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Chemistry-driven mass spectrometry for structural lipidomics at the C=C bond isomer level

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ABSTRACT

As more and more studies have shown that lipid molecules play an important role in the whole biology, in-depth analysis of lipid structure has become particularly important in lipidomics. Mass spectrometry (MS), as the preferred tool for lipid analysis, has greatly promoted the development of this field. However, the existing MS methods still face many difficulties in the in-depth or even comprehensive analysis of lipid structure. In this review, we discuss recent advances in MS methods based on double bond-specific chemistries for the resolving of C=C location and geometry isomers of lipids. This progress has greatly advanced the lipidomics analysis to a deeper structural level and facilitated the development of structural lipid biology.

1. Introduction

Lipids are an important class of biologically active molecules, and increasing evidence suggests that lipids play a critical role not only in cell membrane construction and energy storage, but also in signal transduction and regulation of protein function in cells [1–4]. Living organisms contain thousands of different lipid molecules, which fall into eight main categories, including fatty acids (FAs), glycerolipids (GLs), glycerophospholipids (GPLs), sphingolipids (SLs), sterol lipids, prenol lipids, saccharolipids and polyketides. The biological functions of lipid molecules depend largely on their structures, and the physiological roles of these different lipids and how their content varies with the course of a disease remain largely unknown [5]. The main reason for this is that there are still enormous challenges in structurally resolving and accurately quantifying lipid molecules in complex biological systems [6].

Modern mass spectrometry (MS) has unparalleled advantages in the qualitative and quantitative analysis of molecules, and is therefore also widely used in the analysis of lipidomes [7,8]. MS-based lipidomics can analyze lipids in biological samples on a large scale and obtain information such as their structure and concentration changes, and has been playing an important role in the study of different biological systems [2,9]. However, conventional lipidomics usually uses electrospray ionization (ESI)-based liquid chromatography (LC)-MS analysis to obtain MS/MS spectra of lipids for structural analysis by direct collision-induced dissociation (CID) of underivatized lipid ions [2], which mainly yields information on lipid species and fatty acyl chain composition, and it is difficult to obtain deep structural information related to lipid isomers [7]. Lipid molecules are structurally complex and exist in a variety of isomers [10]. For example, in the case of GPLs, a large number of isomers arise due to differences in C=C bond position, C=C bond geometric configuration, stereospecific numbering (*sn*) of the fatty acyl chain, position of substituents on the fatty chains, and stereo configurations of the chiral centers (Fig. 1).

The C=C bond is present in the fatty chains of unsaturated lipids [11], which gives rise to a number of isomers of unsaturated lipids, including the positional isomers of the C=C bond in the fatty chains and the *cis-trans* isomers of the C=C bond [12]. Disproportionality of the positional isomers of the C=C bond has been demonstrated to be closely associated with the development of certain diseases [13,14], and the transformation of the configuration of the double bond has an important impact on the biological functions of cell membranes [15]. The content of isomers can be affected by dysregulation of lipid metabolism due to the unset or progression of the disease. For example, the proportions of several pairs of C=C position isomers C18:1 (Δ9/Δ11) in phosphatidylcholine (PC) and phosphatidylethanolamine (PE) show significant difference between normal controls and tissue from breast cancer patients and plasma from type 2 diabetes patients [16]. Therefore, the resolving of the fine structure of C=C bond isomers in

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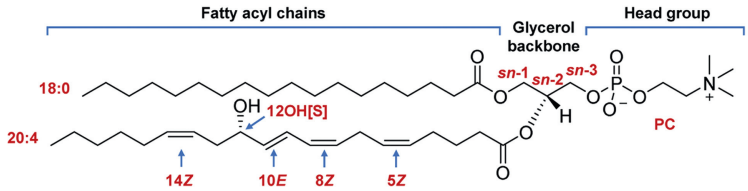

Identified structural levels	Head groups of lipids	Total fatty acyl chain composition	Individual chain compositions	C=C bond locations	C=C bond geometric configurations	*sn*-position of fatty acyl chains	Substitutions at fatty acyl chain	Nomenclature
Subclass	☑							PC
Species level	☑	☑						PC 38:4 (OH)
Molecular species level	☑	☑	☑					PC 18:0_20:4 (OH)
C=C position level	☑	☑	☑	☑				PC 18:0_20:4 (Δ5,8,10,14) (OH)
C=C configuration level	☑	☑	☑	☑	☑			PC 18:0_20:4 (5*Z*,8*Z*,10*E*,14*Z*) (OH)
sn-position level	☑	☑	☑	☑	☑	☑		PC 18:0/20:4 (5*Z*,8*Z*,10*E*,14*Z*) (OH)
Full structure level	☑	☑	☑	☑	☑	☑	☑	PC 18:0/20:4 (5*Z*,8*Z*,10*E*,14*Z*) (12OH[S])

Fig. 1. Hierarchical diagram of complex lipid structure exemplified by PC 18:0/20:4 (5*Z*,8*Z*,10*E*,14*Z*) (12OH[S]). The top chemical structure shows the lipid with complete structure level. The bottom table shows different levels of lipid identification and their nomenclatures. *sn*, stereospecific numbering of the fatty acyl chain.

biological systems and their accurate quantification are key to the study of their functions. Unfortunately, conventional MS-based analytical methods are difficult to characterize C=C bond isomers because they are indistinguishable in molecular weight and tandem MS is difficult to produce characteristic fragments directly.

In recent years, researchers have innovatively developed a variety of chemistry-assisted MS strategies [17–19], which have largely solved the difficult problem of structural resolution of C=C bond isomers, allowing the structural and functional studies of lipids to go deeper into the level of isomers. Here, we will review the chemistry-driven MS methods that have emerged in recent years for the positional and geometrical configuration resolution of lipid C=C bond isomers.

2. Chemical reaction-assisted identification of C=C bond location in lipids

As representatives of soft ionization techniques, ESI-MS and matrix-assisted laser desorption/ionization (MALDI)-MS can provide intact mass information of molecules and are widely used in the analysis of bioactive molecules in complex biological systems. For the resolving of the double bond position in C=C bond isomers, if the lipid ion can be fragmented at the double bond position, the position of the double bond in fatty acyl chain can be inferred from the mass difference between the intact ion mass and the characteristic fragmented ion. However, it is difficult to resolve the structure of the C=C bond isomer because of the high bond energy of the C=C bond, which makes it difficult to break under commonly used MS fragmentation methods such as CID. Thus, researchers have taken advantage of the chemical reactivity of the C=C bond by using double-bond-specific chemical reactions or photochemical reactions to activate or directly break the double bond, thereby generating diagnostic ions that can pinpoint the double bond, and ultimately realizing the precise resolution of the double-bond position isomers.

