Published in final edited form as:

J Mass Spectrom. 2015 December ; 50(12): 1327–1339. doi:10.1002/jms.3698.

Electron-Induced Dissociation (EID) for Structure Characterization of Glycerophosphatidylcholine: Determination of Double Bond Positions and Localization of Acyl Chains

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Abstract

Glycerophospholipids are a highly abundant and diverse collection of biologically relevant lipids and distinction between isomeric and isobaric species is a fundamental aspect for confident identification. The ability to confidently assign a unique structure to a glycerophospholipid of interest is dependent on determining the number and location of the points of unsaturation and assignment of acyl chain position. The use of high energy electrons (>20 eV) to induce gas-phase dissociation of intact precursor ions results in diagnostic product ions for localizing double bond positions and determining acyl chain assignment. We describe a high resolution, tandem mass spectrometry method for structure characterization of glycerophospholipids using electron-induced dissociation (EID). Furthermore, the inclusion of nomenclature to systematically assign bond cleavage sites with acyl chain position and double bond location enables a uniform platform for lipid identification. The EID methodology detailed here combines novel application of an electron-based dissociation technique with high resolution mass spectrometry that facilitates a new experimental approach for lipid biomarker discovery and validation.

Keywords

Electron-Induced Dissociation; tandem mass spectrometry; lipidomics; FT-ICR

INTRODUCTION

The field of lipidomics is addressing the challenge of characterizing the lipidome of a cell, tissue, or organism by a variety of analytical techniques most notably via the use of mass spectrometry.^[1] A crucial aspect of lipidome characterization is confident assignment in structural identification of the lipid(s) of interest. Lipid species are inherently heterogeneous with numerous isobaric and isomeric structures due to the wide range of fatty acid chain length combinations, degree of saturation, and diversity in head groups.^[2–4] The inability to

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accurately describe a lipid's unique structure compromises the extent to which biochemical and biological mechanisms are understood.

An example highlighting the complexity of lipid identification can be seen by examining the accurate mass measurement of three common glycerophosphatidylcholines (PCs), 1-hexadecanoyl-2-octadecanoyl-sn-glycero-3-phosphocholine (PC(16:0/18:0); m/z 762.6007), 1-hexadecanoyl-2-octadecenoyl-sn-glycero-3-phosphocholine (PC(16:0/18:1); m/z 760.5851), and 1-hexadecanoyl-2-octadecadienoyl-sn-glycero-3-phosphocholine (PC(16:0/18:2); m/z 758.5694). In this example, only the protonated ion ($[M+H]^+$) was considered and database searching was performed using LIPID MAPS.^[4,5] When a 1 Da window (~1300 ppm mass accuracy) was selected for the database search criteria, there were 115, 127, and 142 possible lipid structures for PC(16:0/18:0), PC(16:0/18:1), and PC(16:0/18:2), respectively, representing a variety of isobaric and isomeric structures (Figure 1). The number of possible lipid structures was reduced by applying a more stringent m/z search threshold. Beyond the 10 mDa search criteria, the number of identified lipids did not decrease and in fact remained constant at 22 (PC(16:0/18:0)), 36 (PC(16:0/18:1)), and 46 (PC(16:0/18:2)). All lipid structures identified at the 10 mDa and below mass accuracy threshold were isomers and therefore had indistinguishable accurate m/z values. For example, a search criteria of 0.1 mDa (~100 ppb) did not result in reduction in the number of possible lipid structures when compared to the 10 mDa threshold. Thus, it is necessary to include not only accurate mass measurement but also additional structure information for confident lipid structure assignment.

The current mass spectrometry strategies for lipid structure characterization rely heavily on the use of gas-phase dissociation techniques to fragment intact precursor ions to yield structure-specific product ions (i.e., tandem mass spectrometry).^[6,7] The most prevalent gas-phase dissociation technique, low energy (LE) collision-induced dissociation (CID), has proven to be a useful and highly utilized tandem mass spectrometry tool for structure elucidation of lipids (e.g., glycerophospholipids^[6,8,9]) yet its application does not always result in unique product ions that are diagnostic for acyl chain location or double bond positioning. These two key structural features are essential for confident structure identification. As an alternative to LE CID, a number of other types of dissociation techniques including the use high-energy (HE) CID^[10–12], lasers^[13], gases^[14], and electrons^[15,16] have been successfully applied to structurally characterize lipids. Of particular interest is the use of electron-based gas-phase dissociation techniques in which the gas-phase ion chemistry between electrons and the ions of interest induces fragmentation. A range of electron-based dissociation techniques, including electron-capture dissociation (ECD),^[17] hot ECD (HECD),^[18] electron-transfer dissociation (ETD),^[19] electron-detachment dissociation (EDD),^[20] electronic-excitation dissociation (EED),^[21] and electron-induced dissociation (EID),^[22] have been used to provide confident structure assignment of a variety of biological molecules which often times was not obtainable via other gas-phase dissociation platforms.

The application of electron-based dissociation techniques for singly charged gaseous ions, referred to here as EID, has received notable interest of late^[23–35] but has a rich history dating back to the work of Cody and Freiser.^[36,37] We present the use of EID to structurally

characterize glycerophosphatidylcholine (PC) ions produced via matrix-assisted laser desorption/ionization (MALDI) on a Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer. In particular, we describe how EID produced structure-specific fragmentation of diacyl PCs yielding diagnostic product ions for determination of *sn*-1 and *sn*-2 acyl chains and for localization of double bond positions.

METHODS

Reagents and Materials

LC-MS grade methanol and chloroform were purchased from Fisher Scientific (Pittsburg, PA). 2,5-Dihydroxybenzoic acid (DHB) at 99.5% HPLC grade was purchased from Sigma Aldrich (St. Louis, MO).

