Published in final edited form as:

J Am Chem Soc. 2017 November 08; 139(44): 15681–15690. doi:10.1021/jacs.7b06416.

Pinpointing double bond and *sn*-positions in glycerophospholipids via hybrid 193 nm ultraviolet photodissociation (UVPD) mass spectrometry

Peggy E. Williams, Dustin R. Klein, Sylvester M. Greer, and Jennifer S. Brodbelt*

Department of Chemistry, University of Texas at Austin, Austin, Texas 78712, United States

Abstract

Complete structural characterization of complex lipids, such as glycerophospholipids, by tandem mass spectrometry (MS/MS) continues to present a major challenge. Conventional activation methods do not generate fragmentation patterns that permit the simultaneous discernment of isomers which differ in both the positions of acyl chains on the glycerol backbone and the double bonds within the acyl chains. Herein we describe a hybrid collisional activation/UVPD workflow that yields near-complete structural information for glycerophospholipids. This hybrid MS³ strategy affords the lipid's sum composition based on the accurate mass measured for the intact lipid as well as highly specific diagnostic product ions that reveal both the acyl chain assignment (i.e. *sn*-position) and the site-specific location of double bonds in the acyl chains. This approach is demonstrated to differentiate *sn*-positional and double bond positional isomers, such as the regioisomeric phosphatidylcholines PC 16:0/18:1(*n*-9) and PC 18:1(*n*-9)/16:0 and has been integrated into an LC-MS³ workflow.

Graphical abstract

*Corresponding Author: jbrodbelt@cm.utexas.edu.

Supporting Information. Tables showing the names, structures, and masses of all standard glycerophospholipids, as well as masses of HCD/UVPD product ions and diagnostic ions which afford structural definition of PCs, figures showing the CID and HCD spectra of sodium-cationized PG 16:0/18 and PI 16:0/18:1 and the full mass spectra of bovine liver, porcine brain and *E. coli* lipid extracts, and schemes showing possible structures arising from HCD/UVPD of 16:0/18:1 glycerophospholipids and PC 18:0/18:2. This material is available free of charge via the Internet at <http://pubs.acs.org>.

ORCID

Jennifer Brodbelt: 000-0003-3207-0217

Peggy Williams: 0000-0002-9914-3276

INTRODUCTION

Glycerophospholipids are a structurally diverse lipid class that play assorted physiological roles in cellular function, serving as both intra- and intercellular signalling molecules as well as influencing the structure and function of cellular membranes and membrane proteins.^{1–3} The pathophysiology of diseases such as cancer, diabetes, obesity, neurodegeneration and cardiovascular disease have all been associated with aberrant lipid metabolism.^{4–8} In addition, bacteria have been found to selectively modify their membrane lipid composition in response to a particular environmental stimulus.⁹ Lipid remodelling may play a role in dictating the permeability and selectivity of the outer membrane, hence mediating antimicrobial resistance as the membrane lipid bilayer is the first-line of defense against the penetration of antibiotics.^{10–11} When reflecting on the implications of alterations to lipid structure on cellular behavior and function, the impact of achieving detailed molecular level characterization of glycerophospholipids is significant.

Tandem mass spectrometry (MS/MS) has driven great advancements in lipid analysis, such as in shotgun lipidomics, affording characterization of glycerophospholipid structures.^{12–14} Increased resolving power, sensitivity, and speed of modern mass spectrometers have enabled routine, high throughput identification of many hundreds of lipids, providing accurate quantification and insights into the specific functions of the lipid species.^{12, 15–20} Accurate mass and or MS/MS measurement identify a number of structural features of glycerophospholipids: (i) the lipid class, for which the headgroup identity is determined (e.g. phosphatidylcholine (PC)); (ii) the lipid sub-class, meaning the number of carbons and double bonds within the fatty acyl chains, (e.g. PC 34:1); (iii) the lipid sum composition, where the specific type of linkage of the variable components to the lipid backbone is determined, such as O-alkyl vs O-alkenyl ether linkages (e.g. PC O-34:1 and PC P-34:1); and (iv) the molecular lipid, where the identities of individual variable component acyl, alkyl, or alkenyl chains are determined with or without definition of their specific stereospecific numbering (*sn*)-position (e.g. PC 16:0/18:1 and PC 16:0_18:1).^{21–22}

Discerning structural isomers remains challenging, especially in the context of complex lipids in biological mixtures, where the position(s) of the carbon-carbon double bonds in the acyl chains as well as stereochemistry about the double bond are critical features. Isomeric glycerophospholipids are commonplace in biological mixtures, and alterations in their molecular structures, while subtle, impart consequences on their biological function.^{9, 23–25} Many lipidomic studies currently leverage the sensitivity and specificity of LC-MS and LC-MS/MS measurements to resolve isomeric lipids, such as those that differ by only one *sn*-1/*sn*-2 fatty acid position,^{26–27} the positions of the double bonds,^{28–29} or have cis/trans alkenes.³⁰ Coupling LC with ion-mobility has shown potential for facilitating multi-dimensional LC-IMS-MS measurements, permitting isomeric lipids within complex biological extract to be resolved.³¹ Despite these significant advances in lipid isomer separation and analysis, these techniques do not consistently allow isomer identification (e.g. the determination of the location of double bonds, stereochemistry about the double bonds, or *sn*-positions) without requiring analysis of an exhaustive number of standards which may not be commercially or synthetically available.

To address the problems associated with identifying and characterizing lipid isomers, a number of new approaches have been reported.^{32–33} Promising new methods to elucidate double bond position(s) include Paternò-Büchi (PB) reactions,^{34–36} radical-directed dissociation (RDD) via 266 nm photodissociation followed by CID,³⁷ metastable atom activated dissociation (MAD),³⁸ charge transfer dissociation (CTD)³⁹ and charge reversal and charge-switch derivatization.^{40–41} Ozone-induced dissociation (OzID),^{42–43} which utilizes ion-molecule reactions between ionized, unsaturated lipids and neutral ozone within a mass spectrometer, is a clever strategy to assign double bond position, but reaction times of ~0.2 – 10 s are less amenable for high throughput chromatographic application. High-energy CID,⁴⁴ multistage CID,⁴⁵ and electron impact excitation of ions from organics⁴⁶ (EIEIO, an electron-induced dissociation method) have also been employed but have not yet been widely adopted.^{44–46} The lengthy reaction times or spectral acquisition times required by all of these techniques impedes their suitability for high throughput chromatographic workflows. This latter issue has been strategically addressed by implementation of OzID in the high-pressure ion-mobility cell of the Waters Synapt G2-Si platform,⁴⁷ significantly decreasing the residence time required for oxidative cleavage of the double bonds and providing greater compatibility with LC methods. With the exception of high-energy CID and EIEIO, none of the aforementioned methods are capable of simultaneously identifying both double bond positional and *sn*-positional isomers in a single spectrum. While high-energy CID and EIEIO can be used to identify both the *sn*-position and double bond position, like all untargeted fragmentation processes the efficiency of producing the diagnostic fragment ions is low, the product ion spectra produced can be quite complicated, and the ability to identify low abundance *sn*- and double bond positional isomers in complex biological extracts is likely to be poor.^{44, 46} Conversely, combinations of collision-induced and ozone-induced dissociation (CID/OzID) permit the identification of *sn*-positional isomers, but double bond positional isomers cannot unambiguously be identified without performing multiple stages of CID and OzID (e.g. CID/(OzID)² and (CID/OzID)²).⁴⁸

Another activation method that has demonstrated success for characterization of lipids (as well as other biological molecules like peptides and intact proteins) is ultraviolet photodissociation (UVPD).^{49–51} We have recently demonstrated the use of 193 nm UVPD for identification of the position(s) of double bonds in the acyl chains within PCs.⁵² Photo-induced cleavage of the carbon-carbon bonds adjacent to the double bond afforded a diagnostic mass difference of 24 Da enabling differentiation of double bond positional PC isomers, although *sn*-positional isomers could not be distinguished.⁵² Here we present a targeted top-down approach combining higher-energy collisional dissociation (HCD) and 193 nm UVPD in a hybrid MS³ method. This strategy produces abundant diagnostic fragment ions that permit the differentiation of *sn*-positional glycerophospholipid isomers. At the same time, pairs of fragment ions exhibiting a 24 Da mass difference permit the simultaneous characterization of both double bond and *sn*-positional isomers.

