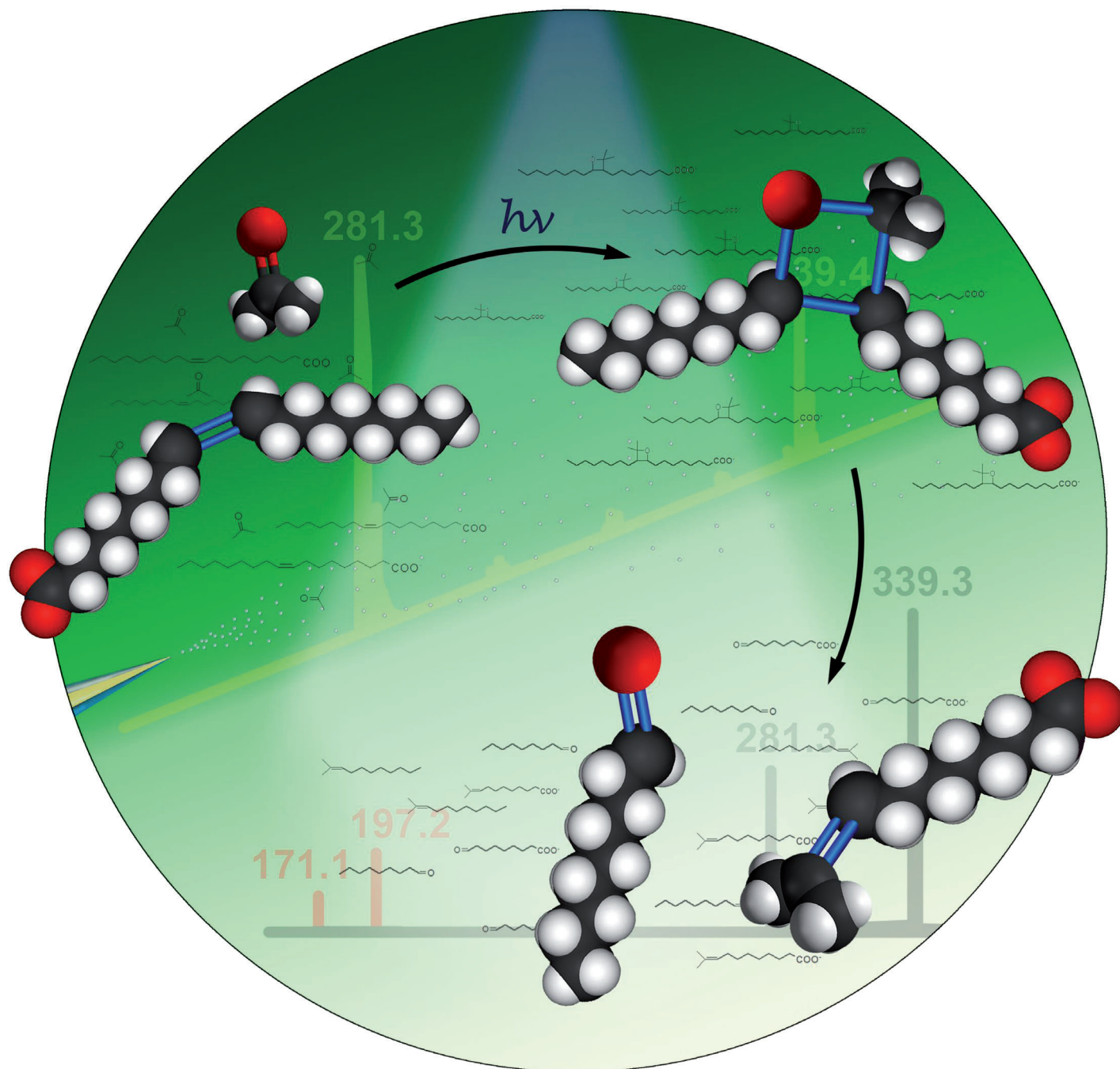


Pinpointing Double Bonds in Lipids by Paternò-Büchi Reactions and Mass Spectrometry**