The following lipid standards were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL): 1-hexadecanoyl-2-octadecanoyl-sn-glycero-3-phosphocholine (PC(16:0/18:0)), 1-octadecanoyl-2-hexadecanoyl-sn-glycero-3-phosphocholine (PC(18:0/16:0)), 1-hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine (PC(16:0/18:1(9Z))), 1-(9Z-octadecenoyl)-2-hexadecanoyl-sn-glycero-3-phosphocholine (PC(18:1(9Z)/16:0)), 1-hexadecanoyl-2-(9Z,12Z-octadecadienoyl)-sn-glycero-3-phosphocholine (PC(16:0/18:2(9Z,12Z))), 1-octadecanoyl-2-(9Z,12Z-octadecadienoyl)-sn-glycero-3-phosphocholine (PC(18:0/18:2(9Z,12Z))), 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine (PC(18:1(9Z)/18:1(9Z))), and 1,2-di-(6Z-octadecenoyl)-sn-glycero-3-phosphocholine (PC(18:1(6Z)/18:1(6Z))).

Sample preparation

Lipid standards were prepared to 1.0 mg/mL in chloroform:methanol (2:1, v/v). Lipid standards were further diluted in chloroform:methanol (1:1, v/v) to a concentration of 0.1 mg/mL. DHB was used as the MALDI matrix and was prepared at a concentration of 10 mg/mL in chloroform:methanol (1:1, v/v). A 1:1 mixture of DHB and lipid standard was prepared and 1 μ L of the mixture was deposited on the MALDI target plate.

Mass Spectrometry

All mass spectrometry experiments were performed on a Bruker 7 Telsa Fourier transform ion cyclotron resonance mass spectrometer (solariX-XR, Bruker Daltonics, Billerica, MA) equipped with a dual electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) source. Instrument calibration was performed using sodium formate via positive ion mode ESI over the mass range of 100-2000 Da. The dataset size was 1 MW (~0.5 second FID) and the detection was in magnitude mode with ramped excitation. All EID and LE CID experiments were performed using the MALDI source with the following parameters: the laser was set to 100 Hz at a laser power of 20-40 % (instrument specific setting), smartwalk with selective accumulation was enabled, 150 laser shots per acquisition, and 50 (CID) or 100 (EID) spectra were summed. The CID tandem mass spectrometry experiments were performed with precursor ion isolation (isolation window set to $\pm$ 2Da) followed by LE CID (Argon gas and collision energy of 25-35 eV) in the front end quadrupole. All ions were then transferred to the ICR cell for ion excitation and detection.

The EID tandem mass spectrometry experiments were performed with precursor ion isolation (isolation window set to ± 2 Da) in the front end quadrupole. Precursor ions were then transferred to the ICR cell for EID, ion excitation, and detection. The electrons were generated using an ECD cathode with the following parameters: 50 ms radiation time, 23 V cathode bias, 1.6 A cathode current and 0 V lens. All data was acquired and processed with DataAnalysis 4.2 (Bruker Daltonics). Product ions were annotated based on satisfying a signal-to-noise ratio greater than 3 and a mass accuracy of less than 5 ppm.

Nomenclature

Nomenclature for describing the product ions generated via gas-phase dissociation of protonated PC precursor ions was adopted from Griffiths et al.^[38] and modified. Although the nomenclature was specific for protonated diacyl PC precursor ions it was designed to describe adducts of positive and negative mode ionization as well as other lipid classes. The nomenclature proposed by Griffiths et al.^[38] was used to describe charge-remote fragmentation (CRF) of fatty acid anions subjected to HE CID. The nomenclature presented herein allowed for naming of product ions generated via both CRF and charge-mediated fragmentation. The following rules outline the proposed nomenclature scheme:

- (i) The capital letters C, H, A, V, D, F, K, and G are used to describe the type of bond cleaved with charge retention at the quaternary nitrogen. PCs readily ionize in the positive ion mode producing an abundant $[M+H]^+$ precursor ion. Upon positive mode ionization, the negatively charged phosphate group of the PC accepts a proton and the quaternary nitrogen of the trimethylamine carries the net positive charge.^[6] Consequently, the chemical structure of the PC $[M+H]^+$ precursor ion sufficiently localizes a “fixed” positive charge at the trimethylamine terminus. A regular C-C single bond was referred to as a C, a homoallyl bond as a H, an allyl bond as an A, a vinyl bond as a V, a C=C double bond as a D, the neutral loss of a fatty acyl as a free fatty acid as a F, the neutral loss of a fatty acyl as a ketene as a K, and a glycerol bond as a G. The bond allyl to one double bond and vinyl to another double bond was referred to as an AV for homoconjugated systems.
- (ii) The fragmentation of the glycerol backbone were referred to as G1 (C1 carbon atom; *sn*-1 acyl chain), G2 (C2 carbon atom; *sn*-2 acyl chain), and G3 (C3 carbon atom; head group) with charge retention at the quaternary nitrogen.
- (iii) A subscript to the right of the letter indicated the number of carbon atoms associated with the neutral loss of the acyl chain, number of double bonds localized, and fatty acyl position if known. The general formula was $X_{n:y-z}$ where the X = letter corresponding to type of bond cleaved, n = number of carbon atoms involved in the neutral loss, y = number of double bonds localized up to product ion being described, and z = acyl chain position if known. For example, $C_{6:0-1}$ referred to the product ion that lost 6 carbon atoms, no double bonds localized, and product ion associated with the *sn*-1 acyl chain. Product ions not assigned to a specific acyl chain position did not carry the “z”

description. For example, C_{6:0} would be the same description as above yet localization to specific acyl chain position was not determined.

- (iv) The superscript to the left referred to the resulting number of H atoms remaining on the fragment ion. No superscript referred to addition of one H atom (even-electron product ion), ‘ referred to no addition of an H atom (odd-electron product ion), “ referred to loss of 2 H atoms (even-electron product ion), ”” referred to loss of 3 H atoms (odd-electron product ion).

