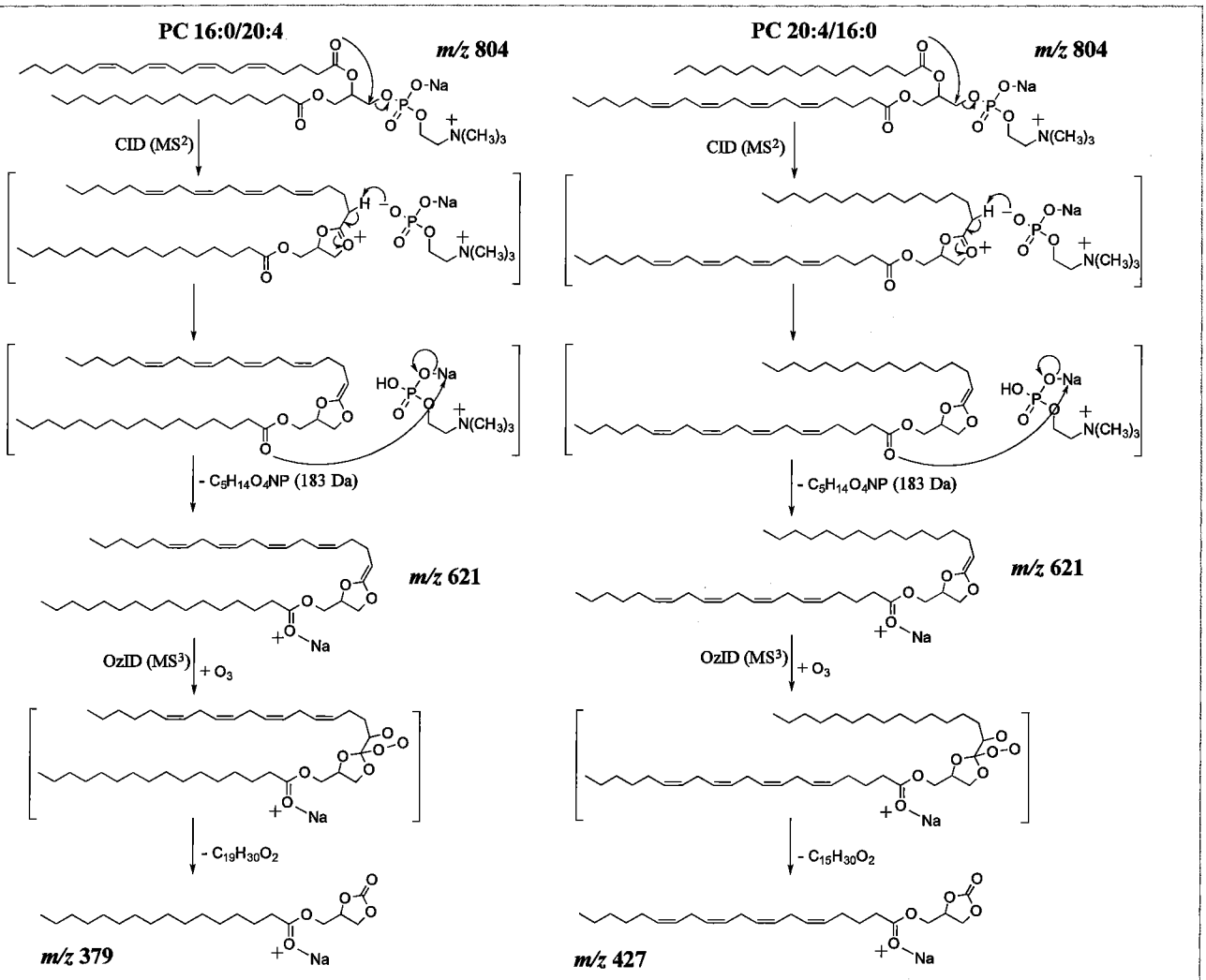


Figure 1. LC-MS analysis of synthetic standard provided as PC 16:0/20:4. (a) Total ion chromatogram for LC-CID/OzID of *m/z* 804. (b) The CID/OzID spectrum obtained from integrating across the chromatographic peak between 44.4 min and 45.4 min. (c) The CID/OzID spectrum obtained from integrating across the chromatographic peak between 46.0 min and 47.0 min. (d) The extracted ion chromatogram for CID/OzID product ions at *m/z* 427 (indicative of PC 20:4/16:0) is shown in green, while the extracted ion chromatogram for *m/z* 379 (indicative of PC 16:0/20:4) is shown in blue.



Scheme 1. CID/OzID dissociation of *sn*-positional isomers PC 16:0/20:4 and PC 20:4/16:0. Aldehyde product ions are shown. Criegee ions are not shown but are 16 Da heavier than the aldehydes (*cf.* Table 1) and arise from incorporation of an additional oxygen atom upon ozonolysis.

is dominated by the product ion at *m/z* 379, corresponding to a neutral loss of 242 Da with a minor product ion also observed at *m/z* 395 (226 Da). Such transitions are indicative of the 20:4 acyl chain occupying the *sn*-2 position and support the assignment of the major chromatographic feature to the expected PC 16:0/20:4. Integration of the chromatographic peaks in Figure 1(a) suggests that the synthetic PC is composed of approximately 88% PC (16:0/20:4) and 12% of the alternate PC (20:4/16:0) isomer, with the latter eluting first upon reversed-phase chromatography. The presence of low-abundance *sn*-positional isomers in synthetic glycerophospholipids has been noted before.[5,6,20] This likely arises from transacylation of the intermediate lysophosphatidylcholine during the preparation.[25] Importantly, however, the LC protocol employed here provides an effective separation of the isomers and the CID/OzID affords rapid identification of the isomeric structure of each chromatographic peak. Indeed, visualization of the extracted ion chromatograms for the diagnostic product ions at *m/z* 427 and *m/z* 379 [see Figure 1(d)] provides a selective means to interrogate the chromatographic data for the presence of each isomer.