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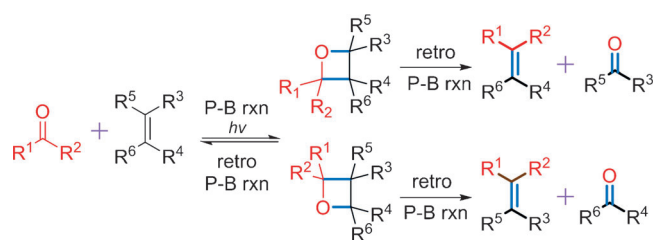


Abstract: The positions of double bonds in lipids play critical roles in their biochemical and biophysical properties. In this study, by coupling Paternò-Büchi (P-B) reaction with tandem mass spectrometry, we developed a novel method that can achieve confident, fast, and sensitive determination of double bond locations within various types of lipids. The P-B reaction is facilitated by UV irradiation of a nanoelectrospray plume entraining lipids and acetone. Tandem mass spectrometry of the on-line reaction products via collision activation leads to the rupture of oxetane rings and the formation of diagnostic ions specific to the double bond location.

Lipids are important structural components of cell membranes and also play critical roles in energy storage and cellular signaling processes.^[1] Unsaturated lipids are a subclass of lipids containing at least one carbon–carbon (C–C) double bond in a fatty acid (FA) chain. A large body of literature has pointed out the significant implications of double bond positions in the chemical, biochemical, and biophysical roles of lipids.^[2] For instance, the *n*-3 polyunsaturated fatty acids (PUFAs) (also called omega-3, where 3 is the double bond position counted from *n*, the terminal methyl group) are essential for the functional development of brain and retina.^[3] By contrast, no such effects have been observed for *n*-6 PUFAs. The efforts to determine double bond positions in lipids, however, are not trivial, largely due to the need of distinguishing the correct structure from a large number of possible double bond position isomers.

Mass spectrometry (MS) has become the method of choice for lipid analysis (such as in shotgun lipidomics)^[4] owing to its high sensitivity, selectivity, and the capability of providing detailed structural information.^[4a] Some notable MS methods capable of C=C bond localization include charge remote fragmentation of intact lipids using high energy collisional-induced dissociation (CID),^[5] CID of dilithiated lipid adduct ions,^[6] ozone-induced dissociation,^[7] and radical-directed dissociation (RDD) of non-covalent lipid complexes by UV irradiation and CID.^[8] Meanwhile, chemistries involving C=C bonds have been utilized to achieve selective chemical derivatizations or reactions prior to MS analysis, such as ozonolysis,^[9] methoxylation,^[10] olefin cross-metathesis,^[11] methylthiolation,^[12] and epoxidation.^[13] Here, we demonstrate a new approach based on the coupling of tandem mass spectrometric analysis with the unique chemistry of Paternò-Büchi (P-B) reaction towards C=C bond for lipid structural characterization.

P-B reaction is a classic [2+2] photochemical reaction widely used in organic synthesis to form compounds containing an oxetane ring (Scheme 1).^[14] The reaction mechanism



Scheme 1. Paternò-Büchi (P-B) reaction between ketone/aldehyde and olefin together with possible retro P-B reactions.

involves activation of the carbonyl group within an aldehyde or ketone to a diradical upon UV excitation, which subsequently reacts with the C=C bond in an olefin.^[15] Depending on the relative positions of the carbonyl and the C=C bond, two position isomers of the oxetanes can be formed as shown in Scheme 1. If energy (such as heating) is applied to the P-B reaction products, retro P-B reactions can happen,^[16] through two possible pathways. One route leads back to the original reactants. The other leads to cleavage of the C–C bond at the initial C=C bond position and the C–O bond of the initial carbonyl group, forming one new olefin and ketone/aldehyde (see Scheme 1). This latter pathway is of special interests in this study since the reaction products carry the C=C bond position information. Therefore, if the P-B reaction and its retro reactions can be coupled to MS analysis of lipids, the C=C bond position should be potentially obtained.