The nomenclature will become clear as examples and corresponding fragmentation are discussed in detail. Refer to the inset of Figures 2-8 and S1-2 for structures of diacyl PCs and labeled fragmentation.

RESULTS AND DISCUSSION

The following details the advantages, challenges, and potential utility associated with the use of EID for localization of acyl chains and determination of double bond positions for PCs. PC standards were chosen as a model glycerophospholipid class due to their general abundance in biological samples (e.g., tissue and plasma), the extensive amount of literature precedence for LE CID of PCs, and accessibility to purchase varying isomers of different acyl chain length and degree of unsaturation. The specific PC lipids used in this study were the following: PC(16:0/18:0), PC(18:0/16:0), PC(16:0/18:1(9Z)), PC(18:1(9Z)/16:0), PC(16:0/18:2(9Z,12Z)), PC(18:0/18:2(9Z,12Z)), PC(18:1(9Z)/18:1(9Z)) and PC(18:1(6Z)/18:1(6Z)).

LE CID of diacyl PCs

LE CID was used as the benchmark dissociation technique to evaluate the performance of EID. The LE CID spectra for the [M+H]⁺ precursor ion for PC(16:0/18:0), PC(18:0/16:0), PC(16:0/18:1(9Z)), PC(18:1(9Z)/16:0), PC(16:0/18:2(9Z,12Z)), PC(18:0/18:2(9Z,12Z)), PC(18:1(9Z)/18:1(9Z)), and PC(18:1(6Z)/18:1(6Z)) were displayed in Figure 2 and Supporting Information (SI) Figure S1. The LE CID spectra of the aforementioned diacyl PCs were consistent with literature^[6,9] and represented the accepted structure information achievable for routine tandem mass spectrometry experiments. The general features associated with the LE CID spectra included the diagnostic phosphocholine ion at *m/z* 184 ([PCho]⁺), several low abundant product ions corresponding to the neutral loss of the acyl chains as free fatty acids or ketenes, and the low abundant product ion corresponding to the loss of phosphoryl choline.

The product ions corresponding to the neutral loss of the acyl chains provided useful structure information as to what fatty acyls were present. The extent to which these product ions assigned acyl chains to specific *sn*-1 or *sn*-2 positions has been reported previously.^[9,39] It was reported the relative abundance of the neutral loss of the acyl chains as ketenes discriminated between the *sn*-1 and *sn*-2 positions for the [M+H]⁺ precursor ion. The preference being the neutral loss of the *sn*-2 ketene was more favorable than the neutral loss of the *sn*-1 ketene. This held true for the PC lipids investigated herein. The extent to which LE CID spectra can assign acyl chain positioning is considered general guidance and warrants a cautious approach. The use of additional mass spectrometry experiments (e.g.,

alternative dissociation techniques, negative ion mode) and/or enzymatic techniques should be incorporated for robust and confident determination of acyl chain positioning. In addition, LE CID does not inform on the location of double bonds.

EID of fully saturated Diacyl PCs

In order to evaluate whether EID could distinguish between acyl chain positioning at the *sn*-1 and *sn*-2 position, we investigated fully saturated diacyl PC (PC(16:0/18:0) and PC(18:0/16:0)). PC(16:0/18:0) and PC(18:0/16:0) were regioisomers differing only in the positioning of the acyl chains. The EID spectra for PC(16:0/18:0) and PC(18:0/16:0) were displayed in Figure 3. Refer to Methods section for description of proposed nomenclature which was adopted and modified from Griffiths et al.^[38]

The most abundant product ion in the EID spectra (Figure 3) corresponded to the phosphocholine ion at m/z 184 (referred to as G_{34:0}) similar to the LE CID spectrum. The EID process resulted in less efficient fragmentation of the precursor ion as compared to LE CID, as evident of the highly abundant [M+H]⁺ peak (m/z 762) and the corresponding low abundant product ions in the m/z region of 450-740, yet produced spectra yielding information-rich, structure-specific product ions. Several general characteristics of the EID spectra of PC(16:0/18:0) and PC(18:0/16:0) were observed. These included the following: EID provided extensive cleavage across the acyl chain backbone (not observed in LE CID), produced both even- and odd-electron product ions (not observed in LE CID), resulted in more favorable formation of the product ion associated with loss of the *sn*-1 ketene (m/z 524) over the product ion associated with loss of the *sn*-2 ketene (m/z 496) in contrast to the LE CID spectrum (*sn*-1 ketene < *sn*-2 ketene), acyl chains at both *sn*-1 and *sn*-2 preferentially lost the fatty acyl as neutral ketenes as opposed to the free fatty acid (not observed in LE CID spectra), and resulted in a series of product ions that were acyl chain position-specific and therefore discriminated the *sn*-1 acyl chain from the *sn*-2 acyl chain.

Systematic cleavage of the acyl terminus was seen in the product ion series in the m/z region of 590 to 750 (Figure 3). The neutral loss from these ions corresponded to the generic formula of C_nH_{2n+2} where n is the number of carbon atoms and were initiated at the acyl terminus. For example, the ion at m/z 732.6 corresponded to the neutral loss of C₂H₆. Of note, the hydrocarbon structure of the neutral loss was not completely understood and could arise from a fully saturated hydrocarbon (alkane) or the neutral loss of an alkene + H₂. The reaction mechanism producing the neutral loss of the alkene + H₂ (referred to as a 1,4-H₂ elimination) was reasoned to be more energetically favorable than the corresponding 1,2-H₂ elimination that resulted in the neutral loss of the alkane.^[40] An alternative fragmentation mechanism for the acyl terminus of fatty acyls was characterized by a homolytic cleavage whereby the C-C bond cleavage resulted in a free radical and a distonic radical ion which subsequently lost the β-hydrogen forming the terminally unsaturated even-electron product ion.^[11] Given the fragmentation in EID was a result of the interaction between energetic electrons (20 eV) and gas-phase ions, resulting in the presence of odd- and even-electron product ions (in the ensuing discussion below), the homolytic fragmentation mechanism appeared to be a more encompassing mechanism. In either case, the fragmentation of the acyl terminus of PCs was considered to involve charge-remote fragmentation (CRF)^[12,40,41]

where the positive charge was localized remotely on the quaternary nitrogen and not considered to be involved in bond cleavage. The product ions in the m/z region of 590 to 750 could not be assigned to specific acyl chains and therefore were not diagnostic for determining acyl chain positioning. The neutral loss of alkanes/alkenes($+H_2$) from the acyl terminus was reported via the use of HE CID from carboxylate anions of saturated and unsaturated fatty acids^[38] and more recently via the use of positive ion adducts of diacyl PCs.^[10,16]

