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Preferential formation of mono-dimethyl disulfide adducts for determining double bond positions of poly-unsaturated fatty acids

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Abstract

Determination of double bond positions of unsaturated lipids is fundamental for understanding their biofunctions and biosynthetic pathways. Derivatizing unsaturated lipids with dimethyl disulfide (DMDS) represents a convenient method to yield characteristic ions for determining double bond positions. However, DMDS adduction of poly-unsaturated lipids often leads to the formation of poly- and/or cyclized DMDS adducts, which yield complex mass spectra that are difficult to interpret in terms of double bond positions. Here, we report a room-temperature, convenient experimental procedure to preferentially form mono-DMDS adducts for poly-unsaturated fatty acid methyl esters (FAMEs) with two to five double bonds (*c*9, *c*12-$C_{18:2}$, *t*9, *t*12-$C_{18:2}$, *c*5, *c*9, *c*12-$C_{18:3}$, *c*7, *c*10, *c*13, *c*16-$C_{22:4}$ and *c*5, *c*8, *c*11, *c*14, *c*17-$C_{20:5}$). Electron-impact ionization mass spectra of these mono-DMDS adducts provide highly diagnostic ions for determining positions of all double bonds. Our approach overcomes the limit of traditional DMDS derivatization methods which are generally limited to mono-unsaturated molecules. In addition, we show gas chromatography and gas chromatography–mass spectrometry analyses of mono-DMDS adducts can also provide useful information about double bond geometry, as *cis* double bonds are prone to isomerize to *trans* isomers.

KEYWORDS
DMDS, double bond geometry, double bond position, isomerization, mono-dimethyl disulfide adducts, poly-unsaturated fatty acids

INTRODUCTION

The reaction between double bonds and dimethyl disulfide (DMDS) in the presence of I_2 represents a convenient one-pot derivatization for the determination of double bond positions in lipids (Buser et al., 1983; Carlson et al., 1989; Dunkelblum et al., 1985; Francis, 1981; Vincent et al., 1987). The resulting DMDS adducts yield highly diagnostic ions related to double bond positions in the electron-impact ionization mass spectrometry (EI-MS). Since 1981, DMDS reaction has been applied to different types of unsaturated compounds including fatty acids (Francis, 1981), alkenes (Carlson et al., 1989), long-chain unsaturated ketones (Liao et al., 2021) and terpenes (Attygalle et al., 1993). Traditionally, unsaturated (including mono- and poly-unsaturated) lipids are reacted with DMDS under heated environments for a prolonged time (typically 40–50°C for at least 4 h) to ensure all double bonds are derivatized (Buser et al., 1983; Carlson et al., 1989; Dunkelblum et al., 1985; Francis, 1981; Nichols et al., 1986; Pepe, Sayer, Dagaut, & Couffignal, 1997; Scribe et al., 1988; Vincent et al., 1987). This approach works well for mono-unsaturated lipids (Buser et al., 1983; Dunkelblum et al., 1985; Francis, 1981; Nichols et al., 1986; Scribe et al., 1988). For unsaturated lipids with multiple double bonds, however, poly- and/or cyclized DMDS adducts are obtained whose mass spectra are generally more difficult to interpret in terms of double bond positions (Carlson et al., 1989; Pepe, Sayer, Dagaut, & Couffignal, 1997). Therefore, the application of DMDS reaction has been largely limited to mono-unsaturated lipids.

One solution to this problem, as demonstrated by Yamamoto et al. (1991), Imbs and Rodkina (2005), and

Tanaka et al. (1997), is to partially hydrogenate poly-unsaturated fatty acid methyl esters (FAMEs) into mono-unsaturated FAMEs with hydrazine, followed by silver nitrate silica gel isolation of mono-unsaturated FAMEs and subsequent DMDS reaction. However, due to the difference in reduction rates for double bonds at different locations (e.g., terminal double bond is often the easiest to reduce) (Ratnayake et al., 1990), it is challenging to obtain sufficient quantity of partially hydrogenated mono-unsaturated FAMEs for every double bond in a poly-unsaturated lipid. Mono-unsaturated FAMEs at certain double bond positions may be missing at each attempt. This would lead to an incomplete elucidation of double bond positions. Additionally, significant efforts in isolating mono-unsaturated products from a mixture of reduced products are needed.

Another solution, as a more convenient one, is to obtain mono-DMDS adducts for poly-unsaturated lipids. One special circumstance, as demonstrated by Shibamoto et al. (2016, 2018), is that mono-DMDS adducts are formed preferentially (and possibly exclusively) for 9,12-$C_{18:2}$ fatty acids (double bonds at Δ^9, Δ^{12} can be either *cis* or *trans*). In both *c*9, *c*12-$C_{18:2}$ and *t*9, *t*12-$C_{18:2}$ fatty acids, DMDS is only (or mostly) added onto one, rather than both, double bond (Shibamoto et al., 2016, 2018). Based on these observations, Shibamoto et al. (2016, 2018) propose that the preferential formation of mono-DMDS adducts for these fatty acids with single methylene-interrupted double bonds is primarily due to steric hindrance (Shibamoto et al., 2016, 2018). In other words, when DMDS is added onto one of the two double bonds on 9,12-$C_{18:2}$, adding DMDS onto the remaining double bond is prohibited due to the proximity of the remaining double bond to the bulky DMDS group already added onto the molecule. Additionally, *cis* double bond reacts preferentially with DMDS, thus in *c*9, *t*12-$C_{18:2}$ or *t*9, *c*12-$C_{18:2}$, only mono-DMDS adducts on *c*9 or *c*12 double bond are formed (Shibamoto et al., 2016, 2018). If the idea of steric hindrance is true, it is possible that similar reaction conditions can also result in the formation of mono-DMDS adducts for other di-unsaturated lipids with one methylene-interrupted double bonds. Alternatively, fatty acids with double bonds that are interrupted with more than one methylene groups would not be hindered sterically and would not preferentially form mono-DMDS adducts for determining double bond positions.

