Identification and quantitation of lipid C=C location isomers: A shotgun lipidomics approach enabled by photochemical reaction

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The field of lipidomics has been significantly advanced by mass spectrometric analysis. The distinction and quantitation of the unsaturated lipid isomers, however, remain a long-standing challenge. In this study, we have developed an analytical tool for both identification and quantitation of lipid C=C location isomers from complex mixtures using online Paternò–Büchi reaction coupled with tandem mass spectrometry (MS/MS). The potential of this method has been demonstrated with an implementation into shotgun lipid analysis of animal tissues. Among 96 of the unsaturated fatty acids and glycerophospholipids identified from rat brain tissue, 50% of them were found as mixtures of C=C location isomers; for the first time, to our knowledge, the quantitative information of lipid C=C isomers from a broad range of classes was obtained. This method also enabled facile cross-tissue examinations, which revealed significant changes in C=C location isomer compositions of a series of fatty acids and glycerophospholipid (GP) species between the normal and cancerous tissues.

Paternò–Büchi reaction | glycerophospholipids | photochemical reaction | lipid biomarkers | cancerous tissue analysis

Lipids play a multitude of crucial roles in biological systems by serving as building blocks of cell membranes, sources for energy storage, and media for signal transduction (1–3). Unveiling the mechanisms and networks behind lipid homeostasis calls for sensitive, quantitative, and molecularly specific lipid analysis (4). The recent advancement in mass spectrometry (MS) for bioanalysis has enabled the field of lipidomics (5, 6) by allowing global identification and quantitation of lipid species at high speed (7–9) and providing information of lipid–lipid (10, 11) and lipid–protein interactions (12, 13) at systems level. These capabilities further expedite research on lipid biomarker discovery and metabolite flux analysis (14–16). Among many analytical figures of merit, high molecular specificity is a distinct feature of the MS-based approaches. Rich structural information of lipids in complex biological samples can now be routinely obtained, including the classes of the lipids, fatty acyl/alkyl composition, and even the *sn* positions of the fatty acyl/alkyl chains (17–19). The locations of the carbon–carbon double bonds (C=C) in the lipids, however, have rarely been identified using commercial MS systems and therefore have been either assumed or not reported in a large body of literatures for lipid study (20).

The MS/MS methods, especially those involving low-energy collision-induced dissociation (CID), have not been effective in locating C=C bond locations, which is due to the high bond dissociation energies associated with cleaving a C=C bond. Without characteristic fragment ions produced, the C=C locations cannot be determined using MS/MS. To tackle this problem, two MS approaches have been explored, each with successes achieved but also with limitations observed. The first one employs C=C specific chemical derivatizations before MS analysis. These reactions either directly cleave the C=C bonds, as in ozonolysis (21), or convert the C=C bonds into functional groups [i.e., alkylthiolation (22) and methoxymercuration (23)] that are fragmentable during ionization or by low-energy CID. The methods based on this approach often require a relatively large amount of samples and additional chromatographic separation for analyzing complex samples. The second approach involves the use of different gas-phase dissociation methods other than lower-energy CID to induce fragmentations at or around the C=C bonds. Well-known methods that use such an approach include the charge remote fragmentation (24), ozone-induced dissociation (25), and radical directed dissociation (26). These methods are of great potentials for mixture analysis, but often require special MS instruments that are not readily available.

Recently, we have explored a method of pinpointing C=C locations of unsaturated lipids by coupling an online photochemical reaction, Paternò–Büchi (PB) reaction, with nanoelectrospray ionization (nanoESI)-MS/MS analysis (27). The PB reaction is a [2+2] cycloaddition reaction, which results in a fast (tens of seconds) and highly specific modification of the C=C bonds in lipids. Acetone was used as the PB reagent for 250-nm UV irradiation. The ions of PB reaction products formed by nanoESI have a mass shift of +58 Da, due to acetone addition to intact unsaturated lipids. Low-energy CID of PB products produces abundant fragment ions by cleavages at the original C=C locations, which, hereafter termed as the C=C diagnostic ions, are used for C=C location determination. Compared with the chemical derivatization methods previously

Significance

Unsaturated lipids constitute a significant portion of total lipids in mammalian cells. They fulfill different biological roles and often exist in isomeric structures that differ only in the locations of carbon-carbon double (C=C) bonds. A long-standing challenge in lipidomics is the identification and quantitation of lipid C=C location isomers at adequate sensitivity. In this study, we have developed a solution to this challenge by implementing online photochemical derivatization (Paternò–Büchi reaction) with tandem mass spectrometry into a shotgun lipidomics workflow. The method is widely applicable for different classes of lipids and is sensitive, fast, and compatible with different MS platforms. It shall serve as an enabling tool for advancing studies in lipid biology and biomarker discovery.

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mentioned, PB reaction has several unique advantages, including fast reaction kinetics suitable for online coupling with ionization, wide applicability to different lipid classes, simplicity of implementation, and compatibility with commercial MS instruments with lower-energy CID capability.