Considering PC(16:0/18:0) (Figure 3a), the product ion series at m/z values 505 (${}^mF_{16:0-1}$), 506 (${}^mF_{16:0-1}$), 522 (${}^mK_{16:0-1}$), 523 (${}^mK_{16:0-1}$), 524 (${}^mK_{16:0-1}$), 565 (${}^mC_{14:0-1}$), and 578 (${}^mC_{13:0-1}$) were consistent with fragmentation of the 16:0 fatty acyl at the *sn-1* position. Conversely, the product ion series at m/z values 477 (${}^mF_{18:0-2}$), 478 (${}^mF_{18:0-2}$), 494 (${}^mK_{18:0-2}$), 495 (${}^mK_{18:0-2}$), 496 (${}^mK_{18:0-2}$), 537 (${}^mC_{16:0-2}$), and 550 (${}^mC_{15:0-2}$) were confidently assigned to the 18:0 fatty acyl at the *sn-2* position. The ion at m/z 578 (${}^mC_{13:0-1}$) which corresponded to the neutral loss of $C_{13}H_{28}$ (C_nH_{2n+2}) could have arisen from either the *sn-1* 16:0 or the *sn-2* 18:0 acyl chain and it could be ruled out that its abundance was a result of mutual fragmentation from both acyl chains. Yet, its relative high abundance indicated the formation of this ion was considerably more favorable in comparison to the much lower abundant product ions that corresponded to the neutral loss of C_nH_{2n+2} . Ion abundance was influenced by a number of factors of which the stability of the product ion justified assigning the m/z value 578 (${}^mC_{13:0-1}$) from Figure 3a to the *sn-1* acyl chain. The neutral loss of $C_{13}H_{28}$ from the 16:0 acyl chain produced a product ion corresponding to $CH=CHCOOR^+$ whereas this same neutral loss from the 18:0 acyl chain yielded a product ion corresponding to $CH=CH(CH_2)_2COO-R^+$. The closer proximity of the bond cleavage in the 16:0 fatty acyl to the ester functional group when compared to the 18:0 fatty acyl provided greater ion stabilization via the electron sharing of the nonbonding orbitals of the oxygen atoms.

Formation of radical cations were viewed in light of the CRF homolytic mechanism whereby the distonic radical cation was a result of a homolytic cleavage of the C-C bond. Examples of these types of ions were m/z values 565 (${}^mC_{14:0-1}$), 537 (${}^mC_{16:0-2}$), 523 (${}^mK_{16:0-1}$), 505 (${}^mF_{16:0-1}$), 495 (${}^mK_{18:0-2}$), and 477 (${}^mF_{18:0-2}$) in Figure 3a. Interestingly, the radical cations associated with the loss of the fatty acyls as ketenes and free fatty acids at the *sn-2* position (m/z 495 (${}^mK_{18:0-2}$) and 477 (${}^mF_{18:0-2}$)) were more abundant than the radical cations at the *sn-1* position (m/z 523 (${}^mK_{16:0-1}$) and m/z 505 (${}^mF_{16:0-1}$)). The preferential formation of radical cations at the *sn-2* position was viewed in the context of charge stabilization by an analogous mechanism to that of Hsu and Turk^[9]. The radical cations (SI Scheme S1) involve a homolytic cleavage of the carbonyl carbon-ester (C-O) bond. The radical retained on the odd-electron cation that lost the *sn-2* acyl chain was charge stabilized through close proximity to the proton of the phosphate group. Likewise, the radical cation formed via the loss of the *sn-1* acyl chain was less charge stabilized and consequently formed a less stable radical cation. This explained the apparent shift in relative abundance observed in the EID spectra, which was in contrast to the CID spectra, where the EID spectra demonstrated a greater preferential neutral loss of the acyl chain at the *sn-1* position over the *sn-2* position. The *sn-2* position involved the loss of the acyl chain via the presence of both the odd-electron (${}^mK_{18:0-2}$ and ${}^mF_{18:0-2}$) and even-electron (${}^mK_{16:0-1}$ and ${}^mF_{16:0-1}$)

ions. Comparatively, the loss of the *sn*-1 acyl chain favored formation of the even-electron product ions (${}^{\circ}\text{F}_{16:0-1}$ and $\text{K}_{16:0-1}$). The formation of the even-electron ions (m/z 478 (${}^{\circ}\text{F}_{18:0-2}$), 496 (${}^{\circ}\text{K}_{18:0-2}$), 506 (${}^{\circ}\text{F}_{16:0-1}$), and 524 ($\text{K}_{16:0-1}$)) corresponding to the neutral loss of the acyl chains as ketenes or free fatty acids, given their abundance, indicated several mechanistic pathways including radical-initiated processes (homolytic cleavage) and non-radical-initiated processes (e.g., heterolytic processes observed in LE CID) contributed to the EID spectra.