EXPERIMENTAL SECTION

Materials

All glycerophospholipids as well as bovine liver polar, bovine heart polar, porcine brain, and *E. coli* total lipid extracts were purchased from Avanti Polar Lipids (Alabaster, Alabama) and used as received without further purification. The structures and molecular weights of all glycerophospholipids included in this study are shown in Table S1. HPLC grade methanol was purchased from EMD Millipore (Billerica, MA). Butylated hydroxytoluene (BHT) and sodium acetate were purchased from Sigma Aldrich (St. Louis, MO). For direct infusion experiments, all samples were diluted in methanol containing 0.01% BHT and 50 μM sodium acetate to 10 μM for individual glycerophospholipids and 12.5 $\mu\text{g/mL}$ for biological extracts. The presence of 50 μM sodium acetate aided in the increased formation of sodium adducts. For LC-MS³ analyses, lipid standards were diluted to an individual lipid concentration of 7.6 ng/ μL in 90:10 acetonitrile/water containing 0.01% BHT.

Mass Spectrometry

All experiments were performed on a Thermo Scientific Instruments Fusion Lumos Orbitrap mass spectrometer (San Jose, CA) that was equipped with a nanospray ion source fitted for off-line static spray and modified to perform UVPD with a 193 nm Coherent Excistar excimer laser (Santa Cruz, CA) as described previously.⁵³ Approximately 10 μL of each sample solution was loaded into a silver-coated pulled tip capillary and individually infused into the mass spectrometer. The lipids were ionized via static nanospray using a spray voltage of 1.4 kV and an ion transfer tube temperature of 275°C. CID spectra were typically acquired with a normalized collision energy of 30-35 (arbitrary units). HCD spectra were typically acquired with a normalized collision energy of 20-28 (arbitrary units). Combinations of CID, HCD and UVPD were performed sequentially by first isolating the desired $[\text{M} + \text{Na}]^+$ species in the quadrupole with an isolation width of 3 m/z units for individual glycerophospholipids and 1.5 m/z for species from the extracts. The quadrupole isolated parent ions were then transferred to the high pressure ion trap for CID or the ion routing multipole for HCD. The headgroup loss fragment ions generated from CID or HCD were subsequently isolated in the high pressure ion trap with an isolation width of 3 m/z , then transferred to the low-pressure ion trap and activated via 193 nm UVPD with 10 laser pulses (4 mJ per pulse). All HCD/UVPD spectra were acquired with an AGC target between 5×10^4 and 1×10^5 at a resolution of 120k. All spectra reported herein as well as in the supporting information are individually normalized, rather than normalized to the same intensity. While 60 scans were acquired and averaged for each mass spectrum (requiring approximately 30 seconds), as few as 8 scans were required to obtain satisfactory signal-to-noise ratios. Both of these averaging options are compatible with chromatographic separations. $[\text{M} + \text{Na}]^+$ ions were selected for this workflow as they produce abundant fragment ions from neutral headgroup loss upon CID and HCD.⁵⁴ The product ions are easily activated and dissociated via 193 nm UVPD in a subsequent activation step.⁵⁵ Conversely, CID and HCD of the $[\text{M} + \text{H}]^+$ ions afforded little to none of these headgroup loss fragment ions.

Data-dependent LC-MS³ Analysis

All LC-MS³ analyses were performed on a Dionex Ultimate 3000 LC system (Thermo Scientific, San Jose, CA) that was configured to deliver high flow rates from the loading pump and coupled to an Orbitrap Fusion Lumos mass spectrometer that was modified to perform UVPD and equipped with a heated ESI source. A mixture of lipid standards (10 μ L) were injected onto an Acclaim C₃₀-column (2.1 $\times$ 150 mm, 3 μ m particle size, Thermo Scientific, San Jose, CA) held at 30 °C and separated using a previously reported⁵⁶ reversed phase LC method capable of separating *sn*-positional phosphatidylcholine isomers. The lipid standards used to optimize and evaluate the LC-MS³ method are reported in Table S2. The lipid mixture was separated over a 31 minute step gradient elution with a 500 μ L/min flow rate (mobile phase A: 90:10 acetonitrile/water containing 10 mM ammonium acetate; mobile phase B: 47.5:45:2.5:5 acetonitrile/methanol/isopropanol/water containing 10 mM ammonium acetate) that began 6 minutes after injection. The gradient ramped from 100% A to 0% A over 6 minutes, was held at 0% A for 25 minutes, and then returned to initial conditions over 1 minute followed by 7 minutes of re-equilibration. Sodium acetate (1 mM) was infused into the flow path post-column using a tee-union at a flow rate of 8 μ L/min to facilitate sodium adduct formation. Heated ESI source conditions consisted of a 4.5 kV spray voltage, sheath gas of 50 (arbitrary units), auxiliary gas of 5 (arbitrary units), ion transfer tube temperature of 300 °C, and vaporizer temperature of 200 °C.

A data-dependent MS³ neutral loss method consisted of a high resolution MS1 scan (60,000 resolution, 5×10^5 AGC target, 50 ms maximum injection time, 1 μ scan/scan, 2×10^4 intensity threshold, profile mode), followed by an MS² HCD event on the top 10 most abundant ions (quadrupole isolation, isolation width = 3, NCE = 25%, 60,000 resolution, 5×10^4 AGC target, 100 ms maximum injection time, 1 μ scan/scan, 2×10^4 intensity threshold, profile mode), and finally an MS³ UVPD event targeting neutral headgroup losses specific for each phospholipid (isolation width = 3, no isolation offset, 20 ms UVPD activation time [corresponding to 10 laser pulses], 60,000 resolution, 5×10^5 AGC target, 200 ms maximum injection time, 1 μ scan/scan, profile mode, ± 0.5 *m/z* targeted inclusion loss, ± 2 *m/z* precursor ion exclusion).

Nomenclature—Lipid structure nomenclature is based on the recommendations of Liebisch *et al.*²¹ The traditional nomenclature “*n* – *x*” is used to indicate the site(s) of unsaturation is *x*-positions from the methyl end, where “*n*” corresponds to the number of carbon atoms in the acyl chain. For example, PC 18:0/18:2 (9, 12) is represented as PC 18:0/18:2 (*n*-6, *n*-9). As the stereochemical configuration of carbon-carbon double bonds was not determined, it is not indicated here.