Relying on the steric hindrance of Δ^n, Δ^{n+3} double bonds, however, exclude the application of DMDS method for lipids with more than two double bonds and/or with variable double bond positions (e.g., more than one methylene interruption) that do not attain similar steric hindrance. Is it possible to establish a convenient and broadly applicable experimental method that will permit the formation of mono-DMDS adducts for lipids with more double bonds, and those with double bonds not located in Δ^n, Δ^{n+3} positions? We have recently demonstrated that, by adjusting reagent amounts and performing the DMDS adduction at low temperatures, sufficient amounts of mono-DMDS alkenone (poly-unsaturated long-chain ketone containing *trans* double bonds) adducts can be obtained for determining their double bond positions (Dillon et al., 2016; Liao et al., 2021; Richter et al., 2017; Zhao et al., 2014), including those possessing five double bonds at Δ^4, Δ^7, Δ^{14}, Δ^{21}, Δ^{28} (Liao et al., 2021). Importantly, our research on alkenones demonstrates that it is not necessary to form mono-DMDS adducts of unsaturated compounds exclusively for the purpose of determining the double bond positions, as appeared to be implied from the studies of Shibamoto et al. (2016, 2018). Even if di- or higher DMDS adducts are also formed, modern gas chromatograph–mass spectrometer can easily separate the mono-DMDS adducts from poly-DMDS adducts, allowing for convenient determination of double bond positions. In addition, it is also possible to adjust the experimental conditions to favor the formation of greater amounts of mono-DMDS adducts (Richter et al., 2017).

The main objective of the present research is to identify a convenient experimental procedure that will permit the formation of sufficient mono-DMDS adducts from poly-unsaturated fatty acids, so that GC–MS can be used to determine the double bond positions. We demonstrate the efficacy of these mono-DMDS adducts in determining double bond positions for fatty acids with two to five double bonds. In addition, we also show DMDS approach can help differentiate *cis* and *trans* double bonds, as only *cis* double bonds can undergo partial isomerization to *trans* double bonds, but not vice versa.

MATERIALS AND METHODS

Chemicals

All solvents were HPLC grade from Fisher Scientific (USA). Diethyl ether (Et_2O, >99%), DMDS (>98%), iodine (>99.8%), sodium thiosulfate (>99%) and sodium sulfate (anhydrous, >99%), methyl linoleate (>99%, methyl (9*Z*,12*Z*)-octadeca-9,12-dienoate, $C_{19}H_{34}O_2$), methyl linolelaidate (>99%, methyl (9*E*,12*E*)-octadeca-9,12-dienoate, $C_{19}H_{34}O_2$), methyl adrenate (>98%, methyl (7*Z*,10*Z*,13*Z*,16*Z*)-docosa-7,10,13,16-tetraenoate, $C_{23}H_{38}O_2$),methyl eicosapentaenoate (>99%, methyl (5*Z*,8*Z*,11*Z*,14*Z*,17*Z*)-icosa-5,8,11,14,17-pentaenoate, $C_{21}H_{32}O_2$) were purchased from Sigma Aldrich (USA). Methyl pinolenate (>98%, methyl (5*Z*,9*Z*,12*Z*)-octadeca-5,9,12-trienoate, $C_{19}H_{32}O_2$) was purchased from Cayman Chemical (USA).

DMDS derivatization reaction

Mono-DMDS adducts of poly-unsaturated FAMEs were prepared following the procedure reported in Richter

et al. (2017) and Liao et al. (2021) with small modifications (Liao et al., 2021; Richter et al., 2017). About 10 mg FAMEs were dissolved in 50 µl hexane. 40 µl iodine solution (60 mg I_2 in 1 ml Et_2O) and 200 µl DMDS were then added to initiate the reaction. The reaction was performed at 0°C for 1–3 h, 25 and 50°C for 1 h for methyl linoleate to study the influence of reaction time and temperature on the yields of corresponding mono-DMDS adducts. For other polyunsaturated FAMEs examined, the reaction was performed at 25°C, but reaction time was adjusted to between 20 min and 1 h to ensure sufficiently high yields of mono-DMDS adducts. The reaction was stopped using 300 µl 5% sodium thiosulfate in deionized water. Products were extracted using hexane. The organic phase was transferred to a clean vial. Anhydrous sodium sulfate was added, and then allowed to sit for 30 s. Average yield for mono-DMDS adducts was ~32%. The resulting hexane solutions were separated and immediately analyzed by GC–FID and GC–MS.

GC analysis

Mono-DMDS adducts of FAMEs were then analyzed on GC–FID (gas chromatography–flame ionization detection) (Agilent 7890B) and GC–EI-MS (gas chromatography–electron impact ionization-mass spectrometry) (Agilent 7890B interfaced to 5977 inert plus MSD) equipped with mid-polarity poly(trifluoropropylmethylsiloxane) RTX-200 columns (105 m (length) × 250 µm (internal diameter) × 0.25 µm (film thickness)) (Liao et al., 2020; Y. Zheng et al., 2017). 4-Tridecanone was used as an internal standard. For the analysis on GC–FID, the carrier gas was hydrogen. Samples were injected under pulsed splitless mode at 320°C. The flow rate (constant flow mode) was 1.5 ml/min. The initial oven temperature was 50°C for 2 min, then increased to 255°C at 20°C/min, then increased to 320°C at 3°C/min and held for 35 min. For the analysis on GC–EI-MS, the carrier gas was helium. The injection and oven program follow the same settings as GC–FID. Samples were analyzed under full-scan mode (*m*/*z* 50–600).

RESULTS AND DISCUSSION

Experimental conditions for the formation of mono-DMDS adducts

In this study we have generally followed the experimental conditions that we used for long-chain alkenones (Dillon et al., 2016; Liao et al., 2021; Richter et al., 2017; Zheng et al., 2016). Poly- and cyclized DMDS adducts are well resolved from mono-DMDS adducts by GC (Figure 1a), hence do not interfere with GC–MS analyses of mono-