The aforementioned observations and rationale were applicable to the corresponding EID spectrum for the regioisomer, PC(18:0/16:0), and provided a blueprint for distinguishing fatty acyls at the *sn*-1 and *sn*-2 position. In contrast to the EID spectrum of PC(16:0/18:0) (Figure 3a), the EID spectrum of PC(18:0/16:0) (Figure 3b) had a product ion series at m/z values 505 (${}^{\circ}\text{F}_{16:0-2}$), 506 (${}^{\circ}\text{F}_{16:0-2}$), 522 (${}^{\circ}\text{K}_{16:0-2}$), 523 (${}^{\circ}\text{K}_{16:0-2}$), 524 ($\text{K}_{16:0-2}$), 565 (${}^{\circ}\text{C}_{14:0-2}$), and 578 (${}^{\circ}\text{C}_{13:0-2}$) that were consistent with fragmentation of the 16:0 fatty acyl at the *sn*-2 position. Conversely, the product ion series at m/z values 477 (${}^{\circ}\text{F}_{18:0-1}$), 478 (${}^{\circ}\text{F}_{18:0-1}$), 494 (${}^{\circ}\text{K}_{18:0-1}$), 495 (${}^{\circ}\text{K}_{18:0-1}$), 496 ($\text{K}_{18:0-1}$), 537 (${}^{\circ}\text{C}_{16:0-1}$), and 550 (${}^{\circ}\text{C}_{15:0-1}$) were confidently assigned to the 18:0 fatty acyl at the *sn*-1 position (Figure 3b). The direct comparison of the isomeric PCs provided a means for generating diagnostic product ions that were characteristic for determining acyl chain positioning (Figure 4). For example, the loss of the acyl chain at the *sn*-2 position favored formation of radical cations and the neutral loss of the acyl chain as a ketene was preferential at the *sn*-1 position.

EID of unsaturated diacyl PCs

In order to evaluate whether EID could determine double bond positioning in unsaturated diacyl PCs, we investigated the following PCs: PC(16:0/18:1(9Z)), PC(18:1(9Z)/16:0), PC(16:0/18:2(9Z,12Z)), PC(18:0/18:2(9Z,12Z)), PC(18:1(9Z)/18:1(9Z)), and PC(18:1(6Z)/18:1(6Z)). The inclusion of a double bond in the acyl chain systematically influenced what product ions were present and their relative abundance in the EID spectra. The EID spectra for PC(16:0/18:1(9Z)), PC(18:1(9Z)/16:0), PC(16:0/18:2(9Z,12Z)), PC(18:0/18:2(9Z,12Z)), PC(18:1(9Z)/18:1(9Z)), and PC(18:1(6Z)/18:1(6Z)) (Figures 5-9) displayed a mixture of odd- and even-electron product ions that corresponded to the cleavage of the acyl terminus with diagnostic product ions characteristic of double bond positioning.

The EID spectrum of PC(16:0/18:1(9Z)) (Figure 5a) contained a series of even-electron product ions at m/z values 730.5 (${}^{\circ}\text{C}_{2:0}$), 716.5 (${}^{\circ}\text{C}_{3:0}$), 702.5 (${}^{\circ}\text{C}_{4:0}$), 688.5 (${}^{\circ}\text{C}_{5:0}$), 674.5 (${}^{\circ}\text{C}_{6:0}$), and 660.5 (${}^{\circ}\text{C}_{7:0}$) corresponding to the neutral loss of $\text{C}_n\text{H}_{2n+2}$ similar to the EID spectra for fully saturated diacyl PCs. These product ions could have resulted from cleavage at either the *sn*-1 or *sn*-2 fatty acyl and as such their descriptive nomenclature did not include acyl chain position. In contrast, odd-electron product ions at m/z values 731.5 (${}^{\circ}\text{C}_{2:0-2}$), 717.5 (${}^{\circ}\text{C}_{3:0-2}$), 703.5 (${}^{\circ}\text{C}_{4:0-2}$), 689.5 (${}^{\circ}\text{C}_{5:0-2}$), 675.5 (${}^{\circ}\text{H}_{6:0-2}$), and 661.5 (${}^{\circ}\text{A}_{7:0-2}$) corresponding to the neutral loss of $\text{C}_n\text{H}_{2n+1}$ were characteristic of homolytic cleavages along the *sn*-2 18:1 acyl chain. The presence of odd-electron product ions at the acyl terminus was not characteristic of the fully saturated diacyl PCs. Acyl radicals removed from a charge site, as was the case in the protonated unsaturated diacyl PC, were resonance stabilized by electron delocalization of the un-paired electron and a π bond of a conjugated

system. Considering PC(16:0/18:1(9Z)), the homolytic cleavage of the acyl chain and the presence of a double bond that provided conjugated resonance stabilization resulted in observation of radical cations originating from the *sn*-2 acyl terminus. Furthermore, allyl radicals were more stable given the proximity of the un-paired electron and the π bond of the C=C bond, which facilitated overall delocalization of the electron density thus stabilizing the allyl radical. The allyl radical resonance stability provided good agreement with the observation of the abundant distal allyl product ion at m/z 661.5 ($'A_{7:0-2}$) substantiating the role conjugative resonance plays in radical cation stability.

The following product ions in the EID spectrum of PC(16:0/18:1(9Z)) were identified as localizing the double bond position: $'A_{7:0-2}$ (m/z 661.5), $V_{8:0-2}$ (m/z 648.5), $''V_{10:1-2}$ (m/z 620.4), and $''A_{11:1-2}$ (m/z 606.5). The product ions observed up to the distal vinyl bond were consistent with radical cation formation (neutral loss of C_nH_{2n+1}) culminating in an abundant $'A$ product ion. These product ions (distal to the double bond) suggested the double bond was allyl to the product ion at m/z 661.5. Next, the m/z value at 648.5 corresponded to the neutral loss of C_nH_{2n} , which was in contrast to the proceeding 6 distal radical cations that corresponded to neutral losses of C_nH_{2n+1} . This product ion was identified at cleavage of a distal vinyl bond giving rise to an even-electron cation. Two more double bond associated product ions, $''V_{10:1-2}$ (m/z 620.4) and $''A_{11:1-2}$ (m/z 606.5), were observed and resulted from cleavage of the proximal vinyl and ally bonds, respectively. The proximal position of these even-electron product ions was substantiated by these ions having a neutral loss of C_nH_{2n} yet yielded an unsaturated acyl cation as indicated in the double dash nomenclature. Taken as a whole, the observation and subsequent identification of the double bond associated product ions localized the point of unsaturation to the C9 position of the *sn*-2 acyl chain for PC(16:0/18:1(9Z)).