Unsaturated lipids constitute a significant portion of total lipids in mammalian cells (28). The lipid C=C location isomers are ubiquitously produced through distinct biosynthetic pathways (29, 30). The lack of an efficient means of distinguishing or quantifying lipid C=C location isomers has caused a long-time knowledge vacancy regarding the differences in biological functions of unsaturated lipids due to the C=C locations. In this study, we aimed to develop a new lipid analysis approach based on PB-MS/MS, which can be readily integrated with existing lipidomics workflows for both identification and quantitation of lipid C=C location isomers from biological samples. We first established PB-MS/MS methods for relative and absolute quantitation of lipid C=C location isomers using fatty acid and glycerophospholipid (GP) standards. These methods were then applied to shotgun analysis of fatty acid and GP extracts from rat tissues. Ninety-six unsaturated fatty acid and GP lipid species from rat brain tissues were successfully identified including specific C=C locations; 50% of them existed as mixtures of C=C location isomers. For the first time, to our knowledge, relative quantitation of a wide variety of unsaturated lipid C=C isomers of fatty acids and GPs was achieved. This quantitative PB-MS/MS method was also applied for cross-tissue analysis and comparison of normal and cancerous mouse breast tissues, which revealed significant differences in concentration ratios of C=C location isomers from several fatty acid and GP species.

Results

PB-MS/MS for Identification and Quantitation of Lipid C=C Location Isomers. There are three unique features of the PB-MS/MS that warrant its further development as a method capable of identification and quantitation of lipid C=C location isomers: (*i*) The 58-Da (mass of acetone) mass increase of the PB reaction products from the original unsaturated lipids allows for a facile identification of the PB products for subsequent MS/MS; (*ii*) the formation of distinct C=C diagnostic ions by lower-energy CID of the PB reaction products leads to an unambiguous identification of the C=C locations of each isomer; and (*iii*) the ion intensities of the diagnostic ions correlate to the amounts of the C=C location isomers, and thus can be used for quantitation. Fig. 1 summarizes the methodology using two hypothesized lipid C=C location isomers, **A** and **B**, as an example. **A** and **B** each consist of one C=C at *Δm* and *Δn* positions, respectively (counting from carboxylic acid end). Because there are two possible orientations for the addition of acetone to a C=C, two regioisomers of PB products are formed from each lipid C=C location isomer. Consequently, a total of four PB products are produced, all of the same mass (58-Da increase from **A** or **B**). These PB products are ionized simultaneously by nanoESI and mass-isolated for subsequent CID. Two pairs of C=C diagnostic ions are produced, i.e., *m1* and *m2* from isomer **A**, *n1* and *n2* from isomer **B**. The two diagnostic ions in each pair bear the structures of an aldehyde and isopropene moiety at the original C=C location, respectively (compare structures of *m1* and *m2* in Fig. 1*A*). Due to the mass difference between "O" and "C_3H_6," the two fragment ions are always separated by 26 Da, which is used as a signature to identify the C=C diagnostic ion pair in an MS/MS spectrum from analyzing complex lipid mixtures. The *m/z* values of C=C diagnostic ions lead to C=C location identification whereas the total intensity of diagnostic ions from each lipid C=C isomer is used for its relative and absolute quantitation (Fig. 1*B*).

The method described above was validated using C=C location isomers of fatty acid and glycerophosphocholine (PC) standards. Details of the experimental setup and procedures can be found in *SI Appendix*, section S1 and Fig. S1. Fig. 2*A* shows the PB reaction spectrum for a mixture of oleic acid [fatty acid 18:1(9Z)] and *cis*-vaccenic acid [fatty acid 18:1(11Z)] (4:1 molar ratio, total concentration: 10.0 μM). The PB reaction products were clearly observed at *m/z* 339, with a 58-Da mass increase from the ions of intact fatty acid 18:1 (*m/z* 281, Fig. 2*A*). PB reactions quickly reached a steady state after 0.3–0.5-min exposure to the UV light (*SI Appendix*, Figs. S2 and S3), with appreciable formation of PB products (20–60% relative ion intensity normalized to the remaining intact lipid ion signal). Subsequent CID of isolated *m/z* 339 produced two pairs of diagnostic ions at *m/z* 171/197 and *m/z* 199/225 (Fig. 2*B*). The empirical formula of each pair was used to deduce the C=C location. For instance, the lower-mass diagnostic ions of fatty acid have a formula of $\mathbf{C_xH_{(2x-3)}O_3^-}$ (*x*: carbon number) because they have aldehyde-terminated carboxylic anion structures, with the *x* corresponding to the C=C location according to the Δ-nomenclature. It is therefore straightforward to conclude that fatty acid 18:1 is a mixture of Δ9 and Δ11 isomers based on the *m/z* values of 171 ($\mathbf{C_9H_{15}O_3^-}$) and 199 ($\mathbf{C_{11}H_{19}O_3^-}$). A limit of identification of 0.3 μM for each fatty acid 18:1 isomer was achieved from the detection of C=C diagnostic ions three times above the noise level.

