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Identification of Double Bond Position Isomers in Unsaturated Lipids by *m*-CPBA Epoxidation and Mass Spectrometry Fragmentation

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Abstract

Lipids are highly diverse biomolecules associated with several biological functions including structural constituent, energy storage, and signal transduction. It is essential to characterize lipid structural isomers and further understand their biological roles. Unsaturated lipids contain one or multiple carbon–carbon double bonds. Identifying double bond position presents a major challenge in unsaturated lipid characterization. Recently, several advancements have been made for double bond localization by mass spectrometry (MS) analysis. However, many of these studies require complex chemical reactions or advanced mass spectrometers with special fragmentation techniques, which limits the application in lipidomics study. Here, an innovative *meta*-chloroperoxybenzoic acid (*m*-CPBA) epoxidation reaction coupling with collision-induced dissociation (CID)-MS/MS strategy provides a new tool for unsaturated lipidomics analysis. The rapid epoxidation reaction was carried out by *m*-CPBA with high specificity. Complete derivatization was achieved in minutes without overoxidized byproduct. Moreover, diagnostic ion pair with 16 Da mass difference indicated localization of carbon–carbon double bond in MS/MS spectra. Multiple lipid classes were evaluated with this strategy and generated abundant fragments for structural analysis. Unsaturated lipid analysis of yeast extract using this strategy took less than 30 min, demonstrating the potential for high-throughput lipidomics analysis by this approach. This study opens a door for high throughput unsaturated lipid analysis with minimal requirement for instrumentation, which could be widely applied in lipidomics analysis.

Graphical Abstract:

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.8b04905. Figures S1–S7 and Tables S1 and S2 (PDF).

The authors declare no competing financial interest.

Lipids are highly diverse molecular components in cellular membranes. The structural isomers and distributions of lipids vary significantly in different biological systems. They have several major functions in cell, including structural constituent, energy storage, and cellular signaling.¹ Numerous diseases are associated with disturbance in the metabolism of lipids, such as type-2 diabetes,² cancer,³ and depression.⁴ Since small structural changes can alter a molecule's biochemical function, it is essential to distinguish and characterize different isomers in order to further understand their biological roles.

Unsaturated lipids are a subclass of lipids containing one or multiple carbon–carbon double bonds. Due to additional double bond position, analysis of unsaturated lipids remains challenging. With advancements in mass spectrometry (MS) instrumentation and chemical strategies, substantial progress has been made during the past decade. Novel fragmentation methods such as ozone-induced dissociation (OzID),^{5–9} ultraviolet photodissociation (UVPD),^{10–15} and chemical reactions, such as ozonolysis^{9,16,17} and cross-metathesis,¹⁸ have been developed to enable pinpointing carbon–carbon double bond position in unsaturated lipids.

Paternò–Büchi (P–B) reaction was introduced to localize carbon–carbon double bond position for unsaturated lipids within the past few years.^{19–23} A four-membered oxetane ring was generated from a carbonyl and an olefin after the photochemical reaction. The oxetane ring could be cleaved upon MS fragmentation to yield diagnostic ion pair for carbon–carbon double bond localization. P–B strategy has been rapidly applied into multiple types of lipid analysis on both electrospray ionization (ESI)^{19–23} and matrix-assisted laser desorption/ionization (MALDI)²⁴ instrument platforms. However, the conversion of P–B reactions is limited. The incomplete reaction creates heterogeneous mixtures with greater complexity due to presence of both unreacted lipids and partially modified species. Therefore, an innovative strategy is needed with high reaction yield, simplified workflow and minimum instrument requirement relying on simple collision-induced dissociation (CID)-MS/MS fragmentation that is widely accessible, rather than requiring multistage or unique fragmentation and additional apparatus.

Recently, epoxidation reaction has been utilized in lipid double bond localization analysis.^{25–27} In situ generated dioxirane by low temperature plasma was used for epoxidation. After derivatization, fatty acid epoxide could generate a pair of fragments with 16 Da difference upon CID fragmentation. Increased conversion with overoxidized byproduct was observed by extending reaction time. Thus, reaction specificity needs to be improved to achieve complete epoxidation without side reaction.

meta-Chloroperoxybenzoic acid (*m*-CPBA) is an oxidant widely used in organic synthesis.²⁸ Epoxidation of olefin with *m*-CPBA in dichloromethane (DCM) offers high specificity, complete conversion and minimal side reaction. The ease of handling as well as rapid reaction make it an ideal candidate to be coupled with MS analysis.