Using a similar LC-MS protocol to that described above, a synthetic standard provided as 1-palmitoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine (annotated here as PC 16:0/22:6) was examined. The resulting LC and mass spectral data are summarized in Figure 2. The total ion chromatogram for ions of *m/z* 828 is illustrated in Figure 2(a) and shows two features with maxima at 41.4 min and 42.8 min, respectively. These features correspond in *m/z* to the $[M + Na]^+$ cation of PCs with a sum composition of PC 38:6. By analogy with the previous example, the early eluting feature would be expected to arise from the less abundant PC 22:6/16:0 isomer while the more abundant, late-eluting feature can be tentatively assigned to PC 16:0/22:6. CID/OzID mass spectra acquired over each chromatographic peak are shown in Figures 2(b) and (c) and among other features, show product ions

Table 1. CID/OzID transitions for common fatty acyl chains when substituted at the *sn*-2 position on the glycerol backbone. Predicted neutral losses for fatty acyl chains upon OzID of the fragment $[PC + Na - 183]^+$ resulting from CID of the $[PC + Na]^+$ precursor ion.

Fatty acid (no. carbon: no. db)	CID/OzID Neutral loss (from $[PC + Na-183]^+$ trapped w/O_3)	
	Aldehyde	Criegee
16:0	194	178
16:1	192	176
18:0	222	206
18:1	220	204
18:2	218	202
18:3	216	200
20:0	250	234
20:1	248	232
20:2	246	230
20:3	244	228
20:4	242	226
20:5	240	224
22:0	278	262
22:1	276	260
22:2	274	258
22:3	272	256
22:4	270	254
22:5	268	252
22:6	266	250

consistent with neutral losses of 194 Da and 266 Da, respectively. These signature transitions indicate that the *sn*-2 acyl chain is 16:0 for the first, earlier-eluting peak and 22:6 for the second (*cf.* Table 1), thus confirming the assignment based on relative retention time. Integration of the chromatographic peaks in Figure 2(a) indicates that this PC sample is composed of approximately 79% PC (16:0/22:6) and 21% of the alternate PC (22:6/16:0) isomer.