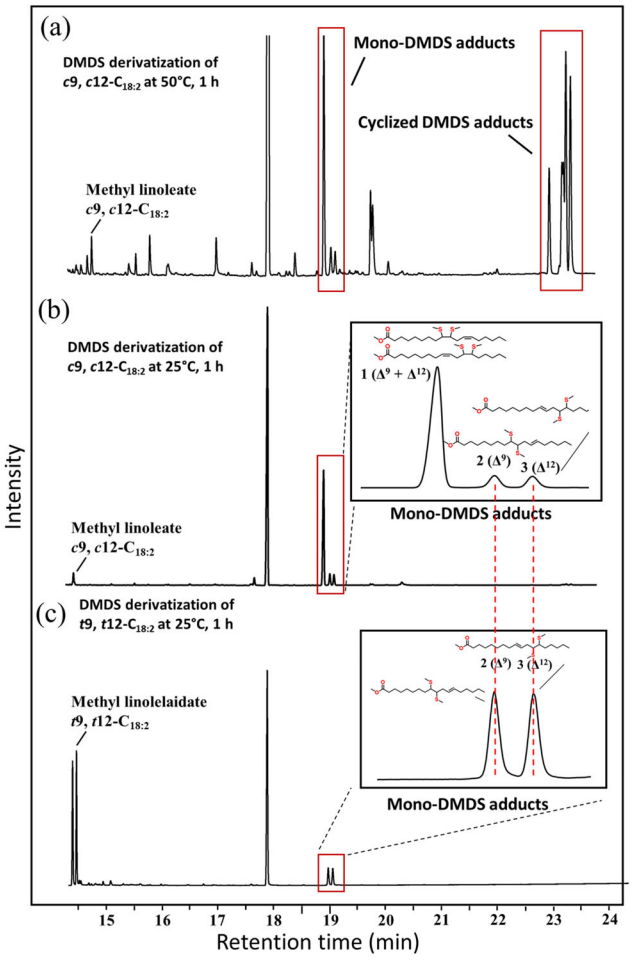

FIGURE 1 Gas chromatograms showing the distribution of products after DMDS reaction of methyl linoleate (*c*9, *c*12-$C_{18:2}$) performed at 50°C for 1 h (a), 25°C for 1 h (b) and methyl linolelaidate (*t*9, *t*12-$C_{18:2}$) performed at 25°C for 1 h (c). Peak numbering $n(\Delta^m)$ in the chromatograms of mono-DMDS adducts denotes compound *n*, with mono-DMDS adducting on the double bond position *m* on the FAME

DMDS adducts. We only made small number of additional tests on reaction time and temperature for methyl linoleate (*c*9, *c*12-$C_{18:2}$) to obtain a more practical and easier-to-perform experimental protocol for yielding ample amount of mono-DMDS adducts. For example, we tested the yield of mono-DMDS adducts for methyl linoleate (*c*9, *c*12-$C_{18:2}$) at 1, 2, and 3 h of reaction time at 0°C, and found an overall relatively low yields of mono-DMDS adducts (ranges from 13% to 22%, Figure S1a). However, performing the reaction at 25°C for 1 h greatly increased the yield from 19% to 92%, with only ~2% cyclized DMDS adducts formed (Figure S1b). A further increase of temperature to 50°C, however, decreased the yield of mono-DMDS adducts to 20% and increased the production of cyclized DMDS products to 58% (Figures 1a,b and S1b). We therefore decided to use 25°C for all our subsequent experiments and did not perform more experiments to identify the optimal reaction

conditions for all compounds. This temperature is close to the room temperature for most chemical laboratories; hence the reaction is more convenient to perform.

We also adjusted the reaction time for different compounds in order to maximize yields at 25°C. While 1 h reaction time also provided high yield of mono-DMDS adducts for di-unsaturated *t*9, *t*12-$C_{18:2}$ (45%), we found a shorter reaction time would generally increase the yield of mono-DMDS adducts for fatty acids containing more double bonds. For example, when we decreased the reaction time from 1 h to 20 min, yield of mono-DMDS adducts for penta-unsaturated *c*5, *c*8, *c*11, *c*14, *c*17-$C_{20:5}$ increased from 22% to 50%. The yields were similar (change from 41% to 44% and 18% to 15%) for tri-unsaturated *c*5, *c*9, *c*12-$C_{18:3}$ and tetra-unsaturated *c*7, *c*10, *c*13, *c*16-$C_{22:4}$, respectively, when reaction time decreased from 1 h to 20 min. In comparison, the yield of mono-DMDS adducts decreased from 45% to 21% for di-unsaturated *t*9, *t*12-$C_{18:2}$ when reaction time decreased from 1 h to 20 min. These observations in reaction yields can be readily explained by the nature (i.e., transient reaction product) of the mono-DMDS adducts.

Overall, as the number of double bonds increases in poly-unsaturated fatty acids, the likelihood of forming cyclized or poly-DMDS adducts increases over prolonged reaction time, at the expense of the mono-DMDS adducts. Therefore, a shorter reaction time is likely more favorable for improving the yields of mono-DMDS adducts as the number of double bonds increases. When applying the procedure to unknown target molecules, we recommend testing a few different reaction times at room temperature to ensure that the yield of mono-DMDS adducts is sufficiently high for GC–MS analysis.

Comparison of mono-DMDS adducts of methyl linoleate (*c*9, *c*12-$C_{18:2}$) and methyl linolelaidate (*t*9, *t*12-$C_{18:2}$)

We retested methyl linoleate (*c*9, *c*12-$C_{18:2}$) and methyl linolelaidate (*t*9, *t*12-$C_{18:2}$) previously studied by Shibamoto et al. (2016) using our modified experimental conditions, and were able to obtain sufficient mono-DMDS adducts for elucidating double bond positions. Different from the results of Shibamoto et al. (2016), however, we observed three peaks (corresponding to four compounds) for mono-DMDS adducts of methyl linoleate (*c*9, *c*12-$C_{18:2}$) (Figure 1b): peak 1 contains two mono-DMDS adducts at Δ^9 and Δ^{12} positions, while peak 2 and peak 3 contain adducts at Δ^9, Δ^{12} position, respectively. In comparison, only two peaks at peak 2 and peak 3 positions were observed for mono-DMDS adducts of methyl linolelaidate (*t*9, *t*12-$C_{18:2}$) (Figure 1c).

The existence of additional mono-DMDS adducts suggests the involvement of other reaction mechanisms during the DMDS reaction. Mass spectra of $C_{18:2}$ mono-DMDS adducts with either *cis* or *trans* double bonds share similar fragmentation patterns ("Interpretation of mass spectra of mono-DMDS adducts of methyl linoleate (*c*9, *c*12-$C_{18:2}$) and methyl linolelaidate (*t*9, *t*12-$C_{18:2}$)" section, Figure 2). This suggests that the underivatized double bonds in adducts corresponding to peak 1 are in *cis* configurations (two compounds are unfortunately not resolved in our GC–FID and GC–MS), whereas double bonds in peak 2 and peak 3 are in *trans* configurations. Although not explicitly discussed, we notice that there are apparent shoulder peaks partially co-eluting with DMDS adducts of *c*9, *c*12-$C_{18:2}$ in Shibamoto et al. (2016) as well. Based on our results, these coeluting peaks are likely isomerized *trans*-mono-DMDS adducts. Our analyses were performed utilizing a 105 m-long RTX-200 column with a gradient temperature program, which may have resulted in different resolutions of isomers.