2.1. Ozonolysis-based MS method for locating C=C bond

Ozone is a very reactive allotrope of oxygen. The reaction of ozone with an olefin leads to the oxidative cleavage of the olefin in a process known as ozonolysis, in which the C=C bond is replaced by a carbon-oxygen double bond, resulting in the desired carbonyl product. Ozonolysis was first used in conjunction with gas chromatography (GC) to characterize the double bond positions of unsaturated FAs by analysis of cleavage products [20,21]. The coupling of the ozonolysis reaction with ESI-MS and MALDI-MS has greatly expanded its application in the analysis of double-bond isomers of unsaturated lipids. In 2006, Blanksby *et al.* demonstrated an on-line approach for the identification of double bond position in intact phospholipids based on ozone-induced dissociation (OzID) (Fig. 2A) [22]. They modified a conventional ESI ion source by using oxygen as the electrospray nebulizing gas in combination with high electrospray voltages to initiate the formation of an ozone-producing plasma. In this method, the C=C bonds present in unsaturated phospholipids are cleaved by ozonolysis to give two chemically induced fragment ions that may be used to unambiguously assign the position of the double bond (Fig. 2A). For ozone electrospray ionization-MS (OzESI-MS), although the *m/z* values of the two product ions are directly correlated with the precursor ions, its ability to analyze complex lipid mixtures is limited because the link between the precursor ions and the two ozonolysis products is difficult to construct when complex lipid mixtures are simultaneously exposed to ozone vapor [23,24]. However, the combination of OzID and mass-selection technique in a quadrupole linear ion trap mass spectrometer could overcome this challenge and enable the identification of C=C bonds locations in intact unsaturated lipids after mass separation [23]. OzID is applicable to a range of lipids such as FA, GPL, triglycerides (TG) and SL [25]. In addition, sodium addition ions are often used to elucidate the structure of lipids such as PC and TG because alkali metal addition ions are more reactive than protonated and deprotonated ions [25]. Combining OzID with CID to produce both CID and OzID product ions enables

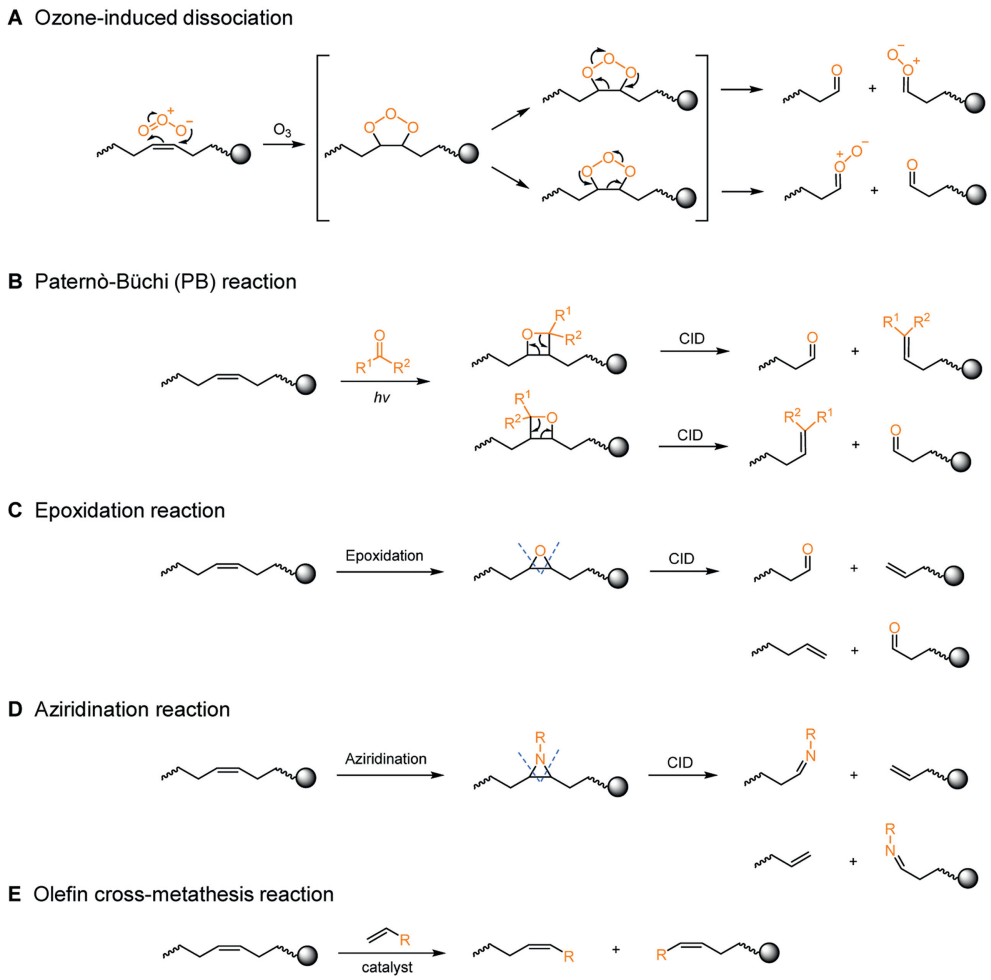

Fig. 2. Chemical reactions for C=C bond activation and location in lipids. (A) Ozone-induced dissociation. (B) Paternò-Büchi reaction. (C) Epoxidation reaction. (D) Aziridination reaction. (E) Olefin cross-metathesis reaction.

the determination of the C=C position of the GPL acyl chain, an important step toward comprehensive top-down lipidomic structure elucidation [26]. Using a similar approach it is also possible to unambiguously identify conjugated double bonds FA methyl esters [27].