Comparative analysis of the EID spectrum of the regioisomer PC(18:1(9Z)/ 16:0) (Figure 5b and 6) demonstrated consistent and reproducible EID product ions that provided bond specific fragmentation for localizing the position of the double bond in the mono-unsaturated diacyl PCs. In addition to determining the position of the double bond location, the EID spectra of monounsaturated diacyl PCs allowed for confident assignment of the fatty acyls to either the *sn*-1 or *sn*-2 position similar to the analysis detailed above for the fully saturated diacyl PCs.

The analysis of di-unsaturated diacyl PCs consisted of investigating PC(16:0/18:2(9Z,12Z)) and PC(18:0/18:2(9Z,12Z)). The EID spectrum for PC(18:0/18:2(9Z,6Z)) was displayed in Figure 7. Systematic cleavage of the acyl terminus fatty acyls proceeded via the *sn*-2 18:2 fatty acyl. Product ions associated with the neutral loss of C_nH_{2n+2} outside of the m/z value at 574.4 ($''C_{15:0-1}$) were not observed. These observations indicated the *sn*-1 fatty acyl (18:0) was left relatively intact and not subject to acyl terminus cleavage. A series of odd-electron product ions, $'C_{2:0-2}$ (m/z 757.6), $'H_{3:0-2}$ (m/z 743.5), $'A_{4:0-2}$ (m/z 729.5), and $'V_{5:0-2}$ (m/z 715.5), were observed. These product ions corresponded to the neutral loss of C_nH_{2n+1} and were identified to be distal to the first double bond. In contrast to the relatively high abundant distal $'A$ product ion in the mono-unsaturated diacyl PCs, the distal $'A$ product ion in the di-unsaturated diacyl PC was of similar abundance as the other distal odd-electron product ions indicating electron density delocalization responded differently for more highly

conjugated systems. The product ion at m/z 704.5 ($D_{6:1-2}$) corresponded to cleavage across the distal double bond. This product ion was associated with a neutral loss of C_nH_{2n-2} (C_6H_{10}). Observation of this relatively high abundant even-electron ion resulting in a fully saturated acyl terminus was not predicted. The neutral loss indicated a net gain of two hydrogen atoms for the product ion. A possible explanation involved a double bond rearrangement towards the acyl terminus, homolytic cleavage across the previous double bond followed by resonance stabilization and β -hydrogen abstraction (SI Scheme S2). The next series of product ions involved fragmentation of the distal allyl/vinyl and proximal allyl/vinyl bonds. These cleavages resulted in abundant doublet peaks at m/z values 690.5 ($AV_{7:1-2}$), 689.5 ($AV_{7:1-2}$) and 676.5 ($AV_{8:1-2}$), 674.5 ($AV_{8:1-2}$) and were consistent with competing homolytic and 1,4- H_2 elimination mechanisms. Cleavage across the proximal double bond produced a product ion at m/z 662.5 ($D_{9:2-2}$). A similar mechanism as the one proposed for $D_{6:1-2}$ involving double bond rearrangement (this time towards the fatty acyl ester), resonance, and β -hydrogen abstraction from product ion explained the unsaturated even-electron product ion. A series of even-electron unsaturated terminal acyl product ions ($A_{11:2-2}$, m/z 634.4; $H_{12:2-2}$, m/z 620.4; $C_{13:2-2}$, m/z 606.4; $C_{14:2-2}$, m/z 592.4; $C_{15:2-2}$, m/z 578.4) associated with neutral loss C_nH_{2n-2} were also detected. The neutral loss of C_nH_{2n-2} combined with identification of the unsaturated even-electron cation confidently assigned these product ions as proximal to both double bonds. The relative abundance of these product ions were viewed as a sliding scale of neutral loss stability and product ion stability. The neutral loss of the abundant $A_{11:2-2}$ ion consisted of a conjugated diene near the bond cleavage site which allowed for conjugated resonance stabilization during the fragmentation mechanism making the neutral loss energetically favorable. As the bond cleavage site moved further from the allyl position, the effect of conjugated resonance stabilization diminished accordingly, as seen in the product ion abundance: $A_{11:2-2} > H_{12:2-2} > C_{13:2-2} > C_{14:2-2}$. Conversely, the relative high abundance of the $C_{15:2-2}$ product ion was due to it being stabilized by its proximity to the ester functional group, whereby electron delocalization of the nonbonding oxygen orbitals facilitated the fragmentation process. Analysis of the di-unsaturated diacyl PC(16:0/18:2(9Z,12Z)) (Figure S2) demonstrated consistent behavior in terms of the appearance and relative abundance of the EID initiated product ions as outlined for PC(18:0/18:2(9Z,12Z)).

Systematic cleavage along the di-unsaturated fatty acyl produced diagnostic product ions characteristic of bond cleavage at or near double bond locations. This resulted in unambiguous assignment of the double bond locations for both PC(18:0/18:2(9Z,12Z)) and PC(16:0/18:2(9Z,12Z)). In addition, the EID spectra revealed similar characteristic product ions as described for mono-unsaturated diacyl PCs that provided a blueprint for distinguishing acyl chain positioning. In particular, the presence of a product ion ($G1$) corresponding to cleavage proximal to the C1 position of the glycerol backbone sharply corresponded to an unsaturated fatty acyl at the *sn*-2 position.