Chromatographic separation of *sn*-positional isomers has previously been shown to become more challenging as the level of unsaturation in the acyl chains is reduced and the difference in the relative lengths of the acyl chains is reduced.[16] Therefore, to challenge the LC-CID/OzID protocol developed here a sample was prepared consisting of an approximately 1 : 1 mixture of synthetic PCs supplied as 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (annotated here as PC 16:0/18:1) and 1-oleoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine (annotated here as PC 18:1/16:0). The chromatographic and mass spectral results from analysis of this mixture are summarized in Figure 3. The total ion chromatogram for all the signals deriving from *m/z* 782, corresponding to the $[M + Na]^+$ of lipids with the sum composition of PC 34:1, is shown in Figure 3(a) and provides only a single broad feature with no resolution between the two components of the mixture. CID/OzID mass spectra averaged across

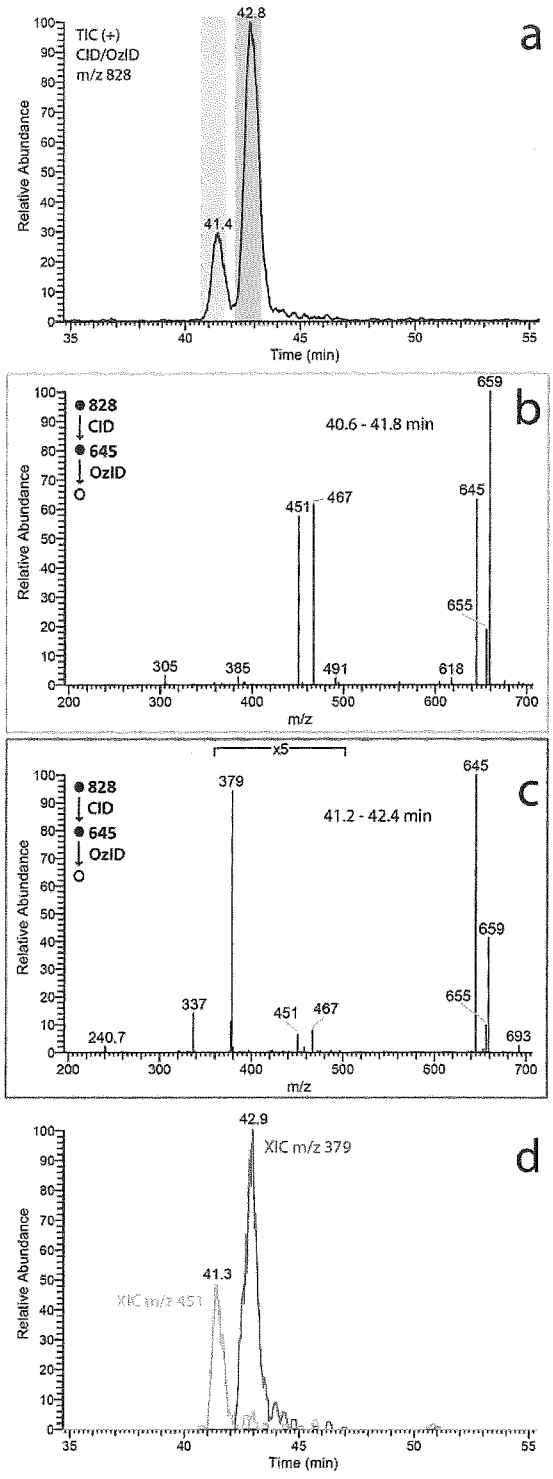


Figure 2. LC-MS analysis of synthetic standard provided as PC 16:0/22:6. (a) Total ion chromatogram for LC-CID/OzID of *m/z* 828. (b) The CID/OzID spectrum obtained from integrating across the chromatographic peak between 40.6 min and 41.8 min. (c) The CID/OzID spectrum obtained from integrating across the chromatographic peak between 41.2 min and 42.4 min. (d) The extracted ion chromatogram for CID/OzID product ions at *m/z* 451 (indicative of PC 22:6/16:0) is shown in green, while the extracted ion chromatogram for *m/z* 379 (indicative of PC 16:0/22:6) is shown in blue.