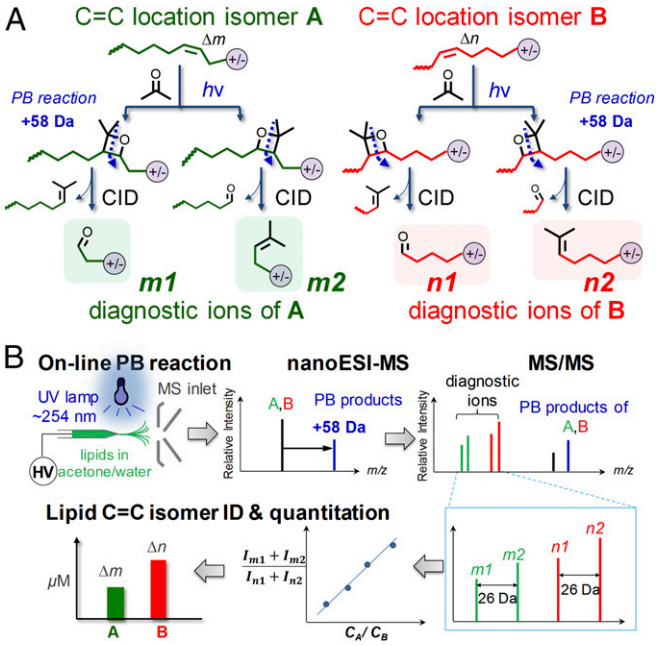

Fig. 1. (*A*) Schematic representation of PB reactions and formation of C=C diagnostic ions from lipid C=C location isomers **A** and **B** (C=C bond located at *Δm* and *Δn* positions, respectively) from MS/MS. (*B*) Analysis flow for the characterization and quantitation of C=C location isomers of lipid.

Quantitation based on diagnostic ion intensities was tested with a series of mixtures of fatty acid 18:1 9Z and 11Z isomers, with the total concentration kept constant (12.5 μM) while the molar ratios (c_{11Z}/c_{9Z}) varied. The ion intensities of each pair of C=C diagnostic ions, i.e., *m/z* 199/225 from the 11Z isomer and *m/z* 171/197 from the 9Z isomer, were summed, and the ion intensity ratios (I_{11Z}/I_{9Z}) were plotted against the concentration ratios (c_{11Z}/c_{9Z}) (Fig. 2*C*). A good linearity (R^2 = 0.9994) and a wide dynamic range of the molar ratios (from 1:19 to 19:1) were obtained, which was equivalent to a detection limit of 5% (mol) for the minor component in the mixture. This linear relationship serves as the basis for both relative and absolute quantitation of the lipid C=C isomers. For absolute quantitation, internal standard (IS) and standard addition methods were developed (details in *SI Appendix*, section S2.2). A proper IS for quantitation should be another C=C location isomer that is absent in the biological system. Because petroselinic acid [fatty acid 18:1(6Z)] has not been observed