The formation of *trans* double bonds in mono-DMDS adducts of methyl linoleate *c*9, *c*12-$C_{18:2}$ suggests a partial isomerization of *cis* double bond into the more stable *trans* double bond during DMDS reaction. The ratio of isomerized mono-DMDS adducts over total mono-DMDS adducts shows a slight increase with longer reaction time and higher reaction temperature. Specifically, isomerization increased from 13% to 18% at 0°C, when reaction time increased from 1 to 3 h (Figure S1). Isomerization increased from 13% to 19% for 1 h reaction time, when reaction temperature increased from 0 to 50°C (Figure S1). The isomerization may be related to the reversible adduction of iodine cation onto double bonds and subsequent rotation of carbon–carbon single bond during the formation of iodine intermediates (proposed reaction mechanism in Figure S2). In our previous DMDS reactions for alkenones containing *trans* double bonds (Dillon et al., 2016; Liao et al., 2021; Richter et al., 2017; Zhao et al., 2014), we did not observe such isomerization. Our results suggest that only *cis* double bonds undergo partial isomerization to *trans* double bonds, but not vice versa. Our preliminary data also suggest that mono-DMDS adduction on fatty acids can provide useful information about double bond geometry.

Interpretation of mass spectra of mono-DMDS adducts of methyl linoleate (*c*9, *c*12-$C_{18:2}$) and methyl linolelaidate (*t*9, *t*12-$C_{18:2}$)

Mass spectra of mono-DMDS adducts have low-abundance molecular ions at *m*/*z* 388, but with strong $[M-48]^+$ ions at *m*/*z* 340 due to a loss of CH_3SH and $[M-95]^+$ ions at *m*/*z* 293 due to a further loss of CH_3S group (Figure 2). For mono-DMDS adduct at Δ^9 position, ions at *m*/*z* 217 and 171 produced through the

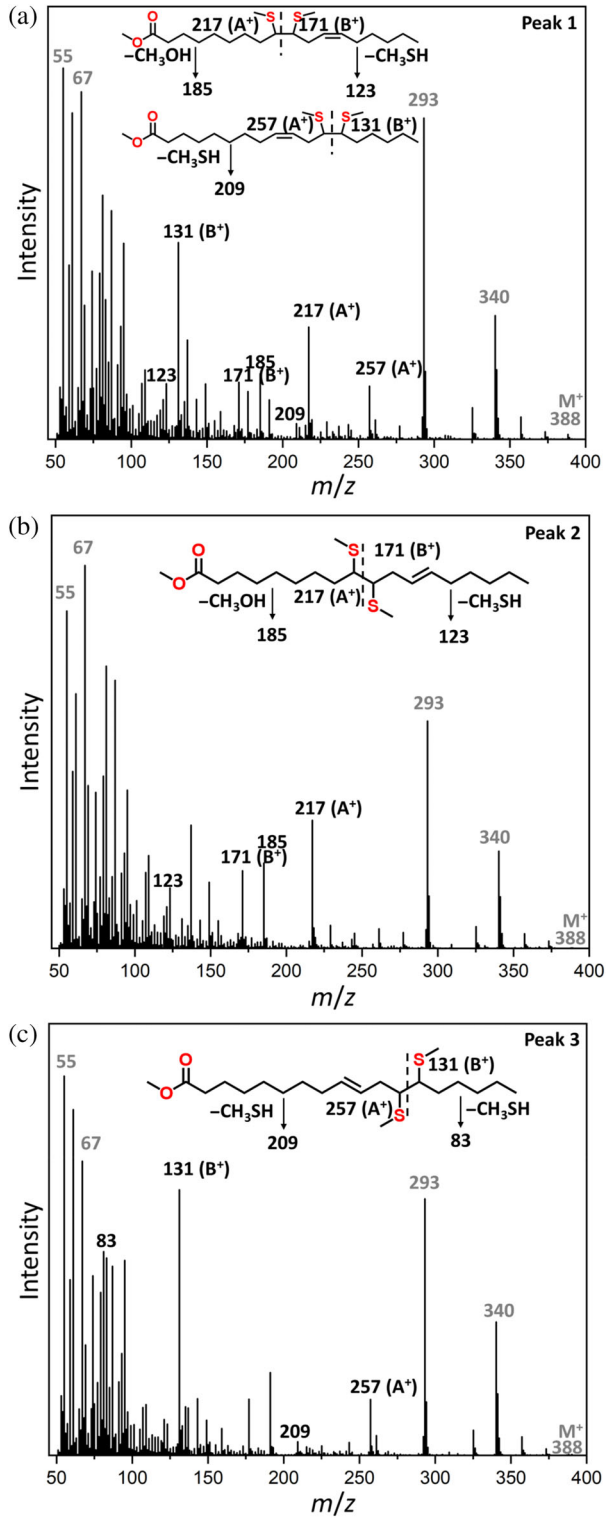

FIGURE 2 Mass spectra of peak 1 (a), peak 2 (b) and peak 3 (c) for mono-DMDS adducts of methyl linoleate (*c*9, *c*12-$C_{18:2}$) shown in Figure 1b. Ion fragments related to double bond positions are highlighted

cleavage of carbon–carbon single bond between two methylthio groups, are diagnostic (Figure 2b). The ion at *m*/*z* 185 can be attributed to a further loss of CH_3OH group from the ion at *m*/*z* 217, while the ion at *m*/*z* 123 to a further loss of CH_3SH group from the ion at *m*/*z* 171 (Figure 2b). On the other hand, DMDS adduct at Δ^{12} position yields two diagnostic ions at *m*/*z* 257 and 131, which subsequently lose CH_3SH groups to yield ions at *m*/*z* 209 and 83 (Figure 2c).

Notably, mono-DMDS adducts of methyl linoleate (*c*9, *c*12-$C_{18:2}$) and methyl linolelaidate (*t*9, *t*12-$C_{18:2}$) are well resolved by GC–MS in this study (Figure 1). However, in our previous applications of mono-DMDS adducts for determining double bond positions in polyunsaturated long-chain alkenones, mono-DMDS adducts for each alkenone were not resolved on the GC (Dillon et al., 2016; Liao et al., 2021; Zhao et al., 2014), possibly related to the long alkyl chains of alkenones (C_{37}–C_{42} carbon atoms). It is important to point out that even without chromatographic separation of mono-DMDS adducts for alkenones, mass spectra of a mixture of mono-DMDS adducts at different double bond positions still provide abundant diagnostic ions for elucidating double bond positions. Similarly, two mono-DMDS adducts coelute in Figure 2a, but the mass spectra of the mixed compounds still provide all the diagnostic ions for elucidating the individual double bond positions. This demonstrates the high efficiency of mono-DMDS adducts in determining double bond positions for different types of lipids.