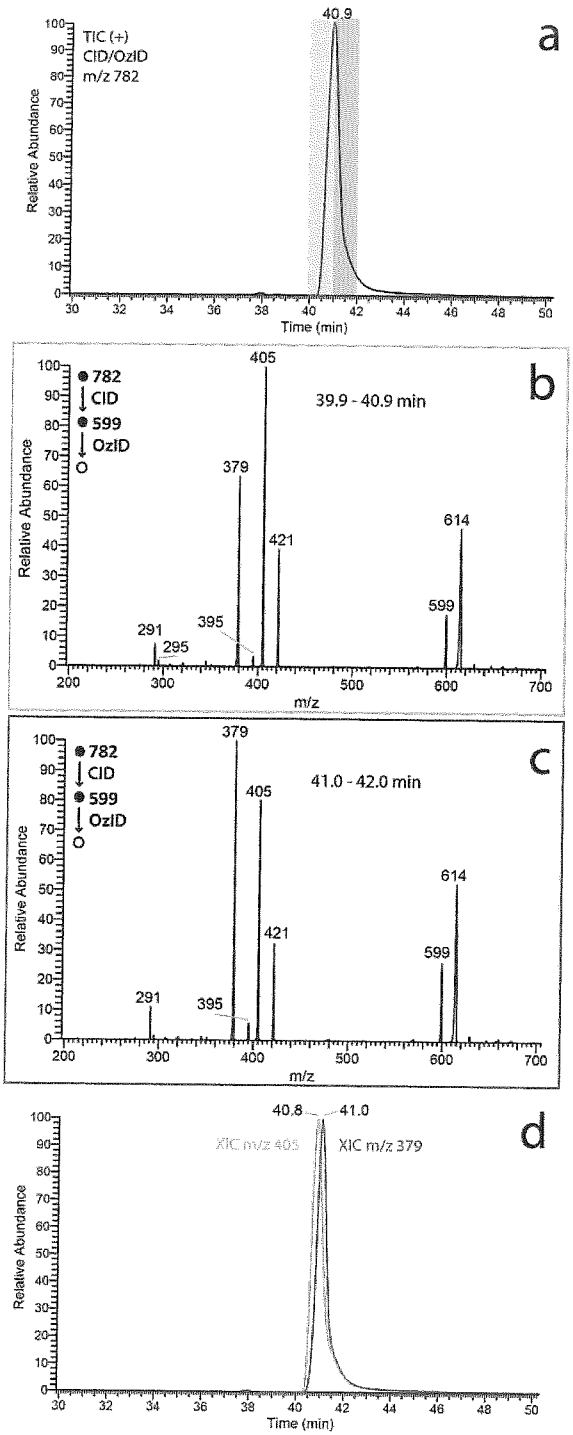