RESULTS AND DISCUSSION

HCD/UVPD analysis of isomeric PCs

The fragmentation patterns produced upon collisional activation of isomeric PCs have been reported previously.⁵⁷ One representative set of CID and HCD spectra for a pair of sodium-cationized *sn*-positional PC regioisomers of *m/z* 782.5 (PC 16:0/18:1(*n*-9) and PC 18:1(*n*-9)/16:0) is shown in Figure S1. The three major product ions of *m/z* 723.5, 599.5 and 577.5

correspond to the neutral loss of trimethylamine (59 Da), the complete phosphocholine headgroup (183 Da), and the sodium-cationized phosphocholine headgroup (205 Da), respectively. Two minor product ions of m/z 526.3 and 500.3 are attributed to losses of 16:0 and 18:1 fatty acids from the precursor ion, respectively. The relative abundances of these ions have been used as an indication of regioisomerism for some time; however, the abundances are heavily dependent upon the headgroup structure^{19, 58} as well as differences in both acyl chain length and the degree of unsaturation,⁵⁹ rendering these ions inadequate for accurate or absolute quantification of regioisomers, especially when isomers are present. Hence, alternative approaches to identify and quantify glycerophospholipid regioisomers that provide abundant product ions largely independent of other structural features are desirable. Previous mechanistic studies suggest that the loss of the complete phosphocholine headgroup occurs first through elimination of trimethylamine,⁵⁵ followed by the elimination of dioxaphospholane to generate a dioxolane-type product containing a newly formed double bond connecting the *sn*-2 fatty acyl chain (Scheme 1, path (b)).⁵⁵ The newly formed double bond in this latter product affords an attractive target for 193 nm UVPD owing to the ability of UVPD to identify the position(s) of double bonds in acyl chains within PCs,⁵² thus potentially facilitating *sn*-position assignment of the acyl chains via a hybrid MS³ strategy. Alternatively, an analogous fragmentation pathway occurring via a six-membered ring intermediate (Scheme 1, path (a)) is also plausible.⁵⁵

HCD afforded more abundant complete headgroup loss for the PCs examined (Figure S1). In an MS³ experiment, the headgroup loss product ions (m/z 599) generated upon HCD were isolated and subjected to 193 nm UV irradiation. The resulting HCD/UVPD (MS³) spectra obtained from two *sn*-regioisomeric PCs, PC 16:0/18:1(*n*-9) and PC 18:1(*n*-9)/16:0, are shown in Figure 1. Importantly, the HCD/UVPD spectra display a significant difference in the major product ions depending upon the *sn*-position of the acyl chains.

For example, product ions of m/z 303.2, 319.3, and 335.3 predominate in the HCD/UVPD spectrum of sodium-cationized PC 16:0/18:1(*n*-9) (Figure 1a), whereas product ions of m/z 277.2, 345.3, and 361.3 are dominant in the HCD/UVPD spectrum of PC 18:1(*n*-9)/16:0 (Figure 1b). Structures of product ions are proposed in Scheme S1 to help guide readers. The major product ion of m/z 319.3 in Figure 1A is assigned as a sodium-cationized 16:0 fatty acid allyl ester arising from photolytic cleavage across the dioxolane moiety. This diagnostic ion suggests that the 16:0 acyl chain is located in the *sn*-1 position of the glycerol backbone. The minor product ion of m/z 303.2 in Figure 1A originates from this same photolytic cleavage with the sodium retained on the opposite side of the cleavage site (Scheme S1), meaning that the ions of m/z 303.2 and 319.3 are a complementary pair. The product ion of m/z 335.3 is generated through a different photolytic cross-ring cleavage across the dioxolane moiety and maps the 18:1 fatty acid in the *sn*-2 position as illustrated in Figure 1A. Similarly, the major m/z 345.3 product ion in Figure 1B is assigned as a sodium-cationized 18:1 fatty acid allyl ester, suggesting that the 18:1 acyl chain is in the *sn*-1 position of the glycerol backbone.

Other HCD/UVPD product ions present in low abundance, such as m/z 345 and m/z 277 in Figure 1A and m/z 319 in Figure 1B, may arise from either (i) the *sn*-isomeric impurity in each PC standard formed *via* the five-membered ring pathway upon HCD (Scheme 1, path

(b)), as similarly evidenced in the CID and HCD spectra (Figure S1), or (ii) the main *sn*-isomer in each PC standard formed *via* the six-membered ring pathway upon HCD (Scheme 1, path (a)). Possible fragmentation pathways for UVPD of the five-membered dioxolane and six-membered dioxane ring structures for both PC 16:0/18:1 and PC 18:1/16:0 and their resulting masses are shown in Scheme S2. In order to discern whether these products originate from the *sn*-isomeric impurity or from a six-membered dioxane ring structure formed upon HCD of the main *sn*-isomer, CID/UVPD analysis of the sodium adduct of a regiopure triacylglycerol standard, TG 18:1/16:0/18:1 (structure shown in Scheme S3), was performed. CID of TG 18:1/16:0/18:1 affords an analogous product ion of m/z 599 to that of PC 18:1/16:0. In the corresponding CID/UVPD spectrum of this regiopure standard, only diagnostic product ions of m/z 361.27, 345.28 and 277.21 were observed (Figure S2). No product ions of m/z 319.26 that would arise from the six-membered dioxane ring structure were observed (*cf.* Scheme 1, path (a)), demonstrating that the five-membered substitution mechanism shown in Scheme 1(b) occurs exclusively (Scheme S3).

The ratios of the diagnostic ions generated by HCD/UVPD are consistent with the relative abundances of the two *sn*-regioisomers determined from other MS/MS approaches, suggesting that the relative abundance of the diagnostic ions for *sn*-position afforded by HCD/UVPD can be used to quantify the regioisomers. To test this concept, we analysed mixtures of the synthetic PC 16:0/18:1 and PC 18:1/16:0 standards prepared in different mole ratios and monitored the relative abundances of the respective m/z 319.3 and m/z 345.3 ions diagnostic for *sn*-1 position assignment. As illustrated in Figure S3, a linear correlation between the relative ratio of the diagnostic ion for *sn*-1 position assignment in PC 16:0/18:1 (m/z 319.3) and the relative molar concentration ratio of PC 16:0/18:1 in solution was found. Further, these studies suggest that the regioisomeric impurities present in PC 16:0/18:1 and PC 18:1/16:0 are 13% and 10%, respectively. These values are in excellent agreement with previous reports^{48, 60–61} of PCs containing percentages of *sn*-positional isomer impurities ranging from 10–25%.

Magnification of the m/z 415 – m/z 495 region of the HCD/UVPD spectra for the two regioisomeric PCs (Figure 1A and 1B) revealed two fragment ions (m/z 461.4 and m/z 485.4, Scheme S1) with a 24 Da mass difference. This diagnostic ion pair indicates the presence and site-specific location of acyl chain C=C double bonds, arising from acyl chain cleavage of the C-C bonds on both sides of the double bond, as recently reported for UVPD of phosphatidylcholines⁵² and sphingolipids.⁶² A second fragment ion pair (m/z 423.4 and m/z 480.4, Scheme S1) with a 57 Da mass difference were also produced. As an abundant neutral sodium loss ion is observed (m/z 576.5), this diagnostic ion pair likely arises from the loss of neutral sodium followed by cleavage of the allylic C-C bonds on both sides of the double bond, serving as a secondary pair of diagnostic ions for double bond position.