Two other di-unsaturated diacyl PC lipids, PC(18:1(9Z)/18:1(9Z)), and PC(18:1(6Z)/18:1(6Z)), were considered to further evaluate the utility of EID diagnosing double bond positioning (Figure 8). In contrast to the previously discussed mono- and di-unsaturated lipids, PC(18:1(9Z)/18:1(9Z)), and PC(18:1(6Z)/18:1(6Z)) contained a single double bond in

each acyl chain and the acyl chains were the same fatty acyl per lipid (e.g., PC(18:1(9Z)/18:1(9Z)) had 18:1(9Z) at the *sn-1* and *sn-2* position). These structure-specific characteristics directly influenced what product ions were observed. The observed product ions were representative of both acyl chains and did not distinguish between the *sn-1* and *sn-2* positions.

A series of even- and odd-electron product ions were present corresponding to cleavage at or near the site of the double bond for both PC(18:1(9Z)/18:1(9Z)) and PC(18:1(6Z)/18:1(6Z)) (Figure 8). These double bond specific cleavages corresponded to fragmentation across the H bond, A bond, V bond, and D bond and resulted in confident assignment of each double bond. The EID spectra of PC(18:1(9Z)/18:1(9Z)) and PC(18:1(6Z)/18:1(6Z)) did not produce systematic acyl terminus cleavages as observed in the other fully saturated, and mono- and diunsaturated diacyl PCs. In addition, a series of neutral losses corresponding to C_nH_{2n-2} were observed in the PC(18:1(9Z)/18:1(9Z)) and PC(18:1(6Z)/18:1(6Z)) EID spectra that were not present in the other lipids. The product ions associated with the neutral loss of C_nH_{2n-2} were a result of fragmentation across both acyl chains. A variety of acyl chain cleavage site combinations were possible due to the indistinguishable *sn-1* and *sn-2* fatty acyls. Further discussion of possible cleavage sites was discussed below.

Comparison of PC(18:1(9Z)/18:1(9Z)) and PC(18:1(6Z)/18:1(6Z)) EID spectra (m/z 450 to 700) revealed a number of product ions that distinguished these two isomeric lipids (Figure 9). PC(18:1(9Z)/18:1(9Z)) was characterized by $'A_{7:0}$, $'V_{8:0}$, $D_{9:1}$, $'V_{10:1}$, and $'A_{11:1}$ double bond specific product ions of which $'A_{11:1}$ was the most abundant. These characteristics were similar to the EID spectra of PC(18:1(9Z)/16:0) and PC(16:0/18:1(9Z)). In contrast, the EID spectrum of PC(18:1(6Z)/18:1(6Z)) yielded an abundant $'V_{11:0}$ product ion followed by lower abundant $D_{12:1}$, $'V_{13:1}$, and $'A_{14:1}$ product ions. Of interest, the most abundant double bond specific product ion for both PC(18:1(9Z)/18:1(9Z)) and PC(18:1(6Z)/18:1(6Z)) resulted from cleavage of the C_{11} - C_{12} bond (counting from acyl terminus), $'A_{11:1}$ and $'V_{11:0}$, respectively. The abundant $'A_{11:1}$ product ion observed for PC(18:1(9Z)/18:1(9Z)) was consistent with EID spectra for PC(18:1(9Z)/16:0), PC(16:0/18:1(9Z)), PC(16:0/18:2(9Z,12Z)), and PC(18:0/18:2(9Z,12Z)) and was explained via the stability of the proximal double bond ally cleavage as explained in previous sections. This rationale did not extend to the EID spectrum of PC(18:1(6Z)/18:1(6Z)) where $'V_{11:0}$ was not only the most abundant double bond associated product ion but resulted from cleavage distal to the double bond. A mechanistic understanding for this observation is still on-going yet it suggested fragmentation at the C_{11} - C_{12} bond was energetically favorable when a double bond regardless of its position in the acyl chain was present, at least when considering the PC lipids under investigation herein.

The product ion series corresponding to the neutral loss of C_nH_{2n-2} further highlighted the role the double bond position had in the presence and abundance of product ions following EID. The EID spectrum of PC(18:1(9Z)/18:1(9Z)) had a product ion series at m/z values 564.4, 550.3, 536.3, 522.3, 508.3, and 494.3 that corresponded to the following neutral losses $C_{16}H_{30}$, $C_{17}H_{32}$, $C_{18}H_{34}$, $C_{19}H_{36}$, $C_{20}H_{38}$, and $C_{21}H_{40}$. On the other hand, the EID spectrum of PC(18:1(6Z)/18:1(6Z)) had a product ion series at m/z values 536.3, 522.3, 508.3, 494.3, 480.3, 466.3, and 452.3 corresponding to the following neutral losses $C_{18}H_{34}$,

$C_{19}H_{36}$, $C_{20}H_{38}$, $C_{21}H_{40}$, $C_{22}H_{40}$, $C_{23}H_{42}$, and $C_{24}H_{46}$. Annotation beyond neutral loss assignment at this stage was tentative. Yet, likely cleavage sites based on accurate mass, ion abundance, and mechanistic rationale detailed *vide infra* suggested fragmentation resulted in product ions of $C_{15:1}$ on one acyl chain and then systematic cleavage starting at the acyl terminus up to the double bond location on the other acyl chain. For example, the product ion at m/z 522.3 was explained via fragmentation of both acyl chains corresponding to product ions of $C_{15:1}$ and $C_{4:0}$.