Here, we couple *m*-CPBA epoxidation with CID-MS/MS for lipid double bond isomer identification. After derivatization, a pair of diagnostic fragments with 16 Da mass difference is generated upon MS/MS fragmentation for multiple types of lipid isomer identification. Specifically, glycerophospholipids including phosphatidylcholine (PC), phosphatidic acid (PA), phosphatidylglycerol (PG), lyso phosphatidylethanolamine (PE), and lyso phosphatidylserine (PS) can generate diagnostic ion pairs in MS/MS spectra, thus, this strategy offers broader instrument accessibility without needing multi-stage fragmentation. Furthermore, no cleanup is required for *m*-CPBA derivatization prior to direct infusion analysis. The entire process including sample preparation, epoxidation, and data acquisition only takes less than 30 min, providing a powerful tool for high-throughput lipidomics analysis.

EXPERIMENTAL SECTION

Materials and Reagents.

Dichloromethane (DCM), water, and acetonitrile (ACN) were purchased from Fisher Scientific (Pittsburgh, PA). *meta*-Chloroperoxybenzoic acid (*m*-CPBA) was purchased from Sigma-Aldrich (St. Louis, MO). All lipid standards and yeast extract total were purchased from Avanti Polar Lipids (Alabaster, AL). All reagents were used without additional purification.

Epoxidation of Unsaturated Lipids and Yeast Extract Total with *m*-CPBA.

The unsaturated lipid standards and yeast extract total sample were reacted with *m*-CPBA reagent, respectively. A total of 10 μ g of unsaturated lipid standards or yeast extract total was mixed with 10 μ L DCM containing 10 μ g/ μ L *m*-CPBA. The reaction was incubated at room temperature for 10 min. After incubation, 500 μ L of 50% ACN was added to quench and dilute reaction system. Diluted sample was used for direct infusion analysis without cleanup.

Direct Infusion Electrospray Ionization (ESI)-MS/MS Analysis.

A 500 μ L Hamilton syringe was used for direct infusion. An Orbitrap Elite mass spectrometer (Thermo Scientific, Bremen, Germany) was used for ESI-MS/MS analyses. The injection rate was set at 10 μ L/min.

The following MS parameters were used for all data acquisitions. Samples were ionized in either positive or negative ion mode. For positive ion mode acquisition, spray voltage was set at 3.6 kV, S-lens RF level was set to be 55, and capillary temperature was set to be 300 °C. For negative ion mode, spray voltage was set at 5 kV, S-lens RF level was set to be 50, and capillary temperature was set to be 160 °C. Full MS scans were acquired at *m/z* 200–1500 with resolving power of 120 K (at *m/z* 400). Maximum injection time of 500 ms,

automatic gain control (AGC) target value of $5e5$, and 1 microscan were used for full MS scans. Data acquisition was set at 1 min for lipid standards and 10 min for yeast extract with top 50 data-dependent MS² analysis. Both full MS and MS/MS acquisitions were performed in the Orbitrap. All collision-induced dissociation MS/MS were operated at a normalized collision energy of 30, while higher-energy collisional dissociation (HCD) was operated at a normalized collision energy of 70 for fatty acids. Dynamic exclusion of acquired precursors was set at 60 s with a ± 10 ppm tolerance for yeast extract analysis.

Unsaturated Lipid Data Analysis.

Unsaturated lipids were identified by accurate mass matching at full MS and diagnostic ion pair with neutral loss fragments matching at MS/MS. Fragment pairs with 18 Da difference could be neutral loss of acyl chain or headgroup for complex lipids. Fragment pairs with 16 Da difference were cleaved from both sides at epoxide. ChemDraw software was used for lipid structural determination and accurate mass calculation.

RESULTS AND DISCUSSION

The mechanism of *m*-CPBA epoxidation with CID-MS/MS analysis for unsaturated lipids is shown in Scheme 1. Double bond can be oxidized by *m*-CPBA to generate an epoxide. After epoxidation, 15.9949 Da (oxygen atom) is added to each double bond. For lipid isomer with one carbon–carbon double bond, the mass of PC 16:0–18:1 (9) increased from 760.5851 to 776.5800 Da. Then, epoxide can be cleaved from both sides to generate a pair of fragments (634.4442 and 650.4391 Da) with 15.9949 Da (oxygen atom) difference upon fragmentation. The generated aldehyde and olefin fragments facilitate double bond localization analysis for unsaturated lipids.

Epoxidation performances including reaction efficiency, specificity, and stability with *m*-CPBA were evaluated with PC 16:0–18:1 (9). For epoxidation reaction, 10 μg of PC 16:0–18:1 (9) was mixed with 100 μg *m*-CPBA in 10 μL of DCM. The reaction was incubated at room temperature for conversion study at different time points. After incubation, 500 μL of 50% acetonitrile (ACN) was added to quench and dilute the reaction system. Diluted sample was used for direct infusion analysis without additional cleanup. Conversion of epoxidation (Figure S1) rapidly increased with extended reaction time and achieved complete conversion at 10 min. Overoxidized byproduct was not observed with *m*-CPBA epoxidation. Moreover, the epoxide of PC 16:0–18:1 (9) was stable for over 1 week in diluted solution.