To further explore the capability of HCD/UVPD to discern double bond positional PC isomers, isomeric PC 18:1(*n*-12)/18:1(*n*-12) and PC 18:1(*n*-9)/18:1(*n*-9) were examined, and the resulting HCD/UVPD spectra are shown in Figure 2. Abundant diagnostic ions of m/z 303.2, 345.3, and 361.3 allowing assignment of the acyl chain in the *sn*-1 position of the glycerol backbone were observed for both PC isomers. Surprisingly, the ratio of the ions of m/z 303.2 and 345.3 differed significantly for the two double bond positional isomers. As

illustrated in Figure 2A, the ion of m/z 345.3 is assigned as an 18:1 fatty acid allyl ester, whereas the ion of the m/z 303.2 is the complementary product. The increased abundance of the m/z 303.2 ion is attributed to the closer proximity of the double bond to the PC headgroup prior to HCD activation and to the dioxolane moiety after headgroup loss resulting in an increased affinity for the sodium ion by the 18:1(*n*-12) fatty acid. Importantly, HCD/VPD of PC 18:1(*n*-12)/18:1(*n*-12) again afforded diagnostic ions of m/z 445.3 and m/z 469.3 with a 24 Da mass difference (Figure 2A) indicating the location of the double bond at the *n*-12 carbons in the acyl chains, whereas HCD/VPD of PC 18:1(*n*-9)/18:1(*n*-9) afforded diagnostic ions of m/z 487.4 and m/z 511.4, also with a mass difference of 24 Da, thus revealing the location of the double bond at the *n*-9 carbons in the acyl chains. No fragment ions were observed that suggested simultaneous cleavage of both double bonds. A second pair of fragment ions (m/z 449.4 and 506.4, Figure 2B) with a 57 Da mass difference arising from loss of neutral sodium and subsequent cleavage of the allylic C-C bonds on both sides of the double bond were also observed for PC 18:1(*n*-9)/18:1(*n*-9). For unknown reasons, these same allylic cleavages are not observed when the double bond is closer in proximity to the dioxolane moiety, as shown by the absence of the diagnostic ions with a 57 Da difference upon HCD/VPD of PC 18:1(*n*-12)/18:1(*n*-12). Rather, more abundant fragment ions (m/z 447.4 and m/z 433.3) corresponding to the loss of neutral sodium and subsequent vinylic or direct cleavage of the double bond are observed. Lastly, a more abundant fragment ion of m/z 393.3 is observed in both Figure 2A and 2B. This fragment ion likely arises from allylic cleavage of the C-C bond adjacent to the newly formed double bond external to the dioxolane moiety. These findings demonstrate that HCD/VPD of phosphatidylcholines can be used to discern both the *sn*-position of the acyl chains as well as the site-specific locations of the C=C double bonds in a single MS³ experiment.

Lithium and potassium are also commonly used for adduction in MS analysis of lipids. Since metal ion adduction can have a pronounced effect on the fragmentation pathways and detected fragment ions of lipids,⁵⁷ we explored the effect of metal ion adduct on HCD/VPD fragmentation. HCD/VPD spectra for lithium, sodium and potassium cationized PC 18:1(*n*-9) are shown in Figure S4. Lithium adduction afforded greater fragmentation of the fatty acyl with the lithium retained in all fragment ions as well as generation of both vinylic and allylic fragment ions diagnostic for double bond position (Figure S4A). Potassium adduction only afforded low abundance fatty acyl chain fragment ions (without potassium attached), and the only double bond positional diagnostic ions arose from allylic cleavage on either side of the double bond (Figure S4C). HCD/VPD of the sodium adduct (Figure S4B) appears to be slightly more selective than the lithium adduct as both sets of vinylic and allylic double bond positional diagnostic ions are generated, but less fragmentation near the tail of the acyl chain is observed. The use of sodium over lithium is also advantageous in optimizing sensitivity during the HCD event: over 50% of the ion population is lost through both ejection of neutral lithium and further allylic fragmentation on both sides of the double bond (Figure S5).

Effect of the degree of unsaturation on HCD/VPD analysis of PCs

To examine the extent to which the degree of unsaturation HCD/VPD of PCs, HCD/VPD experiments were conducted on PCs containing 0-6 double bonds. The resulting MS³ spectra

obtained for the sodium-cationized PCs are shown in Figure 3. The resulting HCD/UVPD spectra of sodium-cationized PC 16:0/18:0 (no double bonds), 16:0/18:2(*n*-6, *n*-9) (two double bonds), 16:0/20:4(*n*-6, *n*-9, *n*-12, *n*-15) (four double bonds), and 16:0/22:6(*n*-3, *n*-6, *n*-9, *n*-12, *n*-15, *n*-18) (six double bonds) are shown in Figure 3. The fragmentation of one of these PCs, specifically PC 16:0/18:2(*n*-6, *n*-9), is described in more detail. Similar to HCD/UVPD of isomeric PC 16:0/18:1 and PC 18:1/16:0 discussed above, HCD/UVPD of PC 16:0/18:2(*n*-6, *n*-9) affords abundant fragment ions diagnostic for identification of the acyl chain present in the *sn*-1 position, as well as lesser abundant fragment ions diagnostic for the location of the double bonds (Figure 3B). The major product ion of *m/z* 319.3 product ion in Figure 3B is a sodium-cationized 16:0 fatty acid allyl ester arising from photolytic cross-ring cleavage of the core dioxolane moiety initially formed during HCD. This diagnostic ion indicates that the 16:0 acyl chain occupies the *sn*-1 position of the glycerol backbone.

The minor *m/z* 301.2 product ion in Figure 3B arises from this same photolytic cleavage with the sodium retained on the opposite side of the cleavage site. The less abundant *m/z* 335.3 product ion originates from another photolytic cross-ring cleavage across the dioxolane moiety. The PC 16:0/18:2(*n*-6, *n*-9) appears to be more regio-pure compared to PC 16:0/18:1 as the major diagnostic ion of *m/z* 343.3, which would be indicative of an 18:2 acyl chain in the *sn*-1 position, was observed in significantly less abundance than the corresponding diagnostic ion for the 18:1 acyl chain in the *sn*-1 position of PC 16:0/18:1, and the other minor diagnostic ions for *sn*-position for PC 18:2/16:0 (*m/z* 277.2 and 359.3) were not observed.

Increased magnification of the *m/z* 450 – *m/z* 530 region of the HCD/UVPD spectrum in Figure 3B revealed several sets of diagnostic fragment ions (*m/z* 459.4 and 461.4; *m/z* 485.4, *m/z* 486.4, 487.4; *m/z* 499.4, 500.4, 501.4; and *m/z* 525.4) that identify the locations of the double bonds in the 18:2 acyl chain. These diagnostic sets of ions are thought to arise from photo-induced excitation of the C=C double bonds in the 18:2 acyl chain to a diradical state, followed by allylic and vinylic cleavages on either side of the double bonds, as illustrated schematically in the structure shown in the right-hand side of the MS³ spectrum. Possible structures of product ions arising from cleavage about the acyl chain double bonds upon HCD/UVPD of PC 16:0/18:2(*n*-6, *n*-9) are shown in Scheme S4. We speculate that the abundances of this series of diagnostic ions may reflect in part the stabilities of the resulting products (i.e. formation of vinylic versus allylic species, concomitant migration of hydrogen atoms, and generation of radical versus non-radical species). All diagnostic ions generated by HCD/UVPD of PC 16:0/18:2(*n*-6, *n*-9) shown in Figure 3B appear to be formed in greater abundance than those generated by HCD/UVPD of isomeric PC 16:0/18:1 and PC 18:1/16:0 (Figure 1), indicating that the degree of unsaturation within the phosphatidylcholine molecules affects the efficiency of UVPD of the headgroup loss ion formed from HCD.