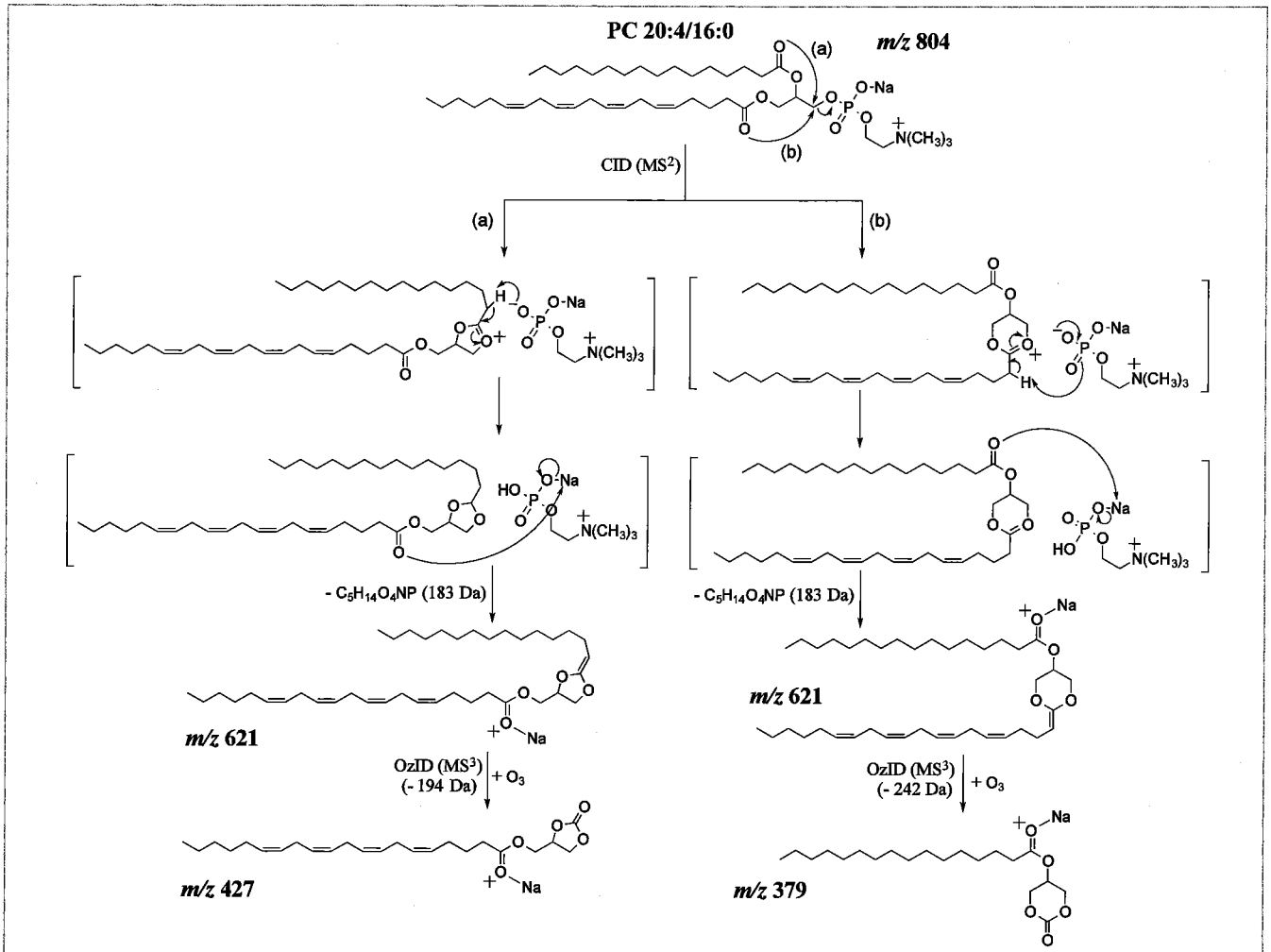