OzID was also combined with MALDI-MS imaging (MSI) on a linear ion trap mass spectrometer, enabling the visualization of multiple isomeric lipids in different spatial regions of the rat brain [28]. However, the ozonolysis typically requires reaction time of up to 10 s to produce sufficient products ions for each pixel [23,28]. Notably, this challenge can be overcome by modifying the ion-mobility mass spectrometer with a higher-pressure compartment, allowing the use of high concentration of ozone for the reaction and reducing the ozonolysis time to the millisecond level [29]. By implementing isomer-resolved MALDI-MSI on an ion mobility mass spectrometer, the acquisition speed was increased by a factor of about 50 and the product ion yields were higher, enabling either imaging of larger areas or higher spatial resolution in practical time frames for both C=C location and *sn*-position isomer resolving [30]. In addition, OzID has been combined with LC equipped with a C18 reversed-phase column to reduce the complexity of lipid analysis of biological samples and to provide additional information (*e.g.*, retention times) that allows the establishment of correlations between precursor and product ions without the need for prior mass selection [31]. Meanwhile, the millisecond timescale OzID can also be incorporated in an integrated and portable MS system [32].

2.2. Paternò-Büchi reaction for locating C=C bond

2.2.1. Ultra-violet light-activated Paternò-Büchi reaction for locating C=C bond

The Paternò-Büchi (PB) reaction is [2 + 2] photocycloaddition between an electronically excited carbonyl compound, such as ketone or aldehyde, and an alkene, forming an oxetane ring (Fig. 2B) [33,34]. The formation of oxetanes usually occurs under ultra-violet (UV) light excitation. The PB reaction can occur between carbonyl compounds and unsaturated lipids, forming an oxetane ring. The resulting oxetane structure serves to chemically activate the lipid double bond, thus making the derivatized product susceptible to fragmentation during CID. The characteristic diagnostic ions can indicate the location of double bond. In 2014, Xia's group reported for first time the combination of PB reaction and MS for the identification of lipid C=C double bond positions [35]. They found that the on-line PB reaction with unsaturated lipids could be performed under UV irradiation using acetone as the carbonyl reagent. Combined with tandem MS, the positions of C=C in various types of lipids could be successfully identified [35]. By combining the PB-tandem MS (PB-MS/MS) approach and shotgun lipidomics analysis, 96 unsaturated FAs and GPLs from rat brain tissue were identified at C=C location level, and 50% of them existed as mixtures of C=C location isomers; relative quantitation of most of the C=C location isomers were obtained [36]. The alterations of compositions of C=C location isomers between healthy and cancerous tissue were observed (Fig. 3).

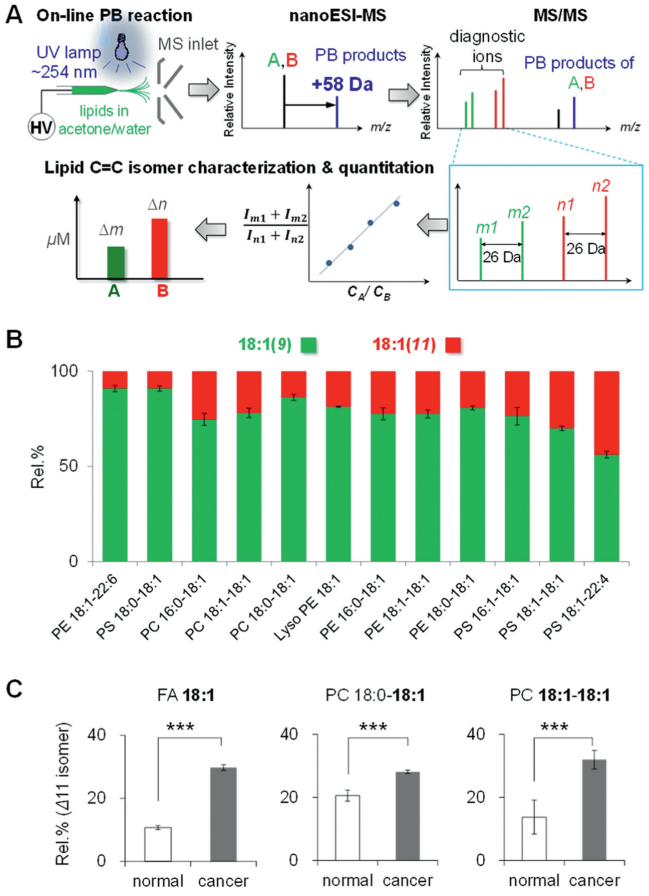

Fig. 3. Analysis of lipid C=C bond positional isomers by using PB reaction and MS. (A) Analysis workflow for the identification and quantitation of C=C location isomers of lipid. (B) The relative content of the Δ9 and Δ11 C=C isomers for GPs containing C18:1 fatty acyl chain in rat brain tissue. (C) Comparison of Rel.% of Δ11 C=C bond positional isomers from C18:1 acyl chain between normal and cancerous mouse breast tissues. Error bars represent SD, $n = 5$. Differences between the two groups were evaluated for statistical significance using the two-tailed Student's *t*-tests (***$P < 0.0005$). Reproduced with permission [36]. Copyright 2016, PNAS.

Although PB reaction can be used for identification and quantitation of C=C location isomers present in biological extracts with direct infusion MS mode, isomeric/isobaric interference and detection challenge for low-abundance lipids compromise its applicability. These challenges can be greatly alleviated by a prior separation step, such as LC with a reserved-phase C18 column. By establishing the LC-PB-MS/MS system with acetone as PB reagent for large scale lipid analysis, more than 200 unsaturated GPLs in bovine liver were identified at C=C location level, among which 55 groups of C=C location isomers were revealed [16].

It is worth noting that side reactions of traditional UV-light-activated PB reaction during lipid derivatization (mainly Norrish type I and II reactions) can affect the detection sensitivity and compromise the structural analysis capability. The Norrish type I side reaction is initiated by the photochemical cleavage or homolysis of ketone or aldehyde into two reactive free radical intermediates [33]. The Norrish II type side reaction results from the abstraction of a hydrogen atom at the allyl position of an olefin by the oxygen atom of excited carbonyl PB reagent. The resulting allyl radical forms a C-C or C-O coupling by-product with the carbonyl compound [37]. In the past several years, a series of alternative PB reagents were developed to reduce the side reactions, such as 2′,4′,6′-trifluoroacetophenone (triFAP) [37], benzophenone [38], and benzaldehyde [39]. These phenyl-containing carbonyl compounds have a more efficient absorption of UV light in the long wavelength region than acetone [40], and the phenyl group can stabilize the T1 excited state of the carbonyl substrate. In these cases, Norrish Type I cleavage was largely diminished; however, the Norrish Type II side reaction was more competitive, producing products isomeric to the PB reaction products [33,41]. Among the acetophenone derivatives, triFAP shows the best performance. It offers a relatively high PB yield (20%−30%) for different types of C=C [41]. Specifically, the PB yields were compared using PC 16:0/18:1 (9Z) as the model compound and showed that triFAP has the highest yield (23%), followed by acetone (18%) and 4-CF_3AP (14%) [37].