Interestingly, the major product ion (*m/z* 319.3) diagnostic for the 16:0 acyl chain in the *sn*-1 position of the glycerol backbone increase from 20% relative abundance for PC 16:0/18:0 (Figure 3A), to 40% relative abundance for PC 16:0/18:2 (Figure 3B), to 80% relative abundance for PC 16:0/20:4 (Figure 3C), and finally to 100% relative abundance for PC 16:0/22:5 (Figure 3D). The abundances of the key ions representative of site-specific double

bond position also increase significantly as the number of double bonds in the PC increase. These results suggest that the photoabsorption cross-section of the PC increases with the number of double bonds. As the number of double bonds is also preserved in regioisomeric impurities (e.g., PC 16:0/18:0 vs PC 18:0/16:0), the abundances of the fragment ions can be used to estimate the proportions of each regioisomeric contribution to a particular PC. Owing to the changes in fragment ion abundance as a function of the degree of unsaturation, this method cannot be employed in a shotgun manner for broad quantitative analysis of lipids containing different numbers of double bonds. However, these preliminary results suggest that this could serve as a sensitive method for the detection of both *sn*- and double bond-positional isomers while also revealing the distribution of each regioisomer.

CID/UVPD and HCD/UVPD analysis of other classes of glycerophospholipids

To explore the generality of the hybrid MS³ strategy, HCD/UVPD and CID/UVPD experiments were conducted on other subclasses of glycerophospholipids, including phosphatidylethanolamine (PE), phosphatidic acid (PA), phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidylglycerol (PG), containing 16:0 and 18:1 fatty acyl chains in the *sn*-1 and *sn*-2 position, respectively. Both HCD and CID were explored as the first stage of the MS³ strategy in order to maximize the abundance of the key headgroup loss species prior to UVPD. Sodium-cationized PE 16:0/18:1, PA 16:0/18:1 and PS 16:0/18:1 generated more abundant headgroup loss ions upon HCD than upon CID (Figure S6), whereas sodium-cationized PI 16:0/18:1 and PG 16:0/18:1 produced more abundant headgroup loss ions upon CID. Hence, the collisional activation method that generated the most abundant diagnostic headgroup loss products was employed for the MS³ strategy for each subclass of glycerophospholipid. Examples of HCD/UVPD and CID/UVPD spectra for sodium-cationized PE, PA, PS, PI and PG 16:0/18:1 are shown in Figure 4. In each case, the first stage of collisional activation results in production of the headgroup loss ions of *m/z* 599.5, the species selected for UVPD in the second stage of activation. Since the acyl chain compositions (location and number of double bonds, and length of chains) of each of these five glycerophospholipids is the same, the HCD/UVPD and CID/UVPD spectra reveal identical product ions to those observed for PC 16:0/18:1 (Figure 1A). UVPD of the selected headgroup loss species results in product ions diagnostic for the *sn*-1 acyl chain position (*m/z* 303.2, 319.3, and 335.3) (Figure 4). Less abundant product ions of *m/z* 277.2 and *m/z* 345.3 reveal the *sn*-1 acyl chain position of regioisomeric impurity ions present in each of the five glycerophospholipids. Magnification of the *m/z* 450 – 500 range highlights two ions (*m/z* 461.4 and *m/z* 485.4 with the characteristic mass difference of 24 Da) diagnostic for the location of the *n*-9 double bond.

The anionic and weakly acidic nature of many phospholipid subclasses, such as PE, PA, PS, PG and PI, renders them more efficiently ionized in the negative-ion mode. Further, the ionization efficiencies of these phospholipid subclasses in the positive-ion mode can be significantly suppressed in the presence of PCs within a complex mixture, even after sodium adduction. To address these potential concerns, the utility of the hybrid MS³ strategy in the negative-ion mode was explored. The [M-H]⁻ ion of PG 16:0/18:1 (*m/z* 747.5) was isolated and subjected to HCD, affording abundant product ions of *m/z* 255.2 and 281.2 which correspond to carboxylate anions of the 16:0 and 18:1 fatty acyl groups, respectively. The

carboxylate anion of the 18:1 fatty acyl group (m/z 281.1) was subsequently isolated and irradiated with 10 pulses of 193 nm photons at an energy of 6 mJ per pulse (Figure S7). While up to 1000X magnification is required, diagnostic ions with the characteristic mass difference of 24 Da (m/z 143.1 and 167.1) are observed, indicating a double bond in the n -9 position. The spectrum shown in Figure S7 is composed of 60 scan averages, however, as few as 6 scan averages are needed for sufficient signal to noise. These results confirm that the broad applicability of the hybrid MS³ strategy for structural identification of sn - and double bond-positional isomers and is independent of both the glycerophospholipid subclass headgroup and fatty acid composition. In essence, this method allows facile identification of sn -regioisomerism and double bond isomerism in a single experiment, a significant advantage over existing approaches to identify sn -regioisomers and double bond-positional isomers.

Integrating HCD/UVPD with high-performance liquid chromatography

The “shotgun” HCD/UVPD method was coupled with LC to enhance the lipid structure elucidation capabilities and demonstration that the workflow is amenable to an LC time scale. The base peak chromatogram of a mixture of synthetic phospholipid standards is shown in Figure S8. The phospholipids in the mixture as well as the masses of the sodium adducted precursor ions and retention times of each lipid are listed in Table S2. Chromatographic separation of sn -positional isomers becomes more challenging as the level of unsaturation and differences in lengths of the acyl chains decreases.⁵⁶ To test if HCD/UVPD coupled with LC can discern such challenging isomers, two PC standards (PC 16:0/18:1(n -9) and PC 18:1(n -9)/16:0) were constituted in a 1:1 molar mixture. The base peak chromatogram (BPC) for ions of m/z 782 (Figure 5A), corresponding to the sodium adduct of PC 34:1, reveals a single broad peak (t_r 20 min) with no resolution between the two isomers. However, when the HCD/UVPD mass spectra are averaged across data points from an early eluting portion of the chromatographic peak (RT 19.7-19.9) an abundant ion of m/z 345.3 consistent with the 18:1 acyl chain occupying the sn -1 position is observed, along with a less abundant ion of m/z 319.3 arising from the other partially co-eluting sn -isomer (Figure 5C).

When the HCD/UVPD mass spectra are averaged across data points from a later eluting portion of the chromatographic peak (RT 20.2 – 20.4), an abundant ion of m/z 319.3 consistent with the 16:0 acyl chain occupying the sn -1 position is observed, with a less abundant ion of m/z 345.3 arising from the other co-eluting sn -isomer (Figure 5D). These findings indicate that the two isomer populations are partially separated with PC 18:1(n -9)/16:0 eluting slightly earlier than PC 16:0/18:1(n -9). While the base peak chromatogram shows no resolution between these two isomer populations, the isomeric resolution is evident in the overlayed extracted ion chromatograms (XICs) for the ions of m/z 345.3 and 319.3 (Figure 5B). Importantly, the LC-HCD/UVPD workflow also permits the discernment of double bond position as less abundant ions of m/z 461.4 and 485.4 diagnostic for a double bond in the n -9 position are observed (Figures 5C and 5D). These data demonstrate the successful deployment of HCD/UVPD on an LC time-scale.