To test the feasibility of this approach, on-line P-B reaction was conducted between acetone and an unsaturated FA (oleic acid, one double bond between C9 and C10). The experimental setup is shown in Figure 1a, where a low-pressure mercury lamp (emission band at 254 nm) was placed in close proximity to a nanoelectrospray ionization (nanoESI) source in front of the sampling interface of a mass spectrometer.^[17] Oleic acid (10 μ M) was dissolved in a mixture of acetone and water (50/50, v/v), and 1% (v) ammonium hydroxide was added to the solution to facilitate ionization in the negative ion mode (–nanoESI). When the lamp was turned on to irradiate the nanoESI plume, a new species at *m/z* 339.4 was observed, with a relative intensity of 60% of the FA signal ($[M-H]^-$, *m/z* 281.3, Figure 1b). A small extent of side reactions were also observed, including sequential lipid oxidation and the addition of acetyl radicals to lipids (Norrish reactions).^[18] Accurate mass measurements (LTQ Orbitrap) revealed a mass increase of 58.0423 Da relative to deprotonated oleic acid, corresponding to C₃H₆O, the elemental composition of acetone (relative error: δ = 0.65 ppm). This result is highly suggestive of the formation of P-B reaction product due to the addition of acetone to a C=C bond. While keeping all other conditions the same, we repeated the reaction by replacing non-labeled acetone with [D₆]acetone (C₃D₆O). The detection of the reaction product that was 64 Da higher in mass than oleic acid (*m/z* 345.4 Da, inset of Figure 1b) further supported the assumption that a whole acetone molecule was added to the lipid ion.

In order to perform retro P-B reactions in the gas phase, we utilized collisional activation in an ion trap mass spec-

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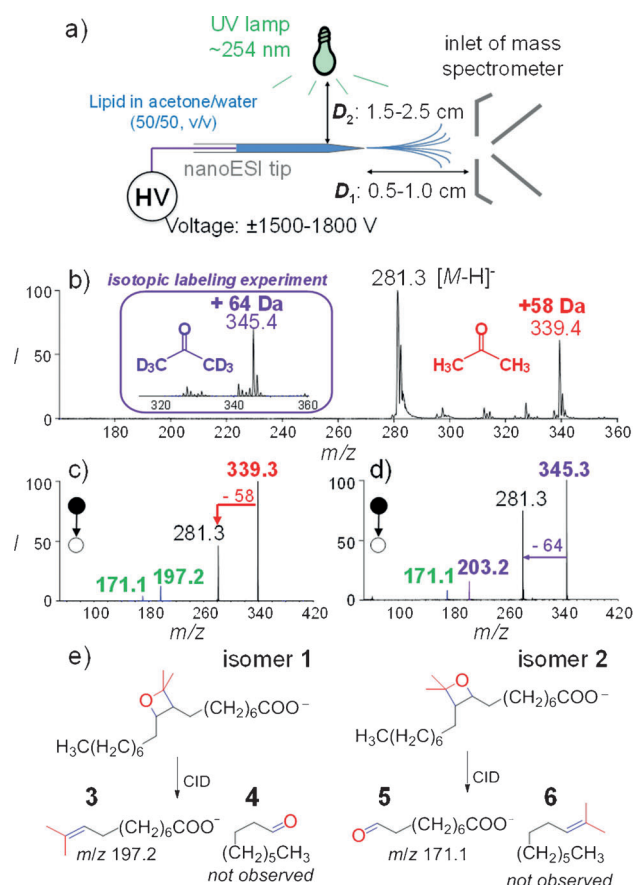


Figure 1. On-line coupling of P-B reactions with MS for lipid analysis. a) Experimental setup. b) P-B reaction mass spectrum of oleic acid and acetone induced by UV irradiation of -nanoESI. Inset: P-B reaction spectrum using D_6 -acetone (C_3D_6O). MS^2 CID of the P-B reaction products at c) m/z 339.3 and d) m/z 345.3. e) Fragmentation Scheme of P-B reaction product isomers.

trometer to mimic thermal dissociation traditionally done in solution. The MS^2 CID mass spectrum of m/z 339.3 is shown in Figure 1 c, where three major fragments are present, including m/z 281.3, 197.2, and 171.1. The product ion at m/z 281.3 results from the retro P-B pathway by losing a neutral acetone (58 Da). Product ions at m/z 197.2 (compound **3**) and 171.1 (compound **5**) result from the other retro P-B pathway via oxetane ring rupturing at the original C=O and C=C sites within P-B reaction products **1** and **2**, respectively (Figure 1 e). Compound **5** still possesses the FA structure however with the original C=C bond transformed to an aldehyde, while compound **3** incorporates the $C(CH_3)_2$ moiety from acetone. Their complementary fragments (**4** and **6**) are not detected due to their existence as neutrals. The above assignments are further supported by CID of the P-B reaction product of oleic acid and $[D_6]$ acetone (Figure 1 d). Note that among the three observed fragments, i.e., m/z 171.1, 203.2, and 281.3, only the fragment at m/z 203.2 has a 6 Da mass increase as compared to products in Figure 1 c, consistent with the incorporation of the two CD_3 groups to form structure **3**.