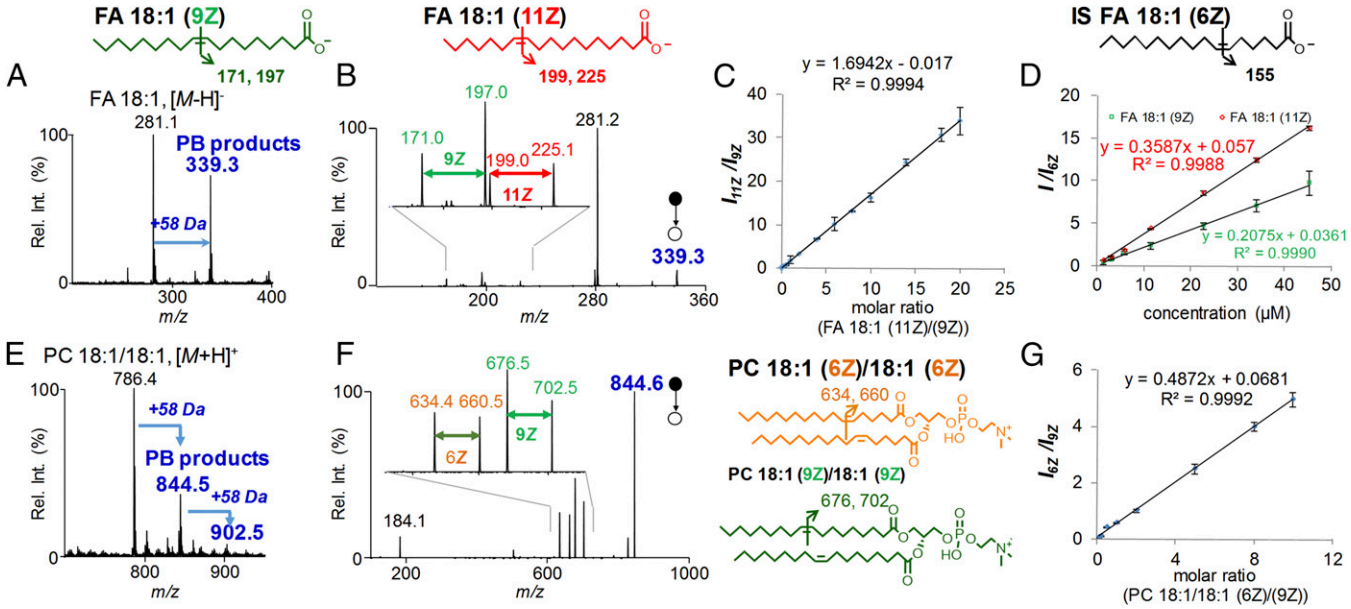

Fig. 2. Method of PB-MS/MS for identification and quantitation of unsaturated lipid C=C location isomers. (*A*) PB reaction MS spectrum of a mixture of fatty acid 18:1(9Z) (8.0 μM) and (11Z) (2.0 μM) isomer mixtures in negative ion mode. (*B*) PB-MS/MS spectrum of *m/z* 339 formed in *A*. (*Inset*) Diagnostic ions of the two fatty acid 18:1 isomers are shown. (*C*) Linear relationship established between diagnostic ion ratio (I_{11Z}/I_{9Z}) and molar ratio (c_{11Z}/c_{9Z}) of the two fatty acid 18:1 C=C location isomers. (*D*) Calibration curves for quantitation of fatty acid 18:1(9Z) and fatty acid 18:1(11Z) using fatty acid 18:1(6Z) as the internal standard. (*E*) PB reaction MS spectrum of a mixture of PC 18:1(6Z)/18:1(6Z) and PC 18:1(9Z)/18:1(9Z) isomers, each at a concentration of 3.2 μM (positive ion mode). (*F*) PB-MS/MS spectrum of PC 18:1/18:1 C=C location isomer mixture. (*Inset*) Diagnostic ions of the two PC isomers are shown. (*G*) Linear relationship established between diagnostic ion ratio (I_{6Z}/I_{9Z}) and molar ratio (c_{6Z}/c_{9Z}) of the PC 18:1/18:1 C=C location isomers.

in animal tissues, it was chosen as the IS to quantify fatty acid 18:1(9Z) and (11Z) isomers. PB-MS/MS of fatty acid 18:1(6Z) produced C=C diagnostic ions at *m/z* 155, distinct from those of the 9Z and 11Z isomers. The intensity ratios of C=C diagnostic ions ($\boldsymbol{I_{11Z}/I_{6Z}}$ and $\boldsymbol{I_{9Z}/I_{6Z}}$) were plotted against concentrations of 11Z and 9Z isomers (1.4–45.4 μM, with IS kept at 22.7 μM) in Fig. 2*D* and good linearity was obtained. When the nonnatural C=C location isomers were not available, the standard addition method could be used. Our results also showed reasonable accuracy (relative error <10%, relative standard deviation <7%, $n = 3$).

With the same principle applied, identification and quantitation of C=C location isomers of PC standards were tested. Fig. 2*E* shows a positive ion mode MS spectrum of PB reaction of an equal molar mixture (3.2 μM each) of PC 18:1(6Z)/18:1(6Z) and PC 18:1(9Z)/18:1(9Z) prepared in 45/45/10 acetone/water/ethanol (vol/vol/v). Because there are two C=C bonds in each PC, PB reaction products were observed at *m/z* 844.6 (major, +58 Da) and *m/z* 902.5 (minor, +116 Da), corresponding to the addition of one and two acetone molecules, respectively. As a general strategy, the reaction conditions were optimized to maximize the yield of the +58-Da products to obtain high-quality MS/MS spectra and to simplify spectrum interpretation. This was achieved by adding 10% of ethanol into the lipid solution. The presence of ethanol slowed down PB reaction through competitive intermolecular photoreduction of acetone (31) and thus rendered less competitive sequential PB reactions. CID of the +58-Da products (*m/z* 844.6) generated two pairs of diagnostic ions at *m/z* 634/660 and *m/z* 676/702, corresponding to C=C cleavage within acyl chains of 18:1(6Z) and 18:1(9Z), respectively (Fig. 2*F*, fragmentation map shown to its right). This allowed for a confident assignment of C=C locations. For quantitation, a good linear relationship was obtained by plotting the ratios of C=C diagnostic ion intensities against the corresponding molar ratios of the two PC isomers (Fig. 2*G*). It is worth pointing out that PB reaction is universally applicable to the C=C bonds at different locations and thereby multiple C=C bonds in one acyl chain could all be derivatized with similar probability. Subsequent CID of the +58-Da products therefore produces diagnostic ions corresponding to each individual C=C. Examples on the analysis of polyunsaturated fatty acids, e.g., fatty acid 20:4 (5, 8, 11, 14) and phosphatidylethanolamine (PE) 18:0/20:4, can be found in discussions below.

Analysis of Unsaturated Lipids in Animal Tissues.