Mono-DMDS adducts of methyl pinolenate (*c*5, *c*9, *c*12-$C_{18:3}$)

Abundant mono-DMDS adducts (44% yield when the reaction was performed at 25°C for 20 min) were also obtained for tri-unsaturated methyl pinolenate (*c*5, *c*9, *c*12-$C_{18:3}$) that contains both two methylene-interrupted (Δ^5 and Δ^9) and one methylene-interrupted double bonds (Δ^9 and Δ^{12}) (Figure 3). This success indicates that our experimental procedure is also applicable for determining the double bond positions that are interrupted by more than one methylene groups. Three dominant peaks (1–3) were observed, with peak 1 corresponding to mono-DMDS adduct at Δ^9 double bond, peak 2 corresponding to adduct at Δ^5 double bond and peak 3 corresponding to adduct at Δ^{12} double bond (Figure 3a). The earlier elution of mono-DMDS adducts at double bonds that lie between other double bonds (Δ^9 for *c*5, *c*9, *c*12-$C_{18:3}$) is also observed for *c*7, *c*10, *c*13, *c*16-$C_{22:4}$ ("Mono-DMDS adducts of methyl adrenate (*c*7, *c*10, *c*13, *c*16-$C_{22:4}$)" section, DMDS adducts at Δ^{10} and Δ^{13} double bonds elute first, Figure 4a) and *c*5, *c*8, *c*11, *c*14, *c*17-$C_{20:5}$ ("Mono-DMDS adducts of methyl eicosapentaenoate (*c*5, *c*8, *c*11, *c*14, *c*17-$C_{20:5}$)" section, DMDS adducts at Δ^8 and Δ^{11} double bonds elute first, Figure 5a). Peak 4 and peak 5 are assigned as mono-DMDS adducts at Δ^5 and Δ^{12} positions with *trans* underivatized double bonds, respectively (Figure 3a).

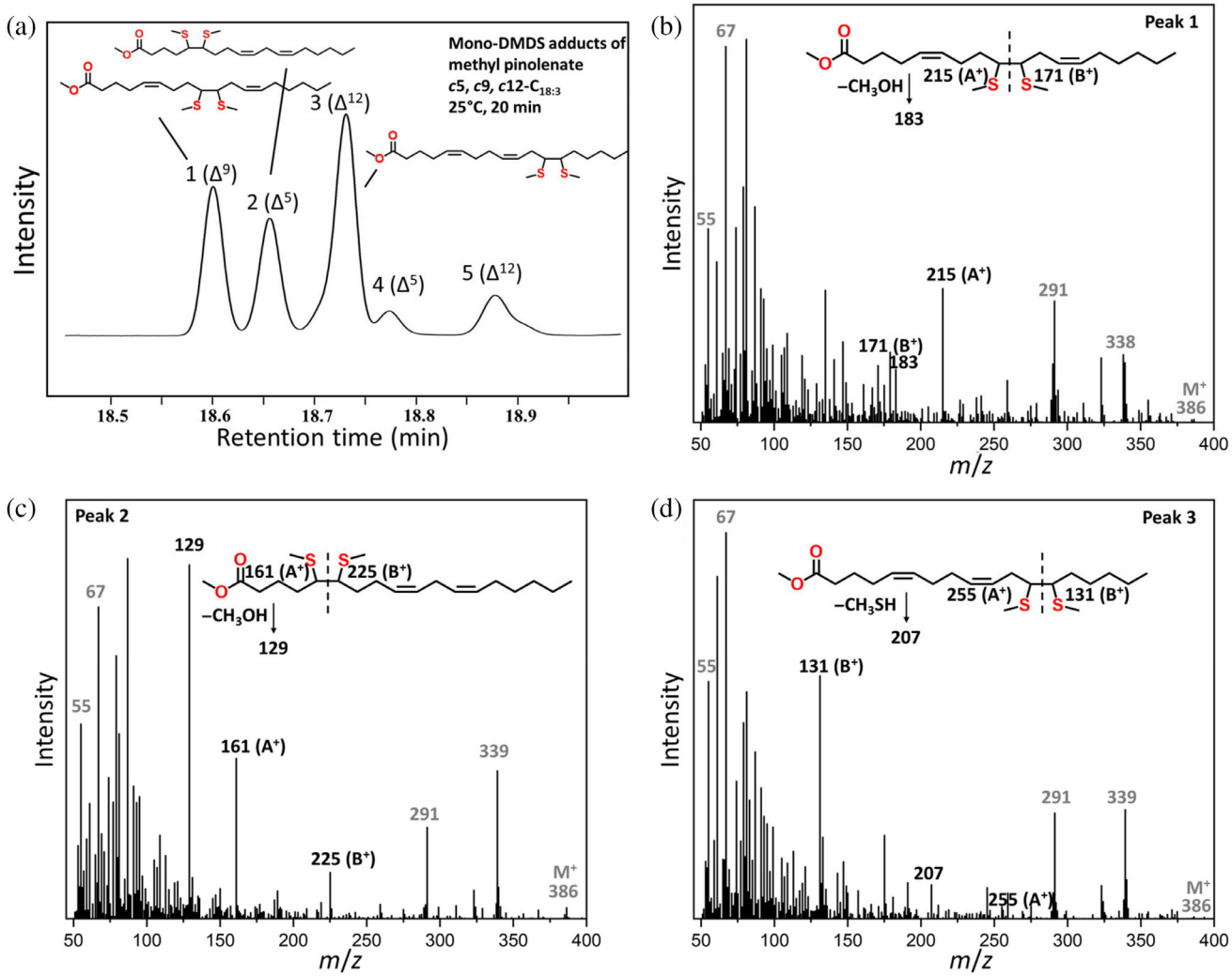

FIGURE 3 Gas chromatogram showing the distribution of mono-DMDS adducts of methyl pinolenate (*c*5, *c*9, *c*12-$C_{18:3}$) reacted at 25°C for 20 min (a). Mass spectra of DMDS adducts of methyl pinolenate at peak 1 (b), peak 2 (c) and peak 3 (d). Peak numbering $n(\Delta^m)$ in the chromatograms denotes compound *n*, with mono-DMDS adducting on the double bond position *m* on the FAME. We did not specifically determine configurations of underivatized double bonds in mono-DMDS adducts. Ion fragments related to double bond positions are highlighted