Based on the results above, we have demonstrated that P-B reaction can be successfully performed on-line and coupled with MS for lipid analysis at reasonable yields. More

importantly, retro P-B reactions can be performed within the framework of tandem mass spectrometry, producing very simple diagnostic fragment ions (i.e. **3** and **5**) that are closely tied to the C=C position in the original lipid. In addition, these “diagnostic ions” have a mass difference of 26 Da due to the use of acetone as the P-B reaction reagent, allowing them to be readily detected and identified from CID spectra.

In order to pinpoint individual double bond positions in different acyl chains of a lipid, we further tested P-B reaction on a phosphatidylserine (PS), PS 16:1(9Z)-18:1(9Z) (m/z 758.6 for deprotonated ions, structure shown in Figure 2 a). The P-B reaction product was formed at m/z 816.5 by -nanoESI. MS^2 CID of m/z 816.5 led to an 87 Da neutral loss (NL) specific to PSs and ions corresponding to the two free acyl chains, R^1COO^- and R^2COO^- at m/z 253.2 and 281.2 (Figure 2 b). Interestingly, the P-B reaction products of the two acyl chains were also observed, at m/z 311.2 and 339.3, respectively. MS^3 CID data of these P-B reaction product ions are shown in Figure 2 c and 2 d. Based on the generic chemical formula of fragment **5**, $C_nH_{(2n-3)}O_3^-$ (structure depicted in Figure 1 e), one can readily deduce that ions at m/z 171 contain 9 carbons ($n=9$) and therefore the double bond is located between C9 and C10 for each acyl chain. The two acyl chain lengths are determined to be C_{16} and C_{18} by considering the general formula ($C_nH_{2n-1}CO_2^-$) of the deprotonated FA ions (m/z 281.2 and 253.2). Putting the information together, it is straightforward to conclude that one acyl chain is C_{16} with a double bond between C9 and C10, and the other one is a C_{18} chain containing a double bond between C9 and C10. The above procedure also forms the basis for structural characterization of unsaturated lipids in a complex lipid mixture shown later in this study.

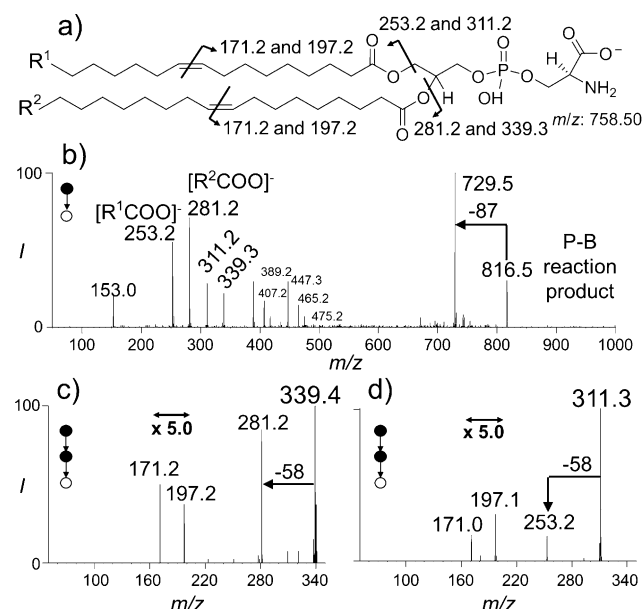


Figure 2. Elucidation of double bond positions in PS 16:1(9Z)-18:1(9Z). a) Structure of PS 16:1(9Z)-18:1(9Z) and the observed cleavage sites. b) MS^2 CID of P-B reaction product of PS at m/z 816.5. MS^3 CID of c) m/z 339.4 and d) 311.3 from P-B reaction product of PS.