Figure 3. LC-MS analysis of a 1 : 1 mixture of two synthetic standards provided as PC 16:0/18:1 and PC 18:1/16:0. (a) Total ion chromatogram for LC-CID/OzID of *m/z* 782. (b) The CID/OzID spectrum obtained from integrating across the chromatographic peak between 39.9 min and 40.9 min. (c) The CID/OzID spectrum obtained from integrating across the chromatographic peak between 41.0 min and 42.0 min. (d) The extracted ion chromatogram for CID/OzID product ions at *m/z* 405 (indicative of PC 18:1/16:0) is shown in green, while the extracted ion chromatogram for *m/z* 379 (indicative of PC 16:0/22:6) is shown in blue.

data points from the early-eluting and late-eluting portions of the chromatographic peak [indicated by the green and blue boxes in Figure 3(a)] are shown in Figures 3(b) and (c), respectively. These mass spectra share the same product ions, with peaks at *m/z* 405 and *m/z* 421 indicative of a 16:0 chain at the *sn*-2 position (*i.e.*, PC 18:1/16:0) and peaks at *m/z* 379 and *m/z* 395 that are consistent with the 18:1 chain occupying the *sn*-2 position (*i.e.*, PC 16:0/18:1). Importantly, the relative abundance of these characteristic product ion pairs changes across the chromatographic peak. Spectra obtained during the early-eluting part of the peak show *m/z* 405 and *m/z* 421 at higher abundances than the *m/z* 379 and *m/z* 395 ion pair [Figure 3(b)], while, in contrast, the latter ions are more abundant in spectra obtained from the peak tail [Figure 3(c)]. The mass spectral data thus indicate that two isomer populations are being partially separated on the reversed-phase column with PC 18:1/16:0 being more concentrated at shorter retention times while PC 16:0/18:1 dominates at longer retention times, consistent with relative retention-time trends for other PCs, as described above (*cf.* Figures 1 and 2). While the total ion chromatogram shows no clear resolution of the isomer populations these can be easily visualized in the extracted ion chromatograms for the ions at *m/z* 405 and *m/z* 379 shown in Figure 3(d). This example serves to highlight the unique capabilities of combining reversed-phase chromatography with the characteristic and abundant mass spectral signatures obtained from CID/OzID analysis.

Implications for dissociation and oxidation mechanisms

Efforts to understand the unimolecular mechanisms for the dissociation of ionized glycerophospholipids in the past have been hampered by the presence of lipid isomers. As illustrated here, the synthetic glycerophospholipids routinely used in such studies typically represent a mixture of both *sn*-positional isomers. The chromatographic separation achieved here, however, enables the tandem mass spectra of each PC isomer to be separately acquired and the mechanisms of the underlying CID/OzID ion-activation processes investigated. Comparing the spectra in Figure 1(b) and (c) reveals several interesting differences. Outside of the characteristic ions already discussed, the product ion apparent at *m/z* 635 is the base peak in Figure 1(b) while it is only a minor contributor to the spectrum in Figure 1(c). This signal has previously been assigned to the addition of oxygen to the mass-selected $[M + Na - 183]^+$.[21] The fragile nature of this ion leads to peak tailing and the assignment of an apparent *m/z* 635 in centroid mode rather than the true *m/z* 637.[26] The greater abundance of this feature correlates with the presence of the polyunsaturated chain at the *sn*-1 position and is consistent with other polyunsaturated PCs [*cf.* *m/z* 659 in Figure 2(b)]. Also of note is that the *m/z* 427 and *m/z* 443 ions that dominate in Figure 1(b) are also present, albeit at low abundance, in Figure 1(c). The converse does not apply, with no significant *m/z* 379 contributions to the spectrum in Figure 1(b). This is best illustrated in the extracted ion chromatograms for the diagnostic ions at