We did not observe isomerized mono-DMDS adduct at Δ^9 position in the GC chromatogram (Figure 3a). It is possible that the isomer may have co-eluted with other mono-DMDS adducts. Alternatively, isomerization may be less favorable for Δ^9 double bond that lies between other double bonds. Thus, while the presence of isomerized mono-DMDS adducts demonstrates *cis* configurations, the absence of isomers cannot always be taken as evidence of *trans* geometry at specific double bond positions. In the case of *c*5, *c*9, *c*12-$C_{18:3}$, the presence of isomerized peaks for mono-DMDS adducts at Δ^5 double bond suggests that either or both Δ^9 and Δ^{12} double bonds must be in *cis* configuration. Similarly, the presence of isomerized peaks for DMDS adducts at Δ^{12} double bond suggests that either or both Δ^5 and Δ^9 double bonds must be in *cis* configuration. The absence of isomerized peaks for mono-DMDS adducts at Δ^9 double bond, however, cannot be taken as evidence for *trans* double bonds at Δ^5 and Δ^{12} positions.

The mono-DMDS adduct at Δ^9 position (peak 1) yields diagnostic ions at *m*/*z* 215 and 171. The ion at *m*/*z* 183 can be attributed to a further loss of CH_3OH group from the ion at *m*/*z* 215 (Figure 3b). Peak 2 corresponds to DMDS adduct at Δ^5 position with characteristic ions at *m*/*z* 161 and 225. Strong ion at *m*/*z* 129 can be attributed to a loss of CH_3OH group from the ion at 161 (Figure 3c). Mass spectrum of peak 3 contains ion at *m*/*z* 131 with high abundance, corresponding to the double bond at Δ^{12} position. On the other hand, the ion at *m*/*z* 255 and correlated ion at *m*/*z* 207 have relatively low abundances (Figure 3d). Notably, compared with DMDS adducts at Δ^5 and Δ^{12} positions, we notice the diagnostic ions in the mass spectrum of the mono-DMDS adduct at Δ^9 position have relatively low abundances. This may suggest that cleavage of carbon–carbon single

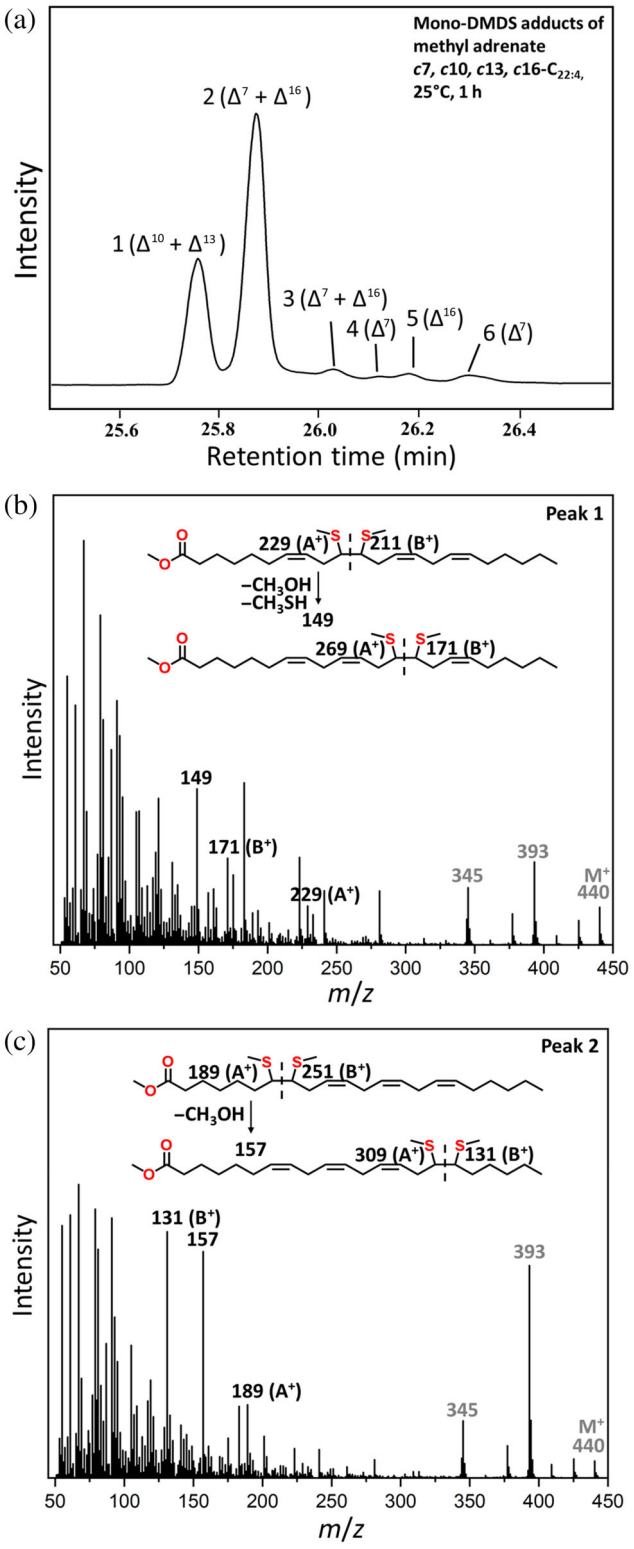

FIGURE 4 Gas chromatogram showing the distribution of mono-DMDS adducts of methyl adrenate (*c*7, *c*10, *c*13, *c*16-$C_{22:4}$) reacted at 25°C for 1 h (a). Mass spectra of DMDS adducts of methyl adrenate at peak 1 (b), peak 2 (c). Peak numbering $n(\Delta^m)$ in the chromatograms denotes compound *n*, with mono-DMDS adducting on the double bond position *m* on the FAME. We did not specifically determine configurations of underivatized double bonds in mono-DMDS adducts. Ion fragments related to double bond positions are highlighted

bond carrying CH_3S groups is less efficient, or the resulting ion fragments are less stable in the ion source when the adducted double bond lies between other double bonds.