Considering that many unsaturated lipids contain multiple double bonds in a single FA chain, the present method was also applied to a lysophosphocholine (LPC), LPC 18:2-(9Z,12Z). Due to the presence of two double bonds, two P-B reaction products were observed at m/z 578.5 and 636.5 (positive ion mode, Figure S4b, Supporting Information), corresponding to the addition of one and two acetone molecules. CID of the reaction product at m/z 578.5 revealed two sets of diagnostic ions at m/z 412.4 and 438.5, 452.4 and 478.6 (Figure S4c). The identification of the diagnostic ion sets was straightforward due to the characteristic 26 Da mass difference within each set. The double bond positions are readily assigned to be between C9 and C10 and between C12 and C13 based on m/z of the diagnostic ions. It is also worth noticing that the intensities of the two pairs of diagnostic ions are comparable with each other, suggesting that P-B reaction is not selective towards methylene-separated double bonds. This feature is beneficial as CID of the first-step reaction product can provide position information of all double bonds.

Although most naturally occurring lipids contain only C=C bond in *cis* (Z) configuration, it is of great importance to unambiguously determine C=C bond configurations. Based on careful evaluation of a series of lipids containing either *cis* or *trans* C=C, little difference was observed either in their reaction yields or relative abundances of diagnostic ions. Therefore, it can be concluded that with the use of acetone as the P-B reaction reagent, the *cis* or *trans* C=C configuration cannot be differentiated. This result may be attributed to the relatively small size and symmetric structure of acetone, making it not suitable for the recognition of double bond configurations.

As a preliminary test for mixture analysis, we analyzed a mock mixture of phosphocholines (PCs) consisting of PC 16:1(9E)-16:1(9E) (M_1), PC 18:1(9Z)-16:0 PC (M_2), and 18:1(6Z)-18:1(6Z) PC (M_3). M_2 has one double bond in the 18:1 acyl chain, while the other two PCs have two identical acyl chains each containing one double bond. P-B reaction products of M_1 , M_2 and M_3 are clearly detected at m/z 802.6, 818.6 and 844.6, respectively (Figure 3a). No specific selectivity towards certain unsaturated lipids was observed. Collisional activation of these isolated products produces abundant diagnostic ions, allowing for confident assignment of C=C location(s) in each lipid (Figure S5). Similarly, using –nanoESI a mixture of FAs including linoleic acid (FA 18:2 (9Z,12Z), M_4), oleic acid (FA 18:1 (9Z), M_5), and stearic acid (FA 18:0, M_6) was analyzed (Figure 3b and S6). Stearic acid, a saturated FA, was deliberately added to test the selectivity of P-B reaction and whether its presence will affect the analysis of its unsaturated counterparts. After reaction, only P-B reaction products of M_4 and M_5 were detected, at m/z 337.3 and 339.3, demonstrating the selectivity of the reaction towards unsaturated lipids.

The efficacy of the method for the analysis of complex real-world mixtures containing unsaturated lipids was demonstrated by analyzing the yeast polar extract (*S. cerevisiae*). Eight major classes of lipids were detected, including FAs, PCs, LPCs, PSs, lysoPSs (LPSs), lysophosphatidylinositols (LPIs), phosphatidylethanolamines (PEs), and lysoPEs (LPEs) (Figures S7–S10). Among them, FAs and LPIs were

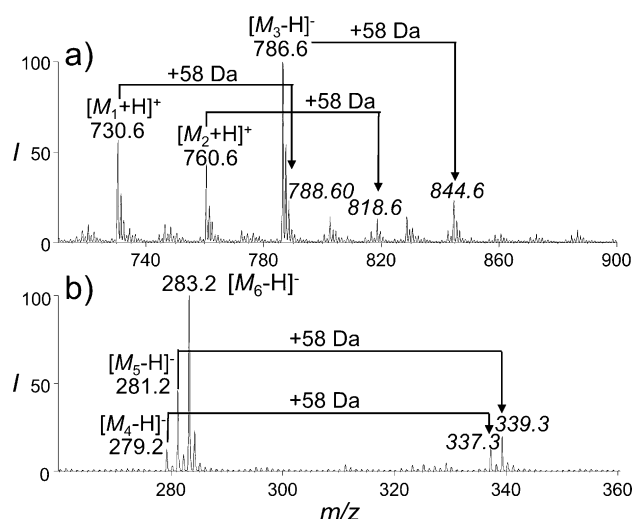


Figure 3. Reaction mass spectra of unsaturated lipid mixtures.