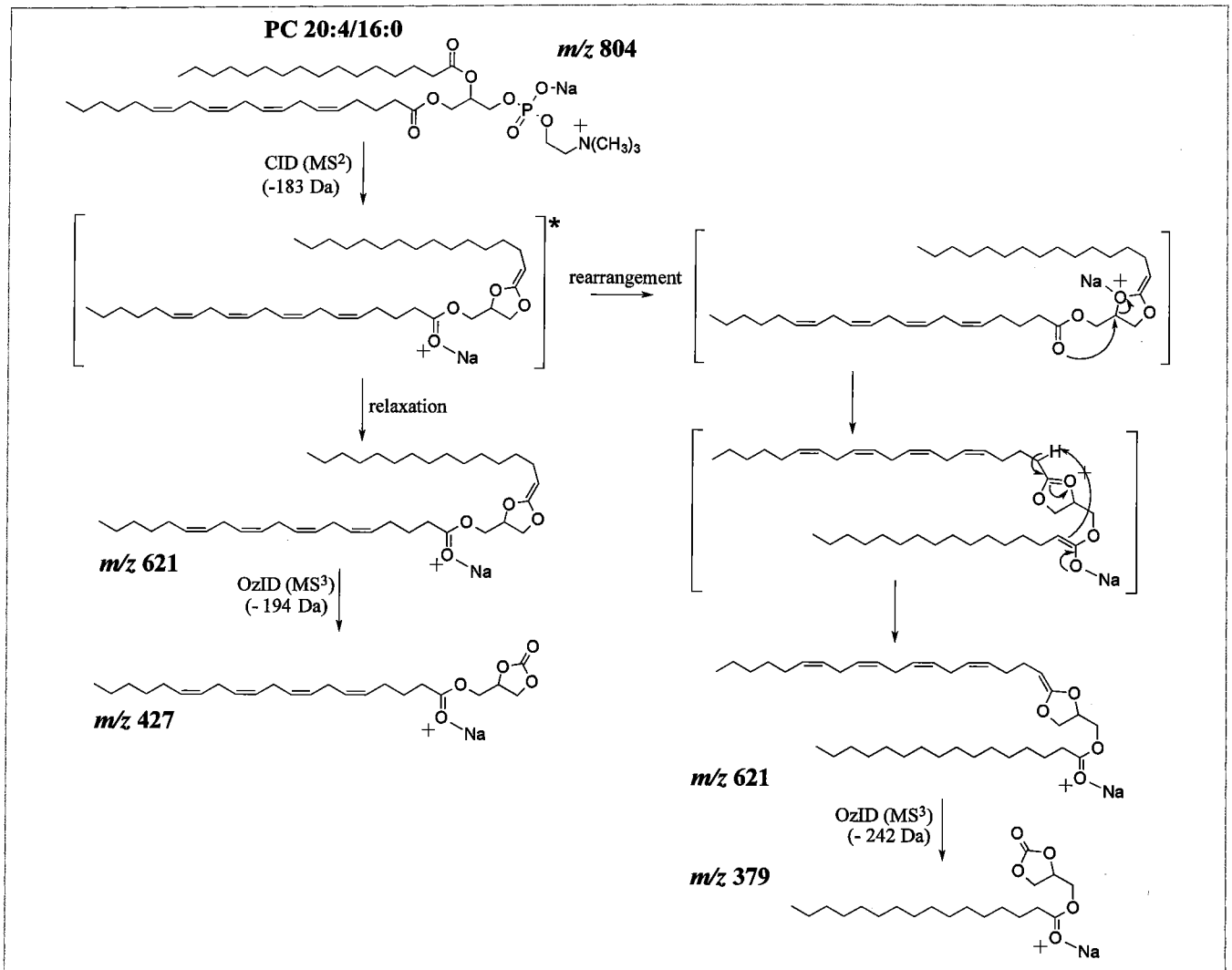


Scheme 2. Competition between five- and six-membered ring formation in the CID of the $[M + Na]^+$ ions of PC 20:4/16:0. This competition could account for the low abundance of *m/z* 379 product ions observed in the CID/OzID spectrum of this isomer.