Double bond positions in tri-unsaturated fatty acids have previously been studied through mass spectra of cyclized DMDS adducts (Pepe, Sayer, & Dagaut, 1997; Pepe, Sayer, Dagaut, & Couffignal, 1997) or DMDS adducts of corresponding mono-unsaturated fatty acids after partial hydrogenation (Tanaka et al., 1997). Notably, in the mass spectra of cyclized DMDS adducts for a series of tri-unsaturated fatty acids (*c*9, *c*12, *c*15-$C_{18:3}$, *c*8, *c*11, *c*14-$C_{20:3}$ and *c*11, *c*14, *c*17-$C_{20:3}$) (Pepe, Sayer, & Dagaut, 1997; Pepe, Sayer, Dagaut, & Couffignal, 1997), we notice ions related to double bonds that are closest to ester groups (e.g., Δ^9 double bond in *c*9, *c*12, *c*15-$C_{18:3}$, Δ^8 double bond in *c*8, *c*11, *c*14-$C_{20:3}$ and Δ^{11} double bond in *c*11, *c*14, *c*17-$C_{20:3}$) have relatively low abundances (~10% of the base peak) compared with ions related to other double bond positions (Pepe, Sayer, & Dagaut, 1997; Pepe, Sayer, Dagaut, & Couffignal, 1997). This significantly increases the difficulty in fully elucidating structures of fatty acids. Such problem, however, was not observed in mass spectra of mono-DMDS adducts obtained in this work (Figure 3).

Mono-DMDS adducts of methyl adrenate (*c*7, *c*10, *c*13, *c*16-$C_{22:4}$)

We observed two major peaks in the chromatogram with peak 1 corresponding o DMDS adducts at Δ^{10} and Δ^{13} double bonds and peak 2 corresponding to DMDS adducts at Δ^7 and Δ^{16} double bonds (Figure 4). Peaks 3–6 are DMDS adducts at Δ^7 and Δ^{16} positions with underivatized double bonds in *trans* configuration. Similar to the scenario of methyl pinolenate (Figure 3a), we did not observe the isomerized DMDS adducts at Δ^{10} or Δ^{13} double bond that lies between other double bonds.

Mass spectrum of peak 1 contains ions at *m*/*z* 229 and 171, which are diagnostic for mono-DMDS adducts at Δ^{10} and Δ^{13} positions. The ion at *m*/*z* 149 can be attributed to a further loss of CH_3OH and CH_3SH groups from the ion at *m*/*z* 229 (Figure 4b). Mass spectrum of peak 2 has high-abundance ions at *m*/*z* 131, 157 and 189. Ions at *m*/*z* 131 and 189 are caused by the cleavage of carbon–carbon single bonds carrying CH_3S groups at Δ^{16} and Δ^7 positions, respectively. The ion at *m*/*z* 157 can be attributed to a further loss of CH_3OH group from the ion at *m*/*z* 189 (Figure 4c). This again, illustrates that partial chromatographic co-elution of mono-DMDS adducts does not prevent mass spectra from providing diagnostic ions for elucidating the individual double bond positions.

Similar to *c*5, *c*9, *c*12-$C_{18:3}$ (Figure 3b), the abundances of diagnostic ions in the mass spectra are

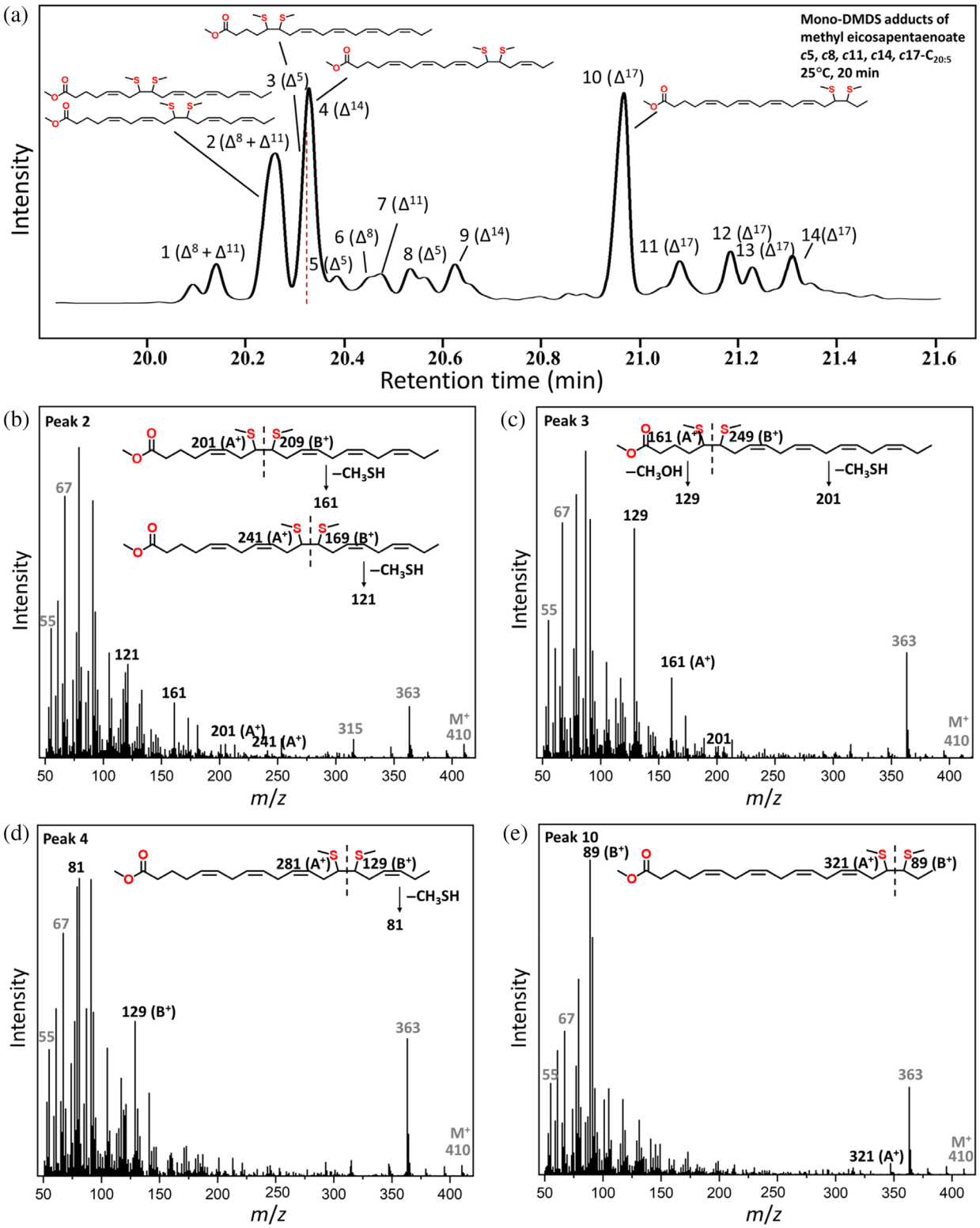

FIGURE 5 (a) Gas chromatogram showing the distribution of mono-DMDS adducts of methyl eicosapentaenoate (*c*5, *c*8, *c*11, *c*14, c17-$C_{20:5}$) reacted at 25°C for 20 min. Mass spectra of DMDS adducts of methyl eicosapentaenoate at peak 2 (b), peak 3 (c), peak 4 (d) and peak 10 (e). Peak numbering $n(\Delta^m)$ in the chromatograms denotes compound *n*, with mono-DMDS adducting on the double bond position *m* on the FAME. We did not specifically determine configurations of underivatized double bonds in mono-DMDS adducts. Ion fragments related to double bond positions are highlighted