To further validate this LC-HCD/UVPD method, the limit of detection for the PC 16:0/18:1(*n*-9) standard was determined. Varying amounts of the PC 16:0/18:1(*n*-9), ranging from 0.5 to 100 pmol, were injected onto the LC column. Calibration curves relating the absolute intensities of diagnostic ions relative to the amounts of analyte injected were constructed using linear regression analysis over the range of 0.5 to 100 pmol for ions diagnostic of the *sn*-position (Figure S11A) and double bond position (Figure S11B). The limit of detection (S/N 3) for *sn*-position assignment was found to be 5 pmol, whereas the limit of detection for double bond position assignment was found to be 25 pmol. The detection limits for other phospholipids will vary depending on the head group attached to the phospholipid (a factor that affects ionization efficiency as well as influencing the loss of the head group during MS/MS) and the number of double bonds present in the molecule (which is expected to influence UV photoabsorption). For example, the absolute detection limit for the *sn*-positional isomer for PC 16:0/18:0 is expected to be different than that of PE 16:0/18:1 or PC 16:0/20:4. These differences occur because the HCD/UVPD product ion abundances are dependent upon the ionization efficiency of the glycerophospholipid as well as the degree of unsaturation.

Application of HCD/UVPD to identify *sn*- and double bond positional regioisomers in biological extracts

Based on the consistent production of diagnostic ions in the HCD/UVPD spectra for each glycerophospholipid, we applied this method to discern both *sn*-positional and double bond positional isomers for lipids found in biological extracts. First, the method was used to characterize several glycerophospholipids in bovine liver and porcine brain extracts. The MS¹ spectra obtained from these extracts via a direct infusion shotgun approach are shown in Figure S9. The HCD/UVPD spectra for two phosphocholine species shown in Figures 6A and 6B reveal the same two sets of diagnostic product ions (*m/z* 303.2, 319.3, 335.3 and *m/z* 277.2, 345.3, 361.3) previously observed for two regioisomeric PCs described earlier, 16:0/18:1(*n*-9) and 18:1(*n*-9)/16:0 (see Figure 1). The presence of both sets of diagnostic ions in the HCD/UVPD spectra from the liver and brain extracts suggests that both *sn*-positional isomers are present in these samples. Magnification of the *m/z* 440 – 500 region of the HCD/UVPD spectrum for the PC 16:0/18:1 and 18:1/16:0 species isolated from the bovine liver extract (Figure 6A) also reveals diagnostic ions corresponding to an *n*-9 double bond (*m/z* 461.4 and *m/z* 485.4). In contrast, the *m/z* 440 – 550 region of the spectrum generated from the porcine brain extract (Figure 6B) exhibits two sets of diagnostic ions: one pair (*m/z* 461.4 and 485.4) which correspond to an *n*-9 double bond, and a second set (*m/z* 489.4, 491.4 and 513.4) which are diagnostic of an *n*-7 double bond. These data suggest that the PC 16:0/18:1 and PC 18:1/16:0 species present in the porcine brain lipid extract are comprised of four isomers, PC 16:0/18:1(*n*-9), PC 18:1(*n*-9)/16:0, PC 16:0/18:1(*n*-7), and PC 18:1(*n*-7)/16:0, and that this protocol can efficiently discern double bond positional isomers of lipids in a biological matrix.

To demonstrate the robustness of this hybrid HCD/UVPD strategy, the PEs present in an *E. coli* lipid extract and PCs present in the bovine liver lipid extract were analysed in a shotgun approach. The MS¹ spectrum obtained for the *E. coli* lipid extract is shown in Figure S10. Specific sodium-cationized PE and PC ions were selected and isolated for subsequent

MS/MS analysis. HCD of the selected precursor ions afforded the diagnostic neutral headgroup loss of 141 Da for PEs and 183 Da for PCs. The headgroup loss ions were subsequently isolated and exposed to 10 laser pulses (4 mJ per pulse). Table 1 summarizes 21 PEs identified in the *E. coli*/lipid extract with localized *sn*- and double bond positions and the corresponding diagnostic fragment ions. Table S3 contains a list of 20 PCs identified in the bovine liver extract. While this hybrid HCD/UVPD shotgun method can definitively localize the *sn*-position of fatty acids in low abundance phospholipids, the key diagnostic ions which can localize the double bond positions diminish significantly with decreasing precursor ion abundance.

CONCLUSIONS

The results presented herein demonstrate that the hybrid MS³ strategy which combines collisional activation with UVPD on selected headgroup loss product ions allows unequivocal assignment of the relative position of acyl chains on the glycerol backbone as well as site-specific localization of double bonds. This method successfully maps both *sn*-positional and double bond positional isomers for all six common classes of glycerophospholipids independent of changes in the headgroup or acyl chain composition. A major advantage of this hybrid MS³ approach over other MSⁿ methods is that both key structural features can be rapidly identified in a single spectrum with high sensitivity. The spectra presented here required approximately 30 seconds per spectrum to acquire at 120k resolution. While this falls well within a chromatographic time scale, further reduction in the scan time can be achieved without significantly decreasing the signal-to-noise ratio of the diagnostic fragment ions by using lower mass resolution. Structural and quantitative analyses of lipids in complex biological samples using the LC-HCD/UVPD methodology is underway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding from the NIH (R01 GM103655, K12GM102745) and the Welch Foundation (F-1155) are gratefully acknowledged. Funding from the UT System for support of the UT System Proteomics Core Facility Network is gratefully acknowledged.

References

1. Dowhan, W., Bogdanov, M., Mileykovskaya, E. Functional Roles of Lipids in Membranes. In: Ridgway, ND., McLeod, RS., editors. Biochemistry of Lipids, Lipoproteins and Membranes. 6th. Elsevier; Boston: 2016. p. 1-40.
2. Barrera NP, Zhou M, Robinson CV. Trends in Cell Biol. 2013; 23:1–8. [PubMed: 22980035]
3. Saliba A-E, Vonkova I, Gavin A-C. Nat Rev Mol Cell Biol. 2015; 16:753–761. [PubMed: 26507169]
4. Beloribi-Djefafli S, Vasseur S, Guillaumond F. Oncogenesis. 2016; 5:1–10.
5. Baenke F, Peck B, Miess H, Schulze A. Dis Model Mech. 2013; 6:1353–1363. [PubMed: 24203995]
6. Fu S, Yang L, Li P, Hofmann O, Dicker L, Hide W, Lin X, Watkins SM, Ivanov AR, Hotamisligil GS. Nature. 2011; 473:528–531. [PubMed: 21532591]