Conventional PB reagents generally do not carry groups that can be easily protonated, so the ionization efficiency of the formed oxetane product is low, which can affect the sensitivity of the lipid isomer analysis. To address this issue, several PB reagents with high ionization efficiencies have been developed to improve the sensitivity of the analysis of low-polarity lipids (*e.g.*, cholesteryl esters (CEs) [41] and GLs [42]) in ESI. For example, Xia *et al.* took 2-acetylpyridine (2-AP) as readily protonated PB reagent to profile C=C location isomer composition of CE at sub-nmol/L range in pooled human plasma [41]. Compared to intact CE detected as ammonium adduct ions $[CE + NH_4]^+$, 2-AP derivatized CE detected as protonated ions $[CE + 2\text{-AP} + H]^+$ detection signal had been improved by 8 times although the reaction conversion rate is 20%−30% in 30 s.

The high sensitivity of 2-AP PB-MS/MS system further allows single-cell deep lipid analysis at the C=C location level whereby the classification of four subtypes of human breast cancer cells was facilitated [42]. This method also allows charge-switching which enables lipids typically best analyzed in negative-ion mode to be detected in positive-ion mode, such as FAs, greatly improving the depth of lipid analysis in a single process [43]. Furthermore, a broad range of PB reagent substitutes allow flexibility in optimal reagent selection for different lipid analysis condition.

PB reaction can also be coupled to MSI. Bednařík *et al.* reported a PB-MALDI-MS/MS procedure, an offline on-tissue PB reaction using benzaldehyde as a MALDI-MSI compatible reagent [39], and observed highly differential expression levels of several double-bond-position isomers of phosphatidylserine and PC in the white and gray regions of the mouse cerebellum. Wäldchen *et al.* reported benzophenone as a MALDI-MSI compatible reagent, revealing GPL double-bond-position isomers in mouse cerebellum and male *Schistosoma mansoni* [44]. Compared to OzID, there is no need for specialized instrumentation for PB reaction coupled to MSI.

2.2.2. Visible-light-activated [2 + 2] cycloaddition reaction for locating C=C bond

Although conventional PB reactions are activated by UV light, researchers have been trying to discover PB-like [2 + 2] cycloaddition reactions that can be activated by mild visible light, which could reduce UV-induced side reactions during lipid double bond derivatization [45–48]. In 2020, Chen *et al.* discovered a new type of visible-light-activated [2 + 2] cycloaddition of carbonyl with C=C bonds. They found that carbonyl in anthraquinone showed great reactivities towards C=C bonds in lipids to form oxetanes under the irradiation of 405 nm visible-light [45]. Combined with tandem MS, this site-specific dissociation of oxetane enabled precisely locating the C=C bonds in various kinds of monounsaturated and polyunsaturated lipids (Fig. 4). Different from the well-studied PB reaction mechanism, Ouyang *et al.* discovered a novel [2 + 2] photocycloaddition reaction system by directly exciting the entire noncovalent complex involving the alkene and carbonyl substrates [46]. The complexes with noncovalent interactions between benzophenone and C=C bonds in unsaturated lipids have been successfully characterized. Density functional theory (DFT) calculations suggest that the entire complexes with dimeric proton-bonded alkenyl and carbonyl substrates can be excited under

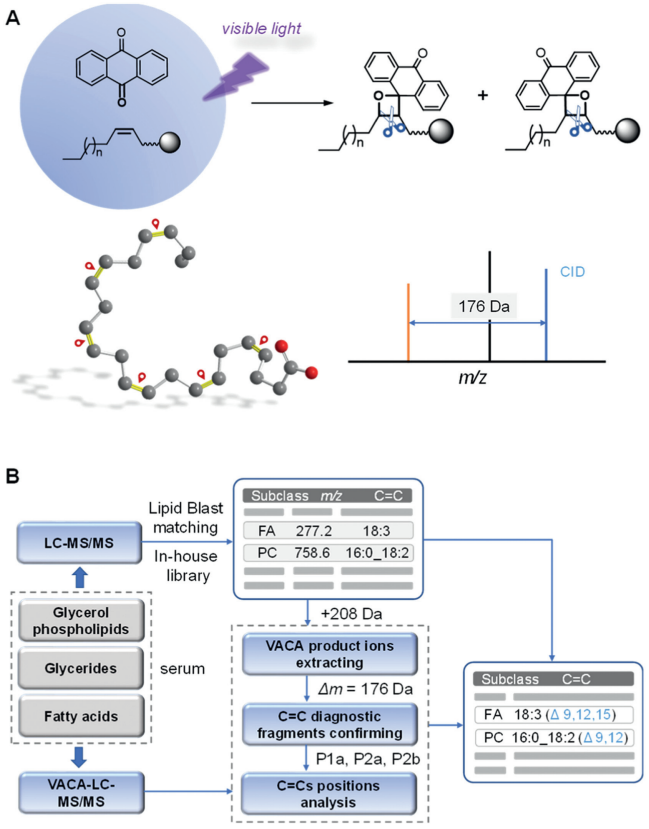

Fig. 4. Visible-light-activated [2 + 2] cycloaddition reaction for locating C=C bond. (A) Schematic representation of the reaction between anthraquinone and unsaturated lipids under visible light irradiation. (B) Workflow for the identification of the positions of C=C bonds *via* the [2 + 2] photocycloaddition reaction and LC-MS/MS. Reprinted with permission [45]. Copyright 2020, Royal Society of Chemistry.

visible light, leading to electron transfer from the alkenyl group in the fatty acyls to the carbonyl group in the complex.

Besides the direct excitation of the carbonyl substrate by light, Chen *et al.* also developed a novel [2 + 2] photocycloaddition system through triplet energy transfer of excited photocatalyst (Fig. 5) [47]. This approach negates the need for both visible-light-absorbing carbonyl substrates and UV light to enable access to a variety of functionalized oxetanes. In this reaction, methyl benzoylformate (MBF) was used as a carbonyl substrate and $Ir[dFppy]_2(dtbbpy)PF_6$ was used as a photocatalyst for the derivatization of unsaturated lipids. The C=C bond positions in unsaturated lipids can be identified by combining this visible-light-activated cycloaddition reaction with tandem MS (Fig. 5). The photocatalyst did not need to be removed prior to MS analysis because LC can separate the catalyst from the derivatized lipids.