relatively low when mono-DMDS adducts occur at Δ^{10} and Δ^{13} positions of *c*7, *c*10, *c*13, *c*16-$C_{22:4}$. However, the corresponding mass spectra are still sufficiently clear for elucidating the double bond positions. We also observed a series of isomerized mono-DMDS adducts for different double bond positions (Figure 4a), consistent with the structure of methyl adrenate that contains *cis* double bonds.

Mono-DMDS adducts of methyl eicosapentaenoate (*c*5, *c*8, *c*11, *c*14, *c*17-$C_{20:5}$)

Gas chromatogram of mono-DMDS adducts of methyl eicosapentaenoate (EPA, *c*5, *c*8, *c*11, *c*14, *c*17-$C_{20:5}$) contains 14 peaks due to the isomerization of double bond configurations (Figure 5a). At least one diagnostic ion is observed for each mono-DMDS adduct of EPA to determine double bond positions (Figure 5b–e). Mass spectrum of peak 2 contains ions at *m*/*z* 121, 161, 201 and 241, corresponding to DMDS adducts at Δ^8 and Δ^{11} double bonds (Figure 5b). The abundances of diagnostic ions for adducts at Δ^8 and Δ^{11} double bonds are lower than those at other double bond positions. This is consistent with the aforementioned observation in the mass spectra of mono-DMDS adducts of *c*5, *c*9, *c*12-$C_{18:3}$ at Δ^9 double bond (Figure 3b) and *c*7, *c*10, *c*13, *c*16-$C_{22:4}$ at Δ^{10} and Δ^{13} double bonds (Figure 4b). The ion at *m*/*z* 121 is diagnostic for the DMDS adduct at Δ^{11} position (Table S1), produced through the loss of CH_3SH group from the ion at *m*/*z* 169. On the other hand, ions at *m*/*z* 161 and 201 are attributed to DMDS adducts at Δ^5 or Δ^8 positions (Table S1). We exclude the possibility that the DMDS adduct at Δ^5 position is responsible for ions at *m*/*z* 161 and 201 in peak 2, because we did not observe a strong ion at *m*/*z* 129 (produced through a further loss of CH_3OH from the ion at *m*/*z* 161) as previously observed in mono-DMDS adducts of methyl pinolenate at Δ^5 position (Figure 3c).

We observe strong ions at *m*/*z* 161 and 129 in the mass spectrum of peak 3, which can be attributed to mono-DMDS adduct at Δ^5 position (Figure 5c). Mass spectrum of adjacent peak 4 contains an ion at *m*/*z* 81 with high abundance, which is diagnostic for adduct at Δ^{14} position and produced through a further loss of CH_3SH group from the ion at *m*/*z* 129 (Figure 5d). Mass spectra for peaks 10–14 all contain the ion at *m*/*z* 89 as the base peak (Figures 5e and S3), consistent with a Δ^{17} double bond.

Determination of double bond positions in highly unsaturated lipids like EPA is challenging. Such determination has previously been achieved through methods including the DMDS derivatization on corresponding mono-unsaturated compounds after partial hydrogenation (Imbs & Rodkina, 2005; Yamamoto et al., 1991), 4,4-dimethyloxazoline (DMOX) derivatization (Toral et al., 2018), Paternò-Büchi Reaction (Murphy et al., 2017) and formation of acetonitrile covalent adduct (Alves et al., 2011). However, it is difficult to control the reaction products for the partial hydrogenation method. Other approaches may yield relatively low abundance of diagnostic ions for certain double bond positions. In comparison, mass spectra of mono-DMDS adducts provide at least one highly abundant diagnostic ion for each double bond in poly-unsaturated fatty acids. Joint applications of mono-DMDS derivatization approach with methods that derivatize the carboxyl groups, such as DMOX (Toral et al., 2018) and pyrrolidides (Andersson, 1978), and/or other double bond derivatization methods (Alves et al., 2011; Murphy et al., 2017), would permit more accurate structural elucidation of poly-unsaturated fatty acids.

CONCLUSIONS

We report a convenient and efficient (room-temperature; 20–60 min) experimental procedure to yield abundant mono-DMDS adducts of poly-unsaturated FAMEs for the purpose of determining double bond positions. The mono-DMDS adducts for the poly-unsaturated FAMEs are well-resolved gas-chromatographically from any cyclized or poly-DMDS products formed in the derivatization reaction, providing highly diagnostic fragment ions for determining the double bond positions through GC–MS analysis. By controlling the reaction conditions, we show abundant mono-DMDS adducts can be formed from poly-unsaturated fatty acids without the reliance on the steric hindrance of one methylene-interrupted double bonds. Notably, elucidating double bond positions in poly-unsaturated fatty acids does not necessarily require complete chromatographic separation of individual mono-DMDS adducts in GC–MS, as the mass spectra of a mixture of mono-DMDS adducts still provide sufficient diagnostic information for elucidating double bond positions.

We also observe, for the first time, extensive *cis* to *trans* isomerization (but not vice versa) for mono-DMDS adducts resulting from our derivatization reactions. Thus, the presence of isomerization provides strong evidence that the corresponding double bond in the fatty acid is in *cis* configuration. However, due to complex chromatographic coelutions of mono-DMDS adducts and different rates of isomerization in different double bond positions in poly-unsaturated fatty acids, absence of certain isomerized mono-DMDS adducts in the GC–MS chromatogram may not be taken as definitive evidence that the corresponding double bond in question is in *trans* configuration. In cases of ambiguity, joint application of mono-DMDS derivatization with other carboxyl and double bond derivatization techniques will permit more accurate structural elucidation of poly-unsaturated fatty acids.

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AUTHOR CONTRIBUTION

YH initiated the research. SL performed experiments. SL and YH wrote the paper jointly.

ETHICS STATEMENT

This research falls outside of human or animal studies and institutional ethical approval was not required.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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SUPPORTING INFORMATION

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