With complete conversion of *m*-CPBA epoxidation, this method allowed increased sensitivity for identification of lipids at low abundance. The limit of detection of PC 16:0–18:1 (9) was examined at 12.9 nM for direct infusion analysis. Furthermore, the *m*-CPBA epoxidation also worked for a broad dynamic range and maintained good linear relationship of signal intensity with lipid concentration ranging from 0.01 to 100 $\mu\text{g}/\text{mL}$ (Figure S2).

After evaluation of rapid and specific epoxidation, fragmentation behavior was compared between CID and HCD fragmentation methods. The mass spectrum in Figure 1 generated by CID shows a simple fragmentation pattern, including acyl chain loss fragments, headgroup loss fragments, and a pair of diagnostic fragments. A normalized collision energy of 30 was

selected to generate abundant fragments after optimization (Figure S3). HCD spectra in Figure S4 also showed acyl chain and headgroup loss fragments. However, the intensity of diagnostic fragments in the HCD spectra was relatively low with additional headgroup loss, which could be problematic in predicting diagnostic fragments for complex lipids containing multiple double bonds.

To further evaluate fragmentation behavior of lipids containing multiple carbon–carbon double bonds, 3 PC isomers, PC 18:1 (6)–18:1 (6), PC 18:1 (9)–18:1 (9), and PC 18:0–18:2 (9, 12) were analyzed by *m*-CPBA epoxidation and ESI-MS/MS analysis. These PC isomers have identical chemical formula and exact masses. They contain total 36 carbons in both acyl chains with 2 double bonds at different positions. Similar to PC 16:0–18:1 (9), PC isomers with multiple double bonds generate distinct diagnostic fragments upon CID fragmentation. Figure 2 shows fragmentation patterns of PC isomers with 2 double bonds at different positions. Acyl chain and headgroup losses with diagnostic fragment pair were observed for all 3 isomers, including epoxide cleavage at 634.4078 and 650.4028 Da for PC 18:1 (6)–18:1 (6), 676.4548 and 692.4497 Da for PC 18:1 (9)–18:1 (9). Due to two double bonds located in a single acyl chain, PC 18:0–18:2 (9, 12) yields two pairs of diagnostic fragments at 662.4755 Da/678.4704 Da, and 718.5017 Da/734.4967 Da. Detailed structure annotations for three lipid isomers were illustrated in Table S1. In contrast, only partial diagnostic fragment pairs were detected with HCD fragmentation, shown in Figure S5. Thus, CID fragmentation was employed for lipid carbon–carbon double bond localization analysis.

Besides PC lipids, several other classes of lipid standards were tested with *m*-CPBA epoxidation and CID-MS/MS strategy. Figure 3 shows fragmentation patterns of fatty acids, lyso PE, lyso PS, PA, and PG after derivatization. Amine group, hydroxyl group, and carboxylic acid were compatible with *m*-CPBA epoxidation. No side reaction or byproduct was observed. Fatty acids (Figure 3a,b) were detected in negative ion mode while other classes of lipids (Figure 3c–f) were detected in positive ion mode. FA 16:1 (9) generated a pair of diagnostic fragments at 155.1111 and 171.1063 Da, while FA 18:2 (9, 12) produced both pairs of fragments at 155.1106 Da/ 171.1057 Da, and 211.1375 Da/227.1327 Da for double bond localization analysis. The normalized collisional energy (NCE) of CID-MS/MS required for different classes of lipids remain constant at 30, which made it easy to generalize this method for complex lipid mixtures consisting of multiple lipid classes. HCD-MS/MS could also generate diagnostic ion pair with higher NCE at 70 for fatty acids shown in Figure S6. However, *cis*- and *trans*-double bond geometry isomers did not show any difference upon either CID or HCD fragmentation. We also performed MS/MS fragmentation for poly unsaturated fatty acid FA 20:4 (5, 8, 11, 14). The epoxide fragments from four double bonds could still be observed while multiple unexpected fragments were also detected (Figure S7). Protonated lyso PE 17:1 (10) yielded diagnostic ions at 368.1838 and 384.3114 Da. For lyso PS 18:1 (9), PA 18:1 (9)–18:1 (9), and PG 18:1 (9)–18:1 (9), sodiated precursor ions were required to generate full pair of diagnostic ions in positive ion mode while no epoxide fragment was detected in negative ion mode.