Rat brain fatty acid and GP analysis. Mammalian cell lipidome consists of many isobaric and isomeric lipid species (32) and thus represents a challenge to both qualitative and quantitative analysis. Only a few studies have been reported for identification of C=C location isomers (33), whereas the quantitation has rarely been achieved in analysis of complex lipid mixtures. In this study, we used the polar lipid extracts from rat brain tissues as a model system to demonstrate that PB-MS/MS could be incorporated into the shotgun lipid analysis workflows for identification and quantitation of unsaturated lipid C=C location isomers. Because the PB-MS/MS method cannot distinguish C=C configurations (*E*/*Z*) or *sn* positions of fatty acyls in GPs, all unsaturated lipids identified in this work are not specified with C=C configuration and a "_" sign is used to indicate a nonspecified *sn* position of the fatty acyl chain (34). Analysis of mono- and diunsaturated fatty acids was achieved via PB-MS/MS in the negative ionization mode. As an example, the PB-MS/MS spectrum of fatty acid 18:1 extracted from rat brain tissue (Fig. 3*A*) clearly shows that fatty acid 18:1 is a mixture of Δ9 and Δ11 C=C location isomers. Using the calibration curve in Fig. 2*C*, fatty acid 18:1 was determined to consist of 27% Δ11 isomer and 73% Δ9 isomer. Analysis of fatty acid 18:2 revealed that it did not contain C=C location isomers and the two C=Cs were located at Δ9 and Δ12 positions, based on the diagnostic ions observed at *m/z* 171/197 and 211/237 (*SI Appendix*, Fig. S6).

As a special case, the application of PB-MS/MS method for fatty acid 20:4 in the negative ion mode was not successful, due to the suppression of the formation of C=C diagnostic ions by a dominant CO_2 loss channel (*SI Appendix*, Fig. S7). However, by performing PB-MS/MS on lithiated adduct in the positive ion mode ($[M+Li]^+$), four pairs of C=C diagnostic ions (*m/z* 123/149,

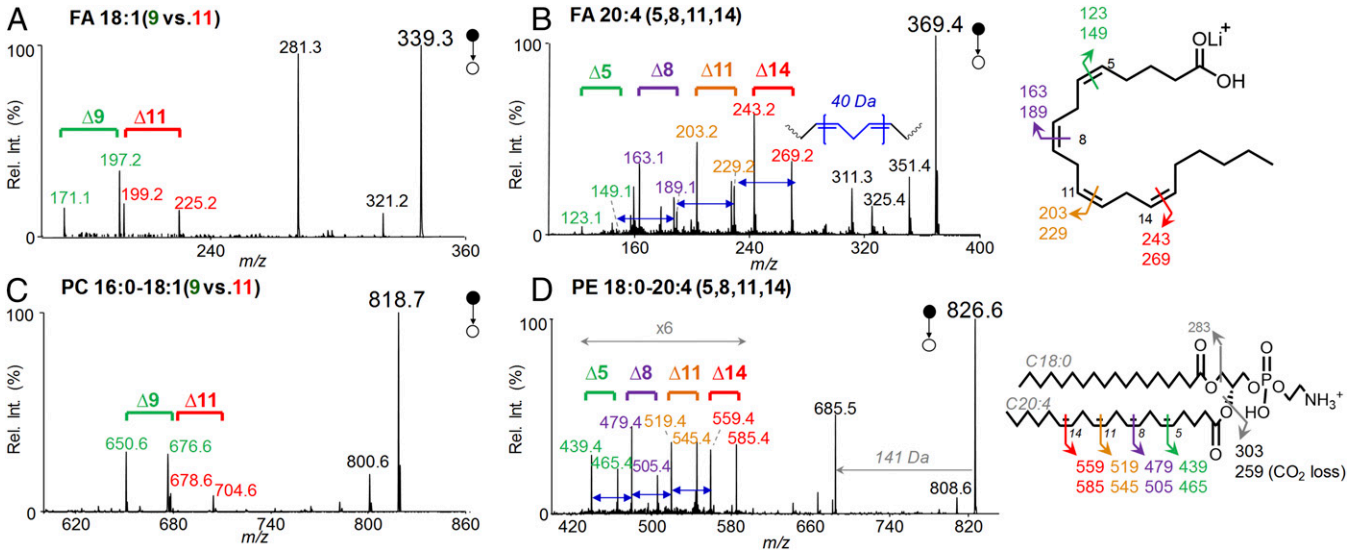

Fig. 3. Analysis of fatty acids and GPs extracted from rat brain. PB-MS/MS spectra of (*A*) fatty acid 18:1, (*B*) lithiated fatty acid 20:4, (*C*) PC 16:0–18:1, and (*D*) PE 18:0–20:4. The fragmentation maps of fatty acid 20:4 and PE 18:0–20:4 are shown on the right.

163/189, 203/229, 243/269) were generated at relatively high abundances (Fig. 3*B*, *Inset* shows the fragmentation map). It is worth noting that the 40-Da (C_3H_6) mass separation between each pair of diagnostic ions corresponds to methylene-separated C=C bonds in fatty acid 20:4. According to these evidence, C=C locations in fatty acid 20:4 were assigned at Δ5, 8, 11, 14 and no C=C location isomers were observed.