In addition, this visible-light-catalyzed novel photocycloaddition reaction of MBF with unsaturated lipids has very high reaction yields. In further study by Xia's group, MBF and its charge-tagging version, pyridylglyoxalate, were found to be the most effective PB reagent so far. Using PC 16:0/18:1 (Δ9) as a model compound, the conversion rate of the PB reaction can reach more than 90% for MBF. They also found the that the conversion rate decreases when the lipid concentration is below 500 μmol/L (92%). For example, conversion rate is only 34% at 50 μmol/L and 8% at 10 nmol/L, suggesting the important role of lipid concentration in the PB reactions. In previous studies, large amounts of Norrish Type II reaction by-products were detected during PB reactions under 254 nm UV excitation using triFAP (36%) and acetone (27%) as PB reagent [37]. However, only less than 3% of Norrish II-type byproducts were produced when utilizing the principle of triplet energy transfer by visible light (405 nm) excitation and MBF as a PB reagent [49].

2.3. Epoxidation reaction for locating C=C bond

Epoxidation of C=C bonds is a common chemical reaction, and epoxidation of lipid double bonds can lead to activation of the double bond, which is further fragmented in tandem MS to produce characteristic diagnostic ions to determine the position of the double bond [50]. Researchers have developed different ways to achieve epoxidation of lipid double bonds to enable the analysis of double bond position isomers in different biological systems (Fig. 2C). In 2017, Zhao *et al.* reported an *in situ* epoxidation method by blowing a low-temperature plasma (LTP) into the surface of unsaturated FAs solution dissolved in acetone/water (50/50, v/v). This reaction owns the high reaction yield and less side reactions [51]. Within 2 min, almost quantitative conversion of monounsaturated FAs can be achieved, which is especially suitable for quantification [51]. The diagnostic ion pairs were produced at relatively high intensities and clearly marked with a 16 Da difference. Using the similar methodology, the C=C bond isomer analysis of phospholipids was also achieved with more lipid-soluble acetonitrile solution [52].

Meta-Chloroperoxybenzoic acid (*m*-CPBA) is a widely used oxidant in organic synthesis. Epoxidation of olefin with *m*-CPBA in dichloromethane can offer high specificity, complete conversion and minimal side reaction. In 2019, Li *et al.* coupled *m*-CPBA epoxidation with tandem MS for lipid C=C bond isomer identification [53]. They found that different kinds of phospholipids can generate diagnostic ion pairs in MS/MS spectra, which means this strategy offers broader instrument accessibility without needing multistage fragmentation. Right after that, Hsu *et al.* comprehensively explore the ability of *m*-CPBA epoxidation for lipid double-bond identification in lipidomics and molecular imaging of multiple classes of monounsaturated and polyunsaturated lipids [54]. The results suggested that C=C isomers are important to cellular lipid homeostasis and thus serve as potential disease biomarkers. This method is also suitable for MALDI-MS for FA C=C location identification [55].

Yan *et al.* also reported an innovative electrochemical epoxidation way for C=C bonds. The on-demand electrochemical epoxidation was incorporated into the standard nanoESI-MS workflow for C=C bond identification [56,57]. On-demand formation of mono/multiple epoxides was achieved at different voltages. Mono/multiple epoxides can be formed on demand at different voltages and then fragmented by tandem MS to produce diagnostic ions to indicate the double bond position. The whole process can be completed in a few seconds and has great potential for high throughput analysis.

In addition, other epoxidation methods for lipid C=C bonds were also developed. For instance, epoxidation with oxone as an oxidant combined with LC-MS and multiple reaction monitoring (MRM) mode strategy for accurate identification and quantification of C=C isomers of FAs [58]. Plasmonic hot-electron [59] and triboelectric nanogenerator [60,61] were also used for C=C epoxidation and identification.

2.4. Aziridination reaction for locating C=C bond

Aziridination of alkenes is a classic reaction for the synthesis of aziridines, which was typically proceeded by transferring nitrenes to C=C bond through a catalyst [40]. Aziridination converts the C=C to a three-membered ring, which owns a relatively high steric strain and a bare lone pair electron of the nitrogen atom. In 2022, Guo and Chen's groups first use the aziridination reaction for the identification of lipid double bond positional isomers (Fig. 2D) [62,63]. In a typical MS/MS spectrum of the aziridination product,

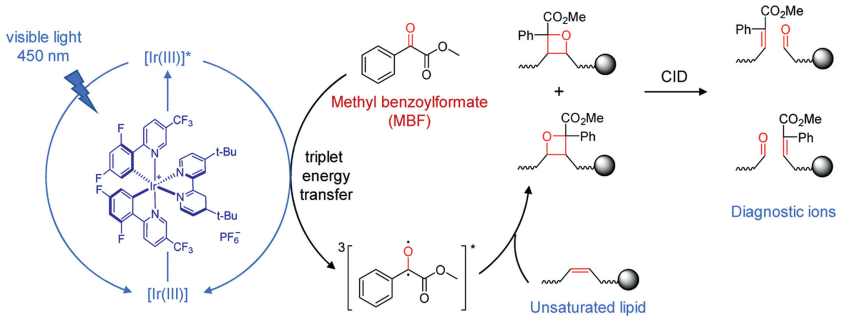

Fig. 5. Schematic illustration of the visible-light-activated photocatalytic reaction system for the analysis of C=C bonds positional isomer. MBF was excited to triplet by the photoexcited Ir[dF(CF$_3$)ppy]$_2$(dtbbpy)PF$_6$ *via* triplet-energy transfer.