a) +nanoESI MS of three PCs after P-B reaction. Concentrations: M_1 , 7 μ M; M_2 , 6 μ M; M_3 , 13 μ M. b) –nanoESI MS of three FAs after P-B reaction. Concentrations: M_4 , 3 μ M; M_5 , 1 μ M; M_6 , 2 μ M.

analyzed by –nanoESI, while all the other lipid classes were analyzed in either or both ionization modes. Due to the addition of acetic acid in the spray solvent, PS, LPS, PC and LPC can also be detected in the forms of $[(L)PS-H]^-$ and $[(L)PC+CH_3COO]^-$ by –nanoESI-MS. Table 1 lists 19 unsaturated lipids whose double bond positions have been

Table 1: 19 unsaturated lipids from yeast polar lipid extract (*S. cerevisiae*) with double bond positions identified.

Names of unsaturated lipids	Diagnostic ions for double bond position (m/z)	Lipid-type specific neutral loss (NL) and/or precursor ions
FA 16:1(9Z)	171.1 and 197.1	
FA 18:1(9Z)	171.1 and 197.1	
LPI 16:1(9Z)	171.0 and 197.2	NL 316 Da (–)
LPI 18:1(9Z)	171.0 and 197.2	NL 316 Da (–)
LPC 16:1(9Z)	420.2 and 438.2	m/z 184.1 (+)
LPC 18:1(9Z)	420.2 and 438.2	m/z 184.1 (+)
LPE 16:1(9Z)	352.2 and 378.2	NL 141 Da
LPE 18:1(9Z)	352.2 and 378.2	NL 141 Da
LPS 16:1(9Z)	229.1 and 255.2	NL 87 Da (–)
		NL 185 Da (+)
LPS 18:1(9Z)	229.1 and 255.2	NL 87 Da (–)
		NL 185 Da (+)
PC 16:0-16:1(9Z)	650.6 and 676.6	m/z 184.1 (+)
PC 16:1(9Z)-16:1(9Z)	648.5 and 674.5	m/z 184.1 (+)
	603.5 and 629.5	
PC 16:1(9Z)-18:1(9Z)	648.5 and 674.5	m/z 184.1 (+)
	676.6 and 702.6	
PC 18:0-16:1(9Z)	678.6 and 704.6	m/z 184.1 (+)
PC 16:0-18:1(9Z)	650.5 and 676.6	m/z 184.1 (+)
PE 16:1(9Z)-16:1(9Z)	465.4 and 491.4	NL 141 Da
PE 16:1(9Z)-18:1(9Z)	465.4 and 491.4	NL 141 Da
	493.4 and 519.4	
PS 16:1(9Z)-16:1(9Z)	465.4 and 491.4	NL 87 Da (–)
		NL 185 Da (+)
PS 16:1(9Z)-18:1(9Z)	465.4 and 491.4	NL 87 Da (–)
	493.4 and 519.4	NL 185 Da (+)

identified. The corresponding mass spectra and structures of diagnostic ions for each lipid were provided in Table S3.

In summary, we have demonstrated the coupling of Paternò-Büchi reactions with MS for confident determination of the double bond locations within unsaturated FA chains of lipids. Collisional activation of the P-B reaction products facilitates retro P-B reactions, producing distinct diagnostic fragment ions. These ions can be unambiguously mapped to their precursor, enabling localization of double bonds in unsaturated lipids. The above method is sensitive in terms of analyte consumption (fmol for FA, Figures S11 and S12) and can be applied to complex lipid mixture analysis. The other analytical advantages include simple experimental setup for reactions, no need for MS instrument modification, easy-to-interpret mass spectra, and inexpensive derivatizing reagents. These characteristics should make this method accessible and attractive to many laboratories. Finally, it should be pointed out that although the P-B reaction yield is reasonable for structural analysis by MS/MS, the reaction itself is not quantitative. Due to this fact and the co-existing side reactions, the reaction mass spectra of complex lipid mixtures can be very complicated, which will lead to reduced detection sensitivity and also increased difficulty in structural analysis. Coupling of on-line P-B reaction with chromatographic separations might be a solution to this issue.

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