A two-step analysis procedure was developed to execute the full structural analysis of unsaturated GPs. As the first step, the classical MS/MS methods, including precursor ion scan (PIS) and neutral loss scan (NLS), were used to identify their specific subclasses (according to the headgroups) and fatty acyl compositions (number of carbons and degrees of unsaturation) (*SI Appendix*, Figs. S12 and S13). In the second step, the unsaturated GPs of each subclasses were further analyzed using the PB-MS/MS method. Fig. 2 *C* and *D* shows the PB-MS/MS spectra of PC 16:0–18:1 and PE 18:0–20:4 (rat brain tissue), following their selective detection by PIS of *m/z* 184 and NLS of 141 Da, respectively. The presence of two pairs of diagnostic ions at *m/z* 650.6/676.6 and 678.6/704.6 suggested that PC 16:0–18:1 exists as a mixture of PC 16:0–18:1 (9) and 16:0–18:1 (11). PE 18:0–20:4 was identified to consist of 20:4 (5, 8, 11, 14) acyl chain from the four pairs of diagnostic ions (*m/z* 439.4/465.4, 479.4/505.4, 519.4/545.4, and 559.4/585.4). No other C=C location isomers of C20:4 were detected.

Coupling PB-MS/MS with shotgun lipid analysis is straightforward because the identity of each unsaturated lipids is well preserved and the mass tag (+58 Da) of PB products facilitates subsequent MS/MS from complex mixture analysis. As a demonstration, the analysis of polar lipid extract from rat brain tissue resulted in the identification of 96 unsaturated fatty acids and GP species with their C=C locations specified; 50% of them actually existed as mixtures of C=C location isomers. Most importantly, relative quantitation of C=C location isomers of a broad range of lipid species was achieved for the first time, to our knowledge. The relative percent, Rel.%, of a specific C=C location isomer was calculated from the percentage of its diagnostic ion intensity from the summed diagnostic ion intensities of all C=C location isomers. The analysis results of unsaturated fatty acids are summarized in Fig. 4*A*. Fatty acids 16:1, 18:2, and 20:4 were identified as fatty acid 16:1 (9), fatty acid 18:2 (9, 12), and fatty acid 20:4 (5, 8, 11, 14), respectively, without the detection of any C=C location isomers. In contrast, fatty acid 18:1 and fatty acid 19:1 existed both as a mixture of Δ9 and Δ11 isomers. Fatty acid 20:1 was a mixture of Δ11 and Δ13 isomers, whereas fatty acid 22:1 consisted of Δ11, Δ13, and Δ15 isomers. Previously, Johnson et al. performed a comprehensive analysis of monounsaturated fatty acids esterified from lipid extracts of rat and human brains using GC-MS. The reported fatty acid C=C isomers in human brain included fatty acid 18:1 Δ9 and Δ11, fatty acid 19:1 Δ9 and Δ11, fatty acid 20:1 Δ11 and Δ13, fatty acid 22:1 Δ11, Δ13, and Δ15 (35). These C=C location assignments are consistent with our findings. GC-MS, however, requires significantly longer analysis time and much higher sample consumption than PB-MS/MS method (35–37). Absolute quantitation of fatty acids (by wet weight of tissue) was also achieved for lipid species where their C=C location isomer standards could be obtained, including fatty acid 18:1 (9) (0.047 ± 0.005 μM/g), fatty acid 18:1 (11) (0.016 ± 0.003 μM/g), and fatty acid 20:4 (0.24 ± 0.02 μM/g) (rat kidney, $n = 3$).

In agreement with the C=C location isomer composition of free fatty acid 18:1, all C18:1-containing GPs were mixtures of Δ9 and Δ11 isomers. These included PCs, lyso PCs, PEs, lyso PEs, phosphatidylinositols (PIs), lyso PIs (LPIs), phosphatidylserines (PSs), and lyso PSs (LPSs). The Rel.% of C=C Δ9 and Δ11 isomers were also measured based on diagnostic ion intensities, as shown in Fig. 4*B*. Interestingly, the same composition of C=C location isomers was consistently observed for fatty acids and their corresponding GPs as well (*viz.*, C18:2, C20:1, C20:4). Close examination of polyunsaturated fatty acyls revealed that C20:4 and C22:4 existed in pure omega 6 (ω-6) form except for C22:6, which was in ω-3 form. Gross and co-workers (38) and Han and co-workers (39) have reported that fatty acid 18:3 in human plasma has two C=C position isomers, with the ω-3 form [fatty acid 18:3 (9, 12, 15)] being more abundant than the ω-6 form [fatty acid 18:3 (6, 9, 12)] (38, 39). We did not observe any C18:3 either as free fatty acid or fatty acyl in GPs, likely due to their low abundances in rat tissues. GP analysis of rat brain led to the identification of 86 GP species with their C=C locations assigned (molecular identities are listed in *SI Appendix*, Figs. S14–S19 and Scheme S1). To our knowledge, this is the first report of a wide range of unsaturated fatty acids and GPs from complex biological samples, with their C=C locations identified and C=C location isomer compositions obtained. This is attributed to the fast and highly specific analysis offered by the PB-MS/MS method coupled with shotgun lipid analysis.