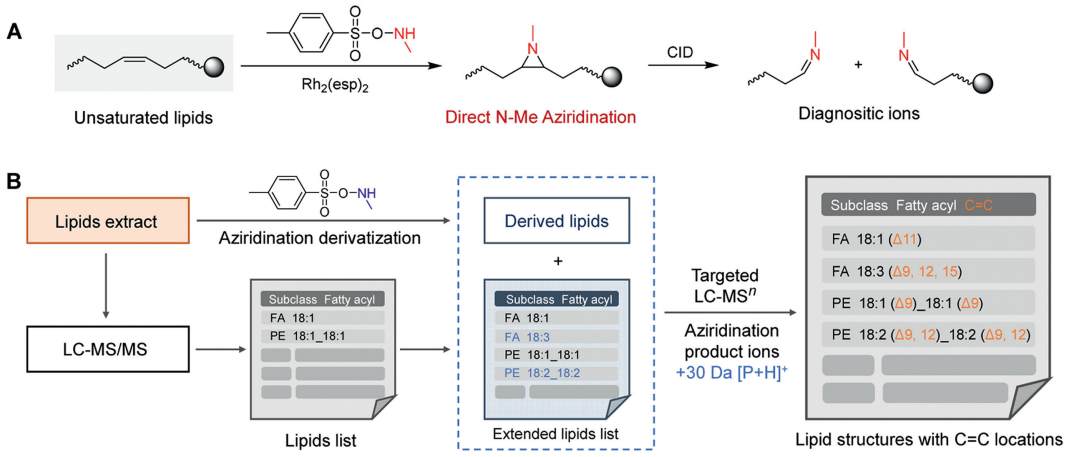

Fig. 6. Aziridination reaction for locating C=C bond. (A) Schematic representation of the reaction between *N*-methyl-*O*-tosylhydroxylamine and unsaturated lipids under the catalysis of Rh$_2$(esp)$_2$. (B) Workflow for the identification of the positions of C=C bonds *via* the direct N-Me aziridination reaction and LC-MS/MS.

a pair of C=C diagnostic fragment ions may be observed for each C=C bond, generated by the cleavage of C-C bond and N-C bond in an aziridine ring denotated as F_M (containing Me-N=C- unit and methyl end) and F_C (containing Me-N=C- unit and acyl group end). The tertiary amine structure allows the derivatized lipids to have a very high proton affinity, which can greatly improve the sensitivity of lipid isomer identification.

Chen *et al.* reported the direct N-Me aziridination of unsaturated lipid using *N*-methyl-*O*-tosylhydroxylamine as the aminating reagent and Rh$_2$(esp)$_2$ as the catalyst, and the reaction yield is over 90% within 5 min. The catalyst does not affect the analysis of derivatized lipids during LC-MS. This direct aziridination method has been successfully utilized in the C=C location identification of FA, GPL, diacylglycerol (DG), TG, and SLs (Fig. 6) [62]. Besides, Guo *et al.* developed a convenient *N*-tosylaziridination method with chloramine-T as aminating reagent for the derivatization and location of C=C bonds in lipids (Fig. 7) [63]. Further studies of C=C isomers showed that *n*-6/*n*-3 ratios were closely associated with human thyroid tumorigenesis, and high ratios of *n*-6/*n*-3 isomers seemed to suffer a high risk of carcinogenesis. The *N*-tosylaziridination derivatization can also be realized by photocatalytic reaction using [(*N*-tosylimino)iodo]benzene (PhI=NTs) as nitrene precursor and Ir[dF(CF$_3$)ppy]$_2$(dtbbpy)PF$_6$ as photocatalyst [64]. Based on this visible-light-activated aziridination reaction, the formed aziridine ring structure for unsaturated lipids could not only be adopted to identify the location of C=C bonds, but also improve the separation of *sn*-position isomers when combined with LC-MS/MS. This strategy enables the dual-resolving of the C=C bond location and *sn*-position isomerism of in lipids. In addition,

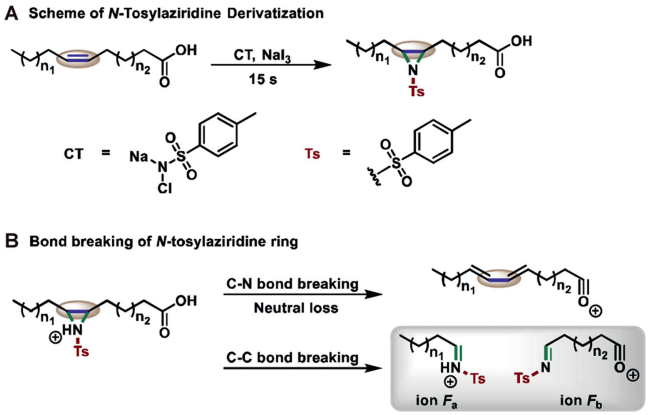

Fig. 7. *N*-Tosylaziridine derivatization for the identification of C=C location. (A) Scheme of *N*-tosylaziridine derivatization. (B) Bond breaking of the *N*-tosylaziridine ring during the CID Process: C–N bond breaking (green) and C–C bond breaking (blue). Reprinted with permission [63]. Copyright 2022, American Chemical Society.

the *N*-tosylaziridination method can also be combined with the carboxylic acid derivatization using 6-plex isobaric positive charge tags for the determination of positional isomers and the quantification of monounsaturated FAs [65].

Yan *et al.* also reported a creative N–H aziridination-based isobaric labelling method that allows identification of C=C bond positions and relative quantification of unsaturated lipid isomers (Fig. 8) [66]. The formed N–H aziridines structure allow further

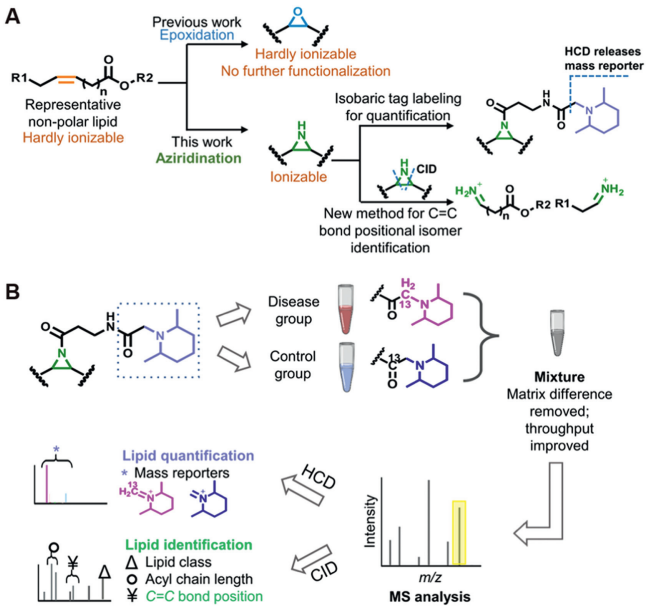

Fig. 8. Aziridine-based isobaric tag labeling for lipid quantification, isomer characterization, and improvement of ionization efficiencies of non-polar lipids. (A) Comparison between functionalization of lipid C=C bond *via* epoxidation and aziridination. (B) Application of aziridine-based isobaric tag labeling in lipid quantification and identification of diseased and control groups. Reprinted with permission [66]. Copyright 2022, Wiley VCH.

derivatization for accurate quantification purpose *via* the amidation of aziridines with *N*-hydroxysuccinimide (NHS) ester-based isobaric tags. Under higher energy C-trap dissociation (HCD), the tagged lipids release charged mass reporters indicating accurate concentration ratio of lipids from different groups. Moreover, under low energy CID, the aziridines is ruptured and releases C=C location diagnostics for isomer identification.