7. Hamilton LK, Dufresne M, Joppe SE, Petryszyn S, Aumont A, Calon F, Barnabe-Heider F, Furtos A, Parent M, Chaurand P, Fernandes KJL. *Cell Stem Cell*. 2015; 17:397–411. [PubMed: 26321199]
8. Wang Z, Klipfell E, Bennett BJ, Koeth R, Levison BS, DuGar B, Feldstein AE, Britt EB, Fu X, Chung Y-M, Wu Y, Schauer P, Smith JD, Allayee H, Tang WHW, DiDonato JA, Lusis AJ, Hazen SL. *Nature*. 2011; 472:57–63. [PubMed: 21475195]
9. Zhang Y-M, Rock CO. *Nat Rev Microbiol*. 2008; 6:222–233. [PubMed: 18264115]
10. Sohlenkamp C, Geiger O. *FEMS Microbiol Rev*. 2016; 40:133–159. [PubMed: 25862689]
11. Hewelt-Belka W, Nakonieczna J, Belka M, Baczek T, Namiesnik J, Kot-Wasik A. *J Proteome Res*. 2016; 15:914–922. [PubMed: 26791239]
12. Blanksby SJ, Mitchell TW. *Annu Rev Anal Chem*. 2010; 3:433–465.
13. Han X, Yang K, Gross RW. *Mass Spectrom Rev*. 2012; 31:134–178. [PubMed: 21755525]
14. Han X, Gross RW. *Mass Spectrom Rev*. 2005; 24:367–412. [PubMed: 15389848]
15. Sethi S, Brietzke E. *Prostaglandins Other Lipid Mediat*. 2017; 128–129:8–16.
16. Ryan E, Reid GE. *Acc Chem Res*. 2016; 49:1596–1604. [PubMed: 27575732]
17. Han, X. *Lipidomics: Comprehensive Mass Spectrometry of Lipids*. John Wiley & Sons, Inc; Hoboken, New Jersey: 2016.
18. Schwudke D, Schuhmann K, Herzog R, Bornstein SR, Shevchenko A. *Cold Spring Harb Perspect Biol*. 2011; 3:a004614. [PubMed: 21610115]
19. Murphy RC, Axelsen PH. *Mass Spectrom Rev*. 2011; 30:579–599. [PubMed: 21656842]
20. Christie, WW., Han, X. *Lipid Analysis: Isolation, Separation, Identification and Lipidomic Analysis*. 4th. Woodhead Publishing; Oxford; 2010.
21. Liebisch G, Vizcaino JA, Kofeler H, Trotzmüller M, Griffiths WJ, Schmitz G, Spener F, Wakelam MJO. *J Lipid Res*. 2013; 54:1523–1530. [PubMed: 23549332]
22. Ekroos, K. *Lipidomics: Technologies and Applications*. Wiley-VCH Verlag & Co; Weinheim, Germany: 2012.
23. Sandra K, Sandra P. *Curr Opin Chem Biol*. 2013; 17:847–853. [PubMed: 23830914]
24. Brown SHJ, Mitchell TW, Oakley AJ, Pham HT, Blanksby SJ. *J Am Soc Mass Spectrom*. 2012; 23:1441–1449. [PubMed: 22669763]
25. Janmey PA, Kinnunen PKJ. *Trends Cell Biol*. 2006; 16:538–546. [PubMed: 16962778]
26. Lee JY, Min HK, Moon MH. *Anal Bioanal Chem*. 2011; 400:2953–2961. [PubMed: 21499968]
27. Dong J, Cai X, Zhao L, Xue X, Zou L, Zhang X, Liang X. *Metabolomics*. 2010; 6:478–488.
28. Lin J-T, McKeon TA, Woodruff CL, Singleton JA. *J Chromatogr A*. 1998; 824:169–174.
29. Damen CWN, Isaac G, Langridge J, Hankemeier T, Vreeken RJ. *J Lipid Res*. 2014; 55:1772–1783. [PubMed: 24891331]
30. Bird SS, Marur VR, Stavrovskaya IG, Kristal BS. *Anal Chem*. 2012; 84:5509–5517. [PubMed: 22656324]
31. Kyle JE, Zhang X, Weitz KK, Monroe ME, Ibrahim YM, Moore RJ, Cha J, Sun X, Lovelace ES, Wagoner J, Polyak SJ, Metz TO, Dey SK, Smith RD, Burnum-Johnson KE, Baker ES. *Analyst*. 2016; 141:1649–1659. [PubMed: 26734689]
32. Hancock SE, Poad BLJ, Batarseh A, Abbott SK, Mitchell TW. *Anal Biochem*. 2017; 524:45–55. [PubMed: 27651163]
33. Mitchell TW, Pham HT, Thomas MC, Blanksby SJ. *J Chromatogr B*. 2009; 877:2722–2735.
34. Ma X, Chong L, Tian R, Shi R, Hu TY, Ouyang Z, Xia Y. *Proc Natl Acad Sci USA*. 2016; 113:2573–2578. [PubMed: 26903636]
35. Stinson CA, Xia Y. *Analyst*. 2016; 141:3696–3704. [PubMed: 26892746]
36. Ma X, Xia Y. *Angew Chem*. 2014; 53:2592–2596. [PubMed: 24500881]
37. Pham HT, Ly T, Trevitt AJ, Mitchell TW, Blanksby SJ. *Anal Chem*. 2012; 84:7525–7532. [PubMed: 22881372]
38. Deimler RE, Sander M, Jackson GP. *Int J Mass Spectrom*. 2015; 390:178–186. [PubMed: 26644782]
39. Li P, Jackson GP. *J Mass Spectrom*. 2017; 52:271–282. [PubMed: 28258643]

40. Bollinger JG, Naika GS, Sadilek M, Gelb MH. *J Lipid Res.* 2013; 54:3523–3530. [PubMed: 23945566]
41. Yang K, Diltz BG, Gross RW. *Anal Chem.* 2013; 85:9742–9750. [PubMed: 24003890]
42. Poad BLJ, Pham HT, Thomas MC, Nealon JR, Campbell JL, Mitchell TW, Blanksby SJ. *J Am Soc Mass Spectrom.* 2010; 21:1989–1999. [PubMed: 20869881]
43. Thomas MC, Mitchell TW, Harman DG, Deeley JM, Nealon JR, Blanksby SJ. *Anal Chem.* 2008; 80:303–311. [PubMed: 18062677]
44. Pittenauer E, Allmaier Gn. *Comb Chem High Throughput Screen.* 2009; 12:137–155. [PubMed: 19199883]
45. Hsu F-F, Turk J. *J Am Soc Mass Spectrom.* 2008; 19:1681–1691. [PubMed: 18771936]
46. Campbell JL, Baba T. *Anal Chem.* 2015; 87:5837–5845. [PubMed: 25955306]
47. Poad BLJ, Green MR, Kirk JM, Tomczyk N, Mitchell TW, Blanksby SJ. *Anal Chem.* 2017; 89:4223–4229. [PubMed: 28252928]
48. Pham HT, Maccarone AT, Thomas MC, Campbell JL, Mitchell TW, Blanksby SJ. *Analyst.* 2014; 139:204–214. [PubMed: 24244938]
49. Reilly JP. *Mass Spectrom Rev.* 2009; 28:425–447. [PubMed: 19241462]
50. Ly T, Julian RR. *Angew Chem Int Ed.* 2009; 48:7130–7137.
51. Brodbelt JS. *Chem Soc Rev.* 2014; 43:2757–2783. [PubMed: 24481009]
52. Klein DR, Brodbelt JS. *Anal Chem.* 2017; 89:1516–1522. [PubMed: 28105803]
53. Klein DR, Holden DD, Brodbelt JS. *Anal Chem.* 2016; 88:1044–1051. [PubMed: 26616388]
54. Domingues P, Domingues MRM, Amado FML, Ferrer-Correia AJ. *Rapid Commun Mass Spectrom.* 2001; 15:799–804. [PubMed: 11344540]
55. Hsu F-F, Turk J. *J Am Soc Mass Spectrom.* 2003; 14:352–353. [PubMed: 12686482]
56. Nakanishi H, Iida Y, Shimizu T, Taguchi R. *J Biochem.* 2010; 147:245–256. [PubMed: 19880374]
57. Murphy, RC. *Tandem Mass Spectrometry of Complex Lipids.* The Royal Society of Chemistry; Cambridge, UK: 2014.
58. Hsu F-F, Turk J. *J Chromatogr B.* 2009; 877:2673–2695.
59. Hvattum E, Hagelin G, Larsen Å. *Rapid Commun Mass Spectrom.* 1998; 12:1405–1409. [PubMed: 9773525]
60. Vernooij EAAM, Brouwers JFHM, Kettenes-Van den Bosh JJ, Crommelin DJA. *J Sep Sci.* 2002; 25:285–289.
61. Ekroos K, Ejlsing CS, Bahr U, Karas M, Simons K, Shevchenko A. *J Lipid Res.* 2003; 44:2181–2192. [PubMed: 12923235]
62. Ryan E, Nguyen CQN, Shiea C, Reid GE. *J Am Soc Mass Spectrom.* 2017; 28:1406–1419. [PubMed: 28455688]