Additional evidence for the proposed annotation for product ions associated with neutral losses of C_nH_{2n-2} was seen in comparison of m/z values 494.3 (PC(18:1(9Z)/18:1(9Z))) and 452.2 (PC(18:1(6Z)/18:1(6Z))) (Figure 9). These m/z values represented the last product ion in the C_nH_{2n-2} series for each lipid when examining the m/z range 450-700 and corresponded to neutral losses of $C_{21}H_{40}$ and $C_{24}H_{34}$. The difference in the number of carbon atoms between these neutral losses was 3 and equated to the difference between the double bond locations in the two lipids (e.g., 9Z versus 6Z). Furthermore, tentative product ion assignments of $C_{15:1}$ and $H_{6:0}$ for m/z 494.3 and $C_{15:1}$ and $H_{9:0}$ for m/z 452.2 was consistent with the double bond location for each lipid. In other words, the double bond location in PC(18:1(6Z)/18:1(6Z)) which was located 3 carbon units proximal from the acyl terminus provided more opportunity for C-C bond fragmentation prior to reaching the C=C position and therefore facilitated the more acyl chain cleavage.

CONCLUSION AND FUTURE DIRECTION

Tandem mass spectrometry is an invaluable analytical technique for structure characterization of lipids. The use of energetic electrons to fragment singly charged gas-phase diacyl PC ions on a FT-ICR mass spectrometer provided a unique platform for gaining comprehensive structural insight into the lipid's chemical composition and structure. The EID spectra yielded information-rich, structure-specific product ions that were diagnostic for determining acyl chain positioning and localization of double bond(s). The combined elucidation of acyl chain positioning and double bond locations allowed for confident structure assignment of saturated, monounsaturated, and di-unsaturated diacyl PC isomers. In addition, the adoption of nomenclature which incorporated a systematic approach for naming even- and odd-electron product ions provided a bond specific lettering system which was descriptive for localizing the double bond location in the fatty acyl and for assignment of the acyl chain to specific positions.

The use of EID to structurally characterize biological lipids with specific localization of acyl chain and double bond positions combined with the use of high resolution mass spectrometry establishes a fundamental technique for confident structure characterization. Indeed, FT-ICR EID is readily adaptable for electrospray ionization and MALDI applications, complementary to LE CID, and applicable to a variety of biological analytes.

Further development of EID fundamentals and applications are warranted and will only broaden the potential use of EID as an advantageous gas-phase fragmentation technique. The incorporation of additional PC structures with varied acyl chain lengths, additional points of unsaturation, and inclusion of double bond stereo-isomers are needed to fully

elucidate EID fragmentation mechanisms and gain complete lipid structure characterization. This insight would provide hypothesis-driven optimization of experimental parameters (e.g., electron energy (irradiation time and intensity) and transient length) to produce the most efficient yet informative EID spectra. Experimentation with other biologically active lipid species including other glycerophospholipids, sphingolipids, glycerolipids, and fatty acids are needed to fully realize the utility of EID for lipid structure characterization. Of note, a recent publication by Campbell et al^[16] highlighted the use of energetic electrons to fragment a variety of phospholipids on a modified quadrupole time-of-flight mass spectrometer showing the potential use of electrons to provide informative phospholipid structure information. In addition, improvement in hardware such as replacement of the hollow ECD cathode with a solid configuration could drastically enhance EID efficiency. It was estimated the use of a solid ECD cathode provided greater than an order of magnitude in signal enhancement for EID fragmentation (unpublished data; communication via Bruker Daltonics). Although the continued investigation and application of electron-induced fragmentation techniques are necessary it is also imperative sophisticated software programs are incorporated for structure annotation, multivariate analysis, and data visualization in order to expedite the data processing of EID spectra and ultimately pave the way for broad accessibility. Ultimately, the future promise of this methodology may be realized in the unique opportunity to couple EID structure characterization with complex biological matrices and innovative mass spectrometry platforms (e.g., mass spectrometry imaging).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

All mass spectrometry experiments were performed at Bruker Daltonics (Billerica, MA). This project has been funded in part with Federal funds from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, under Contract No. HHSN272201000046C. Additional support was provided by the University of Maryland School of Pharmacy Mass Spectrometry Center (SOP1841-IQB2014). The authors would like to acknowledge and thank Michael Easterling (Bruker Daltonics) and all members of the Kane laboratory.

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Mass Accuracy versus Number of Lipid Identifications

Figure 1.

Mass accuracy plotted against the number of lipid identifications using the LIPID MAPS database for $(M+H)^+$ precursor ions of PC(16:0/18:0), PC(16:0/18:1), and PC(16:0/18:2). The search criteria considered all isobaric and isomeric protonated precursor ions tabulated in the LIPID MAPS database.

Figure 2.

CID tandem mass spectra of the $[M+H]^+$ precursor ions of PC(16:0/18:1(9Z)) (a) and PC(18:1(9Z)/16:0) (b). * indicated harmonic.

Figure 4.
EID tandem mass spectra of the $[M+H]^+$ precursor ions of PC(16:0/18:0) and PC(18:0/16:0) covering the m/z range 450-740.

Figure 5.
EID tandem mass spectra of the $[M+H]^+$ precursor ions of PC(16:0/18:1(9Z)) (**a**) and PC(16:0/18:1(9Z)) (**b**). * indicated harmonic.

Figure 6.

EID tandem mass spectra of the $[M+H]^+$ precursor ions of PC(16:0/18:1(9Z)) and PC(16:0/18:1(9Z)) covering the m/z range 450-740.

Figure 7. EID tandem mass spectrum of the $[M+H]^+$ precursor ion of PC(18:0/18:2(9Z,12Z)). * indicated harmonic.

PC 18:1(9Z)/18:1(9Z)
EID m/z 786.6

PC 18:1(6Z)/18:1(6Z)
EID m/z 786.6

Figure 8.

EID tandem mass spectra of the $[M+H]^+$ precursor ions of PC(18:1(9Z)/18:1(9Z)) (a) and PC(18:1(6Z)/18:1(6Z)) (b). * indicated harmonic.

Figure 9.

EID tandem mass spectra of the $[M+H]^+$ precursor ions of PC(18:1(9Z)/18:1(9Z)) and PC(18:1(6Z)/18:1(6Z)) covering the m/z range 450-740.