Accurate relative quantification of individual unsaturated lipid isomers will be an important step in facilitating lipid function studies. However, they suggested that it would be attractive to be able to achieve qualitative and quantitative in one step labeling because it would simplify the process [66].

To address this issue, Chen *et al.* develop a novel stable-isotope N-Me aziridination strategy that enables simultaneous qualification and quantification of unsaturated lipid isomers [67]. The one-step introduction of 1-methylaziridine structure not only serves as an activating group for C=C bond to facilitate positional identification, but also as an isotopic inserter to achieve accurate relative quantification. The high performance of this reaction for the identification of unsaturated lipids was verified by large-scale resolving the C=C positions of 468 lipids in serum. More importantly, by using this bifunctional duplex labeling method, various unsaturated lipids such as FAs, phospholipids, glycerides and CEs were accurately and individually quantified at C=C bond isomeric level during the mouse brain ischemia [67].

2.5. Olefin cross-metathesis reaction for locating C=C bond

Olefin cross-metathesis (CM) is the generation of new carbon-carbon double bonds by intermolecular exchange of alkylene fragments between two olefins under metal-catalyzed conditions (Fig. 2E). In 2011, Kim *et al.* combined this CM reaction with LC-MS for the analysis of double bond positions in unsaturated lipids [68]. The method is based on the cross-metathesis reaction between a target compound and an unsaturated lipid, which results in the formation of fragmented olefinic products. However, the limitation of this method is that it is only applicable to pure lipids or simple mixed systems, as the resulting mixed reaction products can make lipid traceability difficult.

3. Chemical reaction-assisted identification of C=C configuration in lipids

The types of isomers of C=C bonds in lipids includes positional and geometric (*cis-trans* or *E-Z*) isomers, which endow lipids with different structures and functions in living organisms [10,45]. The *cis-trans* conversion of C=C bonds in unsaturated lipids alter the phase transition temperature, rigidity, and permeability of bacterial membranes, which are critical for bacterial survival in response to environmental stimuli [15]. Therefore, accurate identification of the geometrical configuration of lipid double bonds and their isomer-resolved quantification are essential for the study of their biological functions. Previous analytical methods based on GC–MS can only be applied to free FAs, not to complex lipid molecules, and the accurate identification of double bond conformation is problematic due to interferences such as rearrangements during electron ionization [69]. Although the LC and ion mobility spectrometry (IMS) methods may separate the *E*/*Z* isomers under appropriate conditions and provide information by matching their elution times or arrival times with that of the known standard lipids, the acquirement of standards for numerous lipids in complicated biological systems is quite difficult [70,71]. To address these issues, several innovative chemical reaction-assisted methods were developed to identification the C=C bond configurations.

3.1. Visible-light-activated photoisomerization combined LC-MS for identifying cis/trans isomers

Triplet-energy transfer from the photocatalyst to the carbonyl substrates was reported that could trigger the [2 + 2] cycloaddition reaction of carbonyls and alkenes *via* visible-light [72]. Chen *et al.* envision that this process may also induce the photoisomerization of C=C bonds, because the sensitized photoisomerization activated by visible light *via* direct energy transfer or addition-elimination of ketone to the C=C bond was proved feasible [73]. They further proposed that the configurations of C=C bonds could be accurately identified by comparing their LC patterns before and after the photoisomerization reaction. Based on this concept, they established a visible-light-activated photocatalytic reaction system using MBF as carbonyl substrate and Ir[dFppy]$_2$(dtbbpy)PF$_6$ as photocatalyst for unsaturated lipids (Fig. 9) [47].

Upon the excitation of Ir[dF(CF$_3$)ppy]$_2$(dtbbpy)PF$_6$ to triplet state by visible light, this fluorinated Ir(III) complex with high triplet energy would promote the generation of long-lived triplet [MBF]* *via* Dexter energy transfer. Then, ^{3}[MBF]* would undergo subsequent [2 + 2] cycloaddition with C=C bonds in lipids *via* the standard stepwise biradical mechanism of the PB reaction to afford oxetane. Especially, the ^{3}[MBF-lipid]* biradical intermediate may also undergo radical elimination to regenerate ground state MBF and geometrically isomerized lipids. The resulting *cis* or *trans* photoisomerization products have different retention behaviors on chromatograms compared to their reactants. The *trans* product has a longer retention time than the *cis* reactant and will appear as a new peak to its right, while the *cis* product has a shorter retention time than the *trans* reactant and will appear as a new peak to its left. According to this specificity, a second dimension of identity label can be introduced for unsaturated lipid *cis-trans* isomers, enabling their reliable identification and analysis. In addition, the C=C location can be simultaneously identified by the tandem MS analysis of the [2 + 2] photocycloaddition reaction products (Fig. 9) [47].

By using this reaction system, they established an integrated workflow that enables the comprehensively qualitative and