Figure 1. HCD/VPD spectra of two sodium-cationized sn -regioisomeric PCs (A) PC 16:0/18:1(n -9) and (B) PC 18:1(n -9)/16:0. These spectra were obtained by isolating the headgroup loss ions (m/z 599.5) generated by HCD and subjecting them to 10 laser pulses (193 nm) with 4 mJ per pulse.

Figure 2.

HCD/VPD spectra of two double-bond positional PC isomers, sodium-cationized (A) PC 18:1($n-12$)/18:1($n-12$) and (B) PC 18:1($n-9$)/18:1($n-9$). These spectra were obtained by trapping the headgroup loss ions (m/z 625.5) generated by HCD and subjecting them to 10 laser pulses (193 nm) with 4 mJ per pulse.

Figure 3.

HCD/VPD spectra of sodium-cationized (a) PC 16:0/18:0, (b) PC 16:0/18:2(*n*-6, *n*-9), (c) PC 16:0/20:4(*n*-6, *n*-9, *n*-12, *n*-15), and (d) PC 16:0/22:6(*n*-3, *n*-6, *n*-9, *n*-12, and *n*-15). The spectra were obtained by exposing the headgroup loss ions generated by HCD to 10 laser pulses (193 nm) with 4 mJ per pulse.

Figure 4. HCD/UVPD and CID/UVPD spectra of sodium-cationized (a) PE 16:0/18:1, (b) PA 16:0/18:1, (c) PS 16:0/18:1, (d) PI 16:0/18:1 and (e) PG 16:0/18:1 ions.

Figure 5.

LC-HCD/VPD analysis of two synthetic standards (PC 16:0/18:1(*n*-9) and .PC 18:1(*n*-9)/16:0) present within an artificial mixture of standards mixed in a 1:1 ratio. (A) The base peak chromatogram (BPC) for LC-HCD/VPD of m/z 782. (B) The extracted ion chromatogram (XIC) for HCD/VPD product ions of m/z 345, indicative of PC 18:1(*n*-9)/16:0, is shown in blue, while the XIC of m/z 319, indicative of PC 16:0/18:1(*n*-9) is shown in green. (C) The HCD/VPD spectrum obtained by integrating across the chromatographic peak between 19.7 and 19.9 min. (D) The HCD/VPD spectrum obtained by integrating across the chromatographic peak between 20.2 – 20.4 min.

Figure 6. HCD/VPD spectra from sodium-cationized PC 34:1 ions identified in extracts from (A) bovine liver and (B) porcine brain.

Scheme 1.

Fragmentation pathway for complete headgroup loss upon CID of sodium adducted phosphatidylcholine. Adapted from Reference⁵⁵.

Table 1Identified PEs from *E. coli* lipid extract by HCD/UVPD-MS

PE ^a	Precursor [M + Na] ⁺ (<i>m/z</i>)	Headgroup loss ion (<i>m/z</i>)	Diagnostic ions for <i>sn</i> -position (<i>m/z</i>)	Diagnostic ions for double bond position (<i>m/z</i>)
PE 16:0/14:0	686.48	545.45	249.18, 319.26, 335.26	–
PE 14:0/16:0	686.48	545.45	277.21, 291.23, 307.21	–
PE 16:0/16:1(<i>n</i> -7)	712.49	571.47	275.20, 319.26, 335.26	461.36, 485.36
PE 16:1(<i>n</i> -7)/16:0	712.49	571.47	277.21, 317.25, 333.24	461.36, 485.36
PE 16:0/cy17:0	726.51	585.49	289.21, 319.26, 335.26	–
PE cy17:0/16:0	726.51	585.49	277.21, 331.26, 347.26	–
PE 16:0/18:2(<i>n</i> -7, <i>n</i> -10)	738.51	597.49	301.21, 319.26, 335.26	447.35, 471.35, 487.38, 511.38
PE 16:1(<i>n</i> -7)/18:1(<i>n</i> -7)	738.51	597.49	303.23, 317.25, 333.24	487.38, 511.38
PE 16:2(<i>n</i> -7, <i>n</i> -10)/18:0	738.51	597.49	305.25, 315.23, 331.22	447.35, 471.35, 487.38, 511.38
PE cy17:0/cy17:0	738.51	597.49	289.21, 331.26, 347.26	–
PE 18:0/16:2(<i>n</i> -7, <i>n</i> -10)	738.51	597.49	273.18, 347.29, 363.29	447.35, 471.35, 487.38, 511.38
PE 18:1(<i>n</i> -7)/16:1(<i>n</i> -7)	738.51	597.49	275.20, 345.28, 361.27	487.38, 511.38
PE 18:2(<i>n</i> -7, <i>n</i> -10)/16:0	738.51	597.49	277.21, 343.26, 359.26	447.35, 471.35, 487.38, 511.38
PE 16:0/18:1(<i>n</i> -7)	740.52	599.50	303.23, 319.26, 335.26	489.39, 513.39
PE 18:1(<i>n</i> -7)/16:0	740.52	599.50	277.21, 345.28, 361.27	489.39, 513.39
PE 16:0/20:2(<i>n</i> -7, <i>n</i> -10)	766.54	625.52	319.26, 329.25, 335.26	475.38, 499.38, 515.41, 539.41
PE cy17:0/cy19:0	766.54	625.52	317.25, 331.26, 347.26	–
PE cy19:0/cy17:0	766.54	625.52	289.21, 359.29, 375.29	–
PE 18:0/18:2(<i>n</i> -7, <i>n</i> -10)	766.54	625.52	301.21, 347.29, 363.29	475.38, 499.38, 515.41, 539.41
PE 18:1(<i>n</i> -7)/18:1(<i>n</i> -7)	766.54	625.52	303.23, 345.28, 361.27	515.41, 539.41
PE 18:2(<i>n</i> -7, <i>n</i> -10)/18:0	766.54	625.52	305.25, 343.26, 359.26	475.38, 499.38, 515.41, 539.41

^aNote that cy (e.g. cy17:0) refers to the presence of a cyclopropyl group.