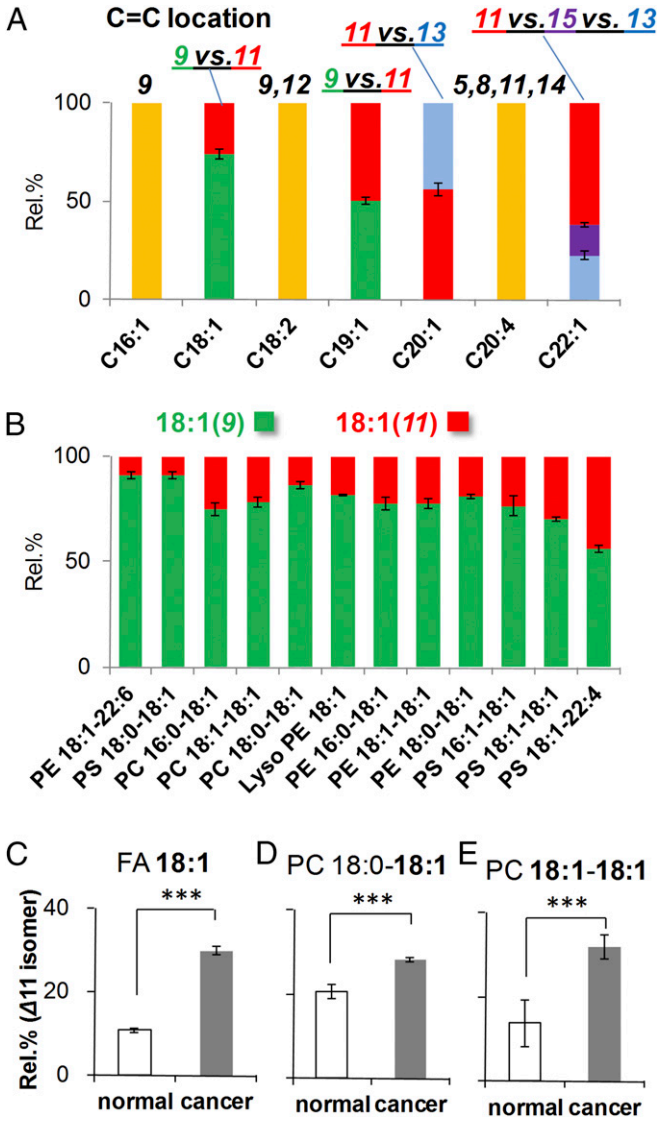

Fig. 4. Fatty acid and GP analysis results from applying PB-MS/MS to shotgun lipid analysis of rat brain tissue. (*A*) The C=C location isomer composition and Rel.% of FAs with chain length ranging from 16 to 22 carbons. (*B*) Rel.% of the Δ9 and 11 C=C isomers for GPs containing C18:1 acyl chains. Comparison of Rel.% of Δ11 C=C location isomers from C18:1 acyl chains between normal and cancerous mouse breast tissues: (*C*) fatty acid 18:1, (*D*) PC 18:0–18:1, and (*E*) PC 18:1–18:1. Error bars represent SD, $n = 5$. Differences between the two groups were evaluated for statistical significance using the two-tailed Student's *t* tests (***$P < 0.0005$).

Cross-tissue analysis of fatty acid and GP C=C location isomers. The PB-MS/MS method makes it possible to compare the compositions of C=C location isomers of any specific lipid species among different types of tissues. Analysis of different types of rat tissue (brain, liver, muscle, adipose, and kidney) all showed that fatty acid 18:1 is composed of Δ9 and Δ11 isomers (*SI Appendix*, Fig. S20). Whereas the Rel.% of the Δ11 isomer was very similar in rat brain, muscle, kidney, and liver (24–26%), it was found to be much lower in rat adipose (13%). Cross-tissue analysis of PC 16:0–18:1 also revealed a constant composition of PC 16:0–18:1 (9) and PC 16:0–18:1 (11) isomers. The Rel.% of the Δ11 isomer however was the highest in the hard tissue such as muscle (59%), compared with other types of tissues (25–35%). Other C18:1-containing GPs also showed a consistency of an increased Rel.% of the Δ11 isomers (59–71%) in rat muscle compared with kidney (20–32%) and liver (36–64%). The variation in Rel.% of the same set of lipid C=C location isomers from different tissue types might be related to their biological functions and could reflect local lipid homeostasis regulated by biosynthetic and metabolic pathways.