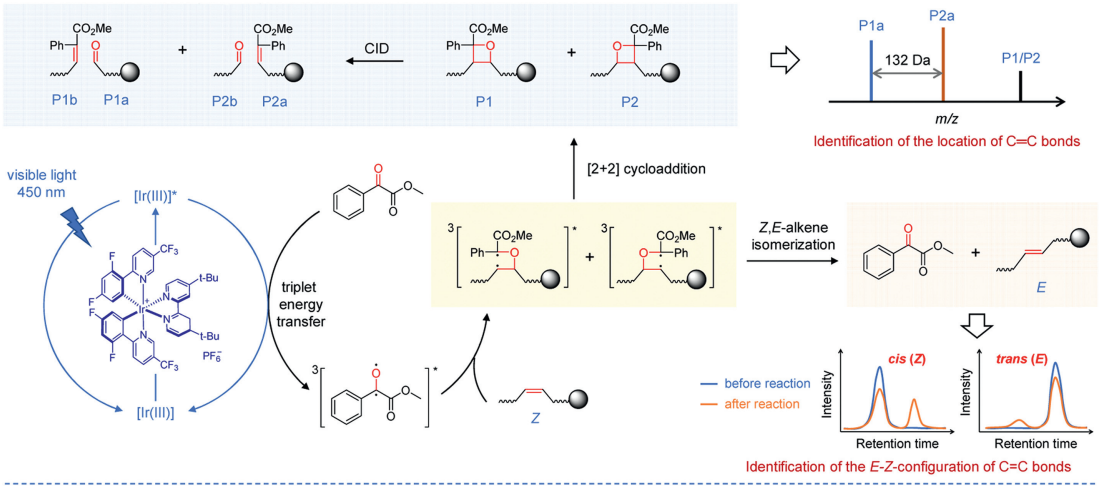

Fig. 9. Schematic illustration of the bifunctional visible-light-activated photocatalytic reaction system. For the analysis of C=C bonds positional isomer, MBF was excited to triplet by the photoexcited Ir[dF(CF$_3$)ppy]$_2$(dtbbpy)PF$_6$ *via* triplet-energy transfer. Then it underwent subsequent [2 + 2] cycloaddition with C=C bonds in lipids *via* the standard stepwise biradical mechanism of the Paternò–Büchi reaction to afford oxetanes P1 and P2. The oxetane isomers could be fragmented to the diagnostic ions P1a, P1b, P2a, and P2b to indicate the locations of C=C bonds *via* CID in tandem MS. For the analysis of *E*-*Z* isomer, the (*Z*)-isomer of lipids prefers to be converted to (*E*)-isomer *via* the triplet-energy transfer from the excited photocatalyst Ir[dF(CF$_3$)ppy]$_2$(dtbbpy)PF$_6$ in the same reaction system. Reprinted with permission [47]. Copyright 2022 Springer Nature.

quantitative analysis of the C=C bonds isomers of lipids with LC-MS. The applicability of this method was validated by deeply analyzing lipid structures of bacteria and revealing their unique patterns of *cis*-*trans*-isomers, as well as tracking the C=C positional isomers changes in brain ischemia with a mouse model [47].

3.2. Ozone-induced dissociation MS for distinguishing cis/trans isomers

OzID can also be used to identify the C=C bond positional isomers of lipids [24]. Blanksby *et al.* found that there were differences in the branching ratio of product ions (aldehyde and Criegee ions) for *cis*- and *trans*-isomers arising from the gas-phase ozonolysis reaction, and suggested that relative ion abundances could be used as markers for C=C bond geometry [74]. Under both positive and negative ion condition, the *trans* isomers have higher aldehyde and Criegee ion abundance up to 2.5-times than *cis*, indicating *trans* C=C owns better OzID reactivity [74]. This is consistent with detailed theoretical studies that show product branching in ozonolysis reactions are sensitive to the structure of the primary ozonide [75] that in turn will be influenced by the double bond geometry. In a later study, they implemented OzID in-line with LC, IMS, and high-resolution MS [31]. Under these conditions, geometric isomers exhibited different IMS arrival time distributions and distinct OzID product ion ratios providing a means for discrimination of *cis*/*trans* double bonds in complex lipids. Although these differences in reactivity and/or branching ratios could be used to determine double bond geometry in biological samples, this would require either careful comparison to appropriate standards (which most often are not available) or, alternatively, spectral comparisons of at least partially resolved isomeric pairs [31].

3.3. Epoxidation combined with tandem MS for distinguishing cis/trans isomers

Epoxidation by *m*-CPBA was successfully coupled with MS by Li's group to identify the location of C=C bonds in lipids [53]. In a recent study, they developed a novel analytical method to investigate FAs for relative quantification, carbon–carbon double-bond localization, and *cis*-/*trans*-geometry differentiation by isobaric multiplex labeling reagents for carbonyl-containing compound (SUGAR) tag conjugation and *m*-CPBA epoxidation [76]. They found that the *cis* or *trans* isomers of SUGAR-labeled FA epoxide isomers had different ion-pair intensity ratios due to hydrazide cleavage at HCD. Thus, in addition to determining the location of carbon-carbon double bonds, the combination of *m*-CPBA epoxidation and SUGAR tag labeling provides additional information for revealing the geometric configuration of monounsaturated FA double bonds. Chen *et al.* also developed a novel approach for the elucidation of C=C bond position and *cis*/*trans* isomers. This approach was achieved by the reaction of ambient water radical cations and double bonds, followed by the fragmentation of epoxide radical cations to generate diagnostic ions in tandem MS [77]. They observed that epoxidation products of 3-hexenol geometry isomers show a preference for the formation of different product ions under CID. The intensity differences of the product ions can be used to identify *cis*/*trans* isomers.

4. Conclusions and perspectives

The discovery of lipid biological functions and the development of lipid analysis tools are mutually reinforcing, and in-depth lipid analysis methods will accelerate the relevant structural lipid biology research. With the emergence of more and more lipidomic analysis methods with isomer resolution capability, the traditional lipidomic analysis has also moved to the deep structural lipidomic level. In particular, the emergence of various chemical reaction-assisted MS methods has brought our understanding of unsaturated lipid isomers to the level of C=C bond positions and configurations, which will greatly contribute to the understanding of the biological functions of lipid double-bond isomers.

However, there are still shortcomings in current structural lipid biology studies. First, the efficiency of chemical reaction derivatization for low-content lipids needs to be improved, and the ability to resolve low-abundance lipid isomers needs to be strengthened. Second, the structure of lipids contains multiple hierarchies, and there is a lack of methods that can comprehensively analyze the isomerization of lipids in various dimensions. Third, although accurate quantification of relative ratios between lipid isomers has been possible, accurate relative and absolute quantification of individual lipid isomers across samples remains a challenge. Fourth, accurate identification of the geometric configuration

of each double bond in unsaturated lipids remains difficult, especially for polyunsaturated lipids containing multiple double bonds. Fifth, although significant differences in the proportion or content of lipid isomers as metabolites in biological processes have been found, there is a lack of studies on the regulation of protein function by different lipid isomers and their differences. Finally, the role of lipid isomers in disease diagnosis or classification has yet to be validated with large-scale clinical samples.

In conclusion, structural lipidomics, represented by chemistry-driven lipid isomer analysis methods, has opened the door to structural lipid biology. In the future, with the emergence and improvement of new analytical methods, our knowledge of the biological functions of lipids will reach an unprecedented level, and our understanding of various diseases and life processes will be more comprehensive and in-depth.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Junmeng Luo: Writing – original draft. **Qiongqiong Wan:** Writing – review & editing. **Suming Chen:** Conceptualization, Supervision, Writing – review & editing.

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