After assessing the *m*-CPBA epoxidation and CID-MS/MS strategy for localizing double bonds in different classes of lipids, this strategy was applied to yeast extract for high

throughput unsaturated lipidomics analysis. Due to same fragmentation condition (CID at 30) required by multiple classes of lipids, a general data-dependent acquisition (DDA) method was used for direct infusion analysis. For epoxidation reaction, 10 μg of lipid extract was used to mix with 100 μg *m*-CPBA in 10 μL DCM. The reaction was incubated at room temperature for 10 min. Then, 500 μL of 50% ACN was added to quench and dilute reaction system for direct infusion analysis without cleanup. Before derivatization, isomers with only one double bond difference were difficult to distinguish: the monoisotopic peak of the ion with *N* double bond(s) overlapped with the third isotopic peak of the ion with *N*+ 1 double bond(s). However, after epoxidation, isomers with different numbers of double bond(s) took up different numbers of oxygen atoms to yield more pronounced mass difference. For instance, the monoisotopic peak of PC 16:0–16:1 (9) overlapped with the third isotopic peak of PC 16:1 (9)–16:1 (9) before epoxidation while 14 Da mass difference was observed after derivatization. Meanwhile, each isomer could be selected for DDA analysis to generate MS/MS spectra for double bond location information. Figure 4 shows comparisons of full MS spectra before and after epoxidation with *m*-CPBA. Four PC isomers are highlighted for comparisons and fragmentation illustrations. With *m*-CPBA and MS/MS strategy, different PC isomers could be separated by mass and fragmented to generate diagnostic ion pairs for double bond localization analysis. In total, 19 unsaturated lipid isomers were identified from yeast extract with *m*-CPBA epoxidation and CID-MS/MS by direct infusion analysis.

CONCLUSIONS

In this study, an innovative strategy has been developed for unsaturated lipid double bond localization using *m*-CPBA epoxidation and CID-MS/MS analysis. The epoxidation reaction conditions and different MS/MS acquisition parameters have been carefully compared and optimized to achieve the best performance for identification. Several classes of unsaturated lipids have been evaluated with *m*-CPBA epoxidation and CID-MS/MS strategy. With CID fragmentation of NCE 30, full pair of diagnostic fragments can be observed for different lipids with one or multiple carbon–carbon double bonds. In addition, this strategy has been applied to yeast extract for complex sample analysis. In total, 20 unsaturated lipid isomers have been identified with their double bond positions localized. This study also demonstrates the utility of MS/MS for complex unsaturated lipid analysis. With rapid epoxidation, minimal instrument requirement and ease for data interpretation, *m*-CPBA epoxidation coupled with CID-MS/MS strategy provides a powerful tool for global unsaturated lipidomics profiling. In conclusion, we anticipate that this new analytical strategy for unsaturated lipids can be widely applied for a variety of lipidomics analyses in many biological systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. CID-MS/MS spectrum of PC 16:0–18:1 (9) epoxide. Fragments of a and b represent PC acyl chain loss. Fragment c represents PC headgroup loss. Red stars marked peak pairs are fragments generated from epoxide to pinpoint carbon–carbon double bond location.

Figure 2. CID-MS/MS spectra of epoxidation products of PC 18:1 (6)–18:1 (6) (a), PC 18:1 (9)–18:1 (9) (b), and PC 18:0–18:2 (9, 12) (c). Red stars demonstrate the fragments of cleavage sites from PC isomers, which are used to differentiate lipids with multiple carbon–carbon double bonds.

Figure 3.

CID-MS/MS spectra of epoxides of FA 16:1 (9) in negative ion mode (a), FA 18:2 (9, 12) in negative ion mode (b), protonated Lyso PE 17:0 (10) in positive ion mode (c), sodiated Lyso PS 18:1 (9) in positive ion mode (d), sodiated PA 18:1 (9)-18:1 (9) in positive ion mode (e), and sodiated PG 18:1 (9)-18:1 (9) in positive ion mode (f). Red stars represent epoxide diagnostic pairs.

Figure 4.

Full MS spectra of (a) lipids from yeast extract before (upper trace) and after *m*-CPBA epoxidation (lower trace). Before epoxidation, the third isotopic peak of PC 16:1 (9)–16:1 (9) overlaps with monoisotopic peak of PC 16:0–16:1 (9). After epoxidation, isomers are separated by different oxygen uptake and selected for MS/MS analysis. MS/MS spectra of epoxides of PC 16:0–16:1 (9) (b), PC 16:1 (9)–16:1 (9) (c), PC 16:0–18:1 (9) (d), and PC 16:1 (9)–18:1 (9) (e) at CID 30. Stars represent epoxide diagnostic ion pairs.

Scheme 1.
Mechanism of *m*-CPBA Epoxidation and CID-MS/MS To Produce Diagnostic Fragment Ion Pair