Application in the analysis of diseased tissue. The new analytical capability enabled by the PB-MS/MS opens up the possibility of studying the correlations of C=C location isomer compositions with various biological states of a subject. As a proof of principle, we analyzed the lipid extracts from normal and cancerous mouse breast tissues (five normal mice as controls and five mice with breast cancer), with a focus on the C=C location isomers of unsaturated fatty acids and GPs. The targets of analysis included fatty acid 18:1 and several C18:1-containing PCs, such as PC 16:0–18:1, PC 18:0–18:1, and PC 18:1–18:1. As expected, all of these lipids were found to consist of C18:1 Δ9 and Δ11 isomers (*SI Appendix*, Fig. S21 and Table S1). The Rel.% of the Δ11 isomer showed no significant change for PC 16:1–18:1 between normal and cancer breast tissues; however, Δ11 isomers were significantly elevated (***$P < 0.0005$) in the cancerous tissues for fatty acid 18:1 (29.8 ± 0.9% vs. 10.7 ± 0.6%, cancer vs. normal), PC 18:0–18:1 (28.1 ± 0.5% vs. 20.6 ± 1.7%), and PC 18:1–18:1 (32.0 ± 2.9% vs. 13.8 ± 5.4%) (Fig. 4 *C*–*E*). Other than uncovering the C=C location isomers, PB-MS/MS also revealed that the Rel.% of PC 18:0–18:2 (9, 12) within the mixture of PC 36:2 (consisting of PC 18:1–18:1 isomers) was significantly decreased in breast cancer tissues. The capability of monitoring the quantitative changes of unsaturated lipids with high molecular specificity of a lipidome, such as lipid C=C isomer compositions, offers a new perspective to identifying malignancies and diseased state.

Discussion

Choice of PB Reagents. Acetone as the PB reagent has several advantages including good solubility for a variety of polar or nonpolar lipids, high miscibility with water and other organic solvents, and compatibility with ESI. The use of acetone as both solvent and the PB reaction reagent allows a huge stoichiometric excess relative to unsaturated lipids, which assures a reasonable degree of reaction to be achieved for unsaturated fatty acids and GPs, albeit rather limited quantum yield of acetone for PB reactions. For instance, we did not observe appreciable difference in PB reaction yield when varying acetone from 20% to 80% (vol/vol) in aqueous solutions. There are also limitations associated with the use of acetone. Most notably, spectrum complexity is increased due to nonquantitative conversion of unsaturated lipids to their PB reaction products and several side reactions. The mass increase of 58 Da through the PB reactions with acetone can be used as a signature for identifying the products; however, they often overlap with other lipid species in shotgun analysis due to the complexity of the lipid samples. Several issues of chemical interference in PB-MS/MS are discussed in *SI Appendix*, section S5. Monitoring of the changes in intensities as well as the mass shift might help to identify the PB reaction products. For the future development of PB-MS/MS method, it is beneficial to survey a wide variety of carbonyl compounds as PB reagents by exercising the following considerations. The potential PB reagent should have (*i*) high PB reaction yield, (*ii*) fast reaction kinetics, (*iii*) good solubility in the solvent system commonly used for ESI, (*iv*) relatively large molecular weight (~200 Da), and (*v*) preferential formation of C=C diagnostic ions from low energy CID of the PB reaction products.

Implication of Lipid C=C Location Isomer Composition to Lipid Biology and Biomarker Discovery. Naturally occurring changes associated with unsaturated lipids have been reported to contribute both positive and negative effects on the development and progression of cancer (40), cardiovascular disease (41), and type 2 diabetes (42). Additionally, increasing evidence supports that the composition of lipid C=C location isomers, especially ω-3 and ω-6 fatty acid ratios, plays important roles in the development of

a series of chronic diseases (43). For instance, α-linolenic acid (fatty acid 18:3 ω-3) is found to prevent cardiovascular disease (41, 43), whereas γ-linolenic acid (fatty acid 18:3 ω-6) worsens the disease condition (41). Our findings add to this growing body of evidence that depending on the health stages and type of tissues, C=C location isomer compositions can vary and serve as potential biomarkers.

Conclusion

The composition of a cell lipidome is under a precise regulation, which is an integral part of a range of critical cellular processes. Systematic monitoring and profiling unsaturated lipid compositions with the C=C location specificity shall provide extremely valuable information for biological studies. The PB-MS/MS approach developed in this study is not only fast and sensitive, but also can provide high-confidence identification and quantitation of a wide variety of unsaturated lipid species. The experimental setup is simple and does not require any modifications of a mass spectrometer. It is also highly compatible with existing lipid analysis workflows using ESI-MS/MS. The unique capability of profiling lipid C=C location isomer compositions should enable new investigations on a range of enigmatic problems in lipid biology related to the lipid unsaturation and C=C locations. Our results clearly show the significant differences in C=C location isomer compositions of several fatty acid and GP species between normal and cancerous tissues. Other than the demonstrated application for shotgun lipid analysis herein, the PB-MS/MS method can be potentially applied to other lipid analysis platforms including liquid chromatography MS, MS imaging, ambient MS, and direct sampling MS. With further development, PB-MS/MS should serve as a simple and generally applicable tool for the exploration of the roles of lipid C=C location isomers in biological processes and biomarker discovery for disease diagnosis.

Materials and Methods

Fatty acid and GP standards (Avanti Polar Lipids, Inc.) and polar lipid extracts from rat or mouse tissue samples were dissolved in 50/50 (vol/vol) acetone/water for MS analysis unless otherwise mentioned. A low-pressure mercury lamp with emission around 254 nm was placed 1.0 cm away from the nanoESI emitter (pulled from borosilicate glass capillaries) to initiate PB reactions. All MS experiments were performed on a 4000 QTRAP triple quadrupole/linear ion trap hybrid mass spectrometer (Sciex). See *SI Appendix*, section S1 for experimental detail.

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