m/z 427 and *m/z* 379 shown in green and blue, respectively, in Figure 1(d). While the *m/z* 379 trace follows exactly the chromatographic profile of the peak at 46.4 min, the *m/z* 427 trace shows features arising from both isomers. A similar—although less pronounced—phenomenon is observed for the extracted ion chromatograms shown in Figure 2(d), where the *m/z* 379 signal is exclusive to the peak at 42.9 min, while the *m/z* 451 signal is dominant for the peak at 41.3 min, but also has some abundance for the feature at 42.9 min. This is better illustrated in the chromatograms provided as supporting information (Figure S1) where a higher concentration of the lipid was loaded onto the column. Taken together, these observations provide new insight into the possible mechanisms of ion dissociation for the different *sn*-positional isomers. That is, upon CID the $[(PC\ 16:0/20:4) + Na]^+$ and $[(PC\ 16:0/22:6) + Na]^+$ cations undergo loss of the headgroup exclusively driven by the ester of the *sn*-2 acyl chain (*cf.* Scheme 1). In contrast, the isomeric $[(PC\ 20:4/16:0) + Na]^+$ and $[(PC\ 22:6/16:0) + Na]^+$ cations may exhibit competition between nucleophilic attack from the *sn*-2 and *sn*-1 acyl chains resulting in isomeric 5- and 6-membered dioxalane structures (Scheme 2, pathways a and b, respectively). Alternatively, a small proportion of energetic $[M + Na - 183]^+$ ions may isomerize *via* a transacylation pathway such as that shown in Scheme 3. Differentiating the mechanistic possibilities in Schemes 2 and 3 is not possible based on the available data; however, these results suggest that the mechanism of dissociation can be influenced by the relative *position* and *identity* of the acyl chains. These experimental observations are consistent with recent computational studies of the unimolecular dissociation of triacylglycerols where both the relative energetics and entropy were found to play a role.[27]

Conclusions

Consistent with prior work, the separation of *sn*-positional isomers of PCs can be achieved using reversed-phase HPLC, provided that the acyl chains are of sufficiently different lengths and degrees of unsaturation. In general, the isomer with the unsaturated chain occupying the *sn*-1 (*e.g.*, PC 20:4/16:0) position elutes first, while the isomer with the unsaturated



Scheme 3. Possible isomerization of energetic [M + Na – 183]$^+$ ions via a unimolecular trans-acylation pathway following CID of [PC 20:4/16:0 + Na]$^+$. This isomerization may account for the low abundance of *m/z* 379 product ions observed in the CID/OzID spectrum of this isomer

chain at the *sn*-2 position is more strongly retained on the reversed-phase column (*e.g.*, PC 16:0/20:4). The novel CID/OzID ion-activation approach yields tandem mass spectra with abundant product ions diagnostic of the relative position of the acyl chains on the glycerol backbone. The data presented indicate that CID/OzID spectra can be readily obtained on the chromatographic timescale and enable the simple identification of the isomers and thus unequivocal assignment of chromatographic features. In the future, these protocols could be programmed into data-dependent workflows with consecutive CID and CID/OzID events triggered on abundant lipid ions as they exit the column and enter the mass spectrometer.

The product ions obtained in the LC-CID/OzID mass spectra are generally of higher abundance and more selective (although not exclusive) than conventional CID mass spectral signatures used for this purpose.[5] This is illustrated by the fact that extracted ion chromatograms can be obtained from these diagnostic ions, which can assist in resolving isomers that are not completely separated on LC [*cf.* Figure 3(d)]. While it is tempting to suggest that these extracted ion chromatograms could provide a convenient means to quantify the relative proportions of *sn*-positional isomers, we note that the reaction efficiencies of the different isomeric forms can be substantially different; compare, for example, the peak abundances in Figure 1(a) with the extracted ion chromatograms in Figure 1(d). Future work will endeavor to better understand and calibrate these response factors to enable relative quantification in complex mixtures.

Finally, undertaking CID/OzID analysis of chromatographically resolved *sn*-positional isomers has provided new insight into the differences in dissociation behavior of ionized phospholipids. Our prior work had implied that the CID of [PC + Na]$^+$ was largely independent of the acyl chain identity.[21] These new data, however, suggest that the nature of the acyl chains, as well as their position on the glycerol backbone, can affect the mechanisms of dissociation and thus, potentially, the structure and branching ratio of the product ions.

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Supporting Information

Additional chromatographic and mass spectral information for PC 38:6 are provided in the online version at http://dx.doi.org/10.1255/ejms.1300.

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