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RESEARCH ARTICLE

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Elucidation of double-bond positions of polyunsaturated alkenes through gas chromatography/mass spectrometry analysis of mono-dimethyl disulfide derivatives

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Rationale: Derivatization with dimethyl disulfide (DMDS) followed by gas chromatography/mass spectrometry (GC/MS) analysis is a well-established method for locating double-bond position on the alkyl chain of mono-unsaturated compounds such as alkenes. For alkenes containing more than one double bond, however, the conventional DMDS derivatization approach forms poly- or cyclized DMDS adducts whose mass spectra are difficult to interpret in terms of double-bond positions. In this study, we report an efficient experimental procedure to produce mono-DMDS adducts for polyunsaturated alkenes with two to six double bonds. GC/MS analyses of these mono-DMDS adducts yield highly characteristic mass fragments, allowing unambiguous assignments of double-bond positions on the alkyl chain. We also apply our new approach (i.e., preferential formation of mono-DMDS adducts during derivatization with DMDS) to determine the double-bond positions of unsaturated alkenes produced by laboratory cultured *Isochrysis litoralis*, a haptophyte algal species.

Methods: Alkenes from different sources were derivatized with DMDS at 25°C for 20 to 160 min. The mass spectra of mono-DMDS adducts were obtained by GC/EI-MS analysis of reaction products which contain chromatographically resolved mono-DMDS adducts.

Results: Mass spectra of corresponding mono-DMDS adducts contain prominent diagnostic ions that allow a conclusive elucidation of double-bond positions. In culture samples of *Isochrysis litoralis*, a series of novel mono- to tri-unsaturated C_{31} alkenes (9-$C_{31:1}$, 6,9-$C_{31:2}$, 6,22-$C_{31:2}$, 6,25-$C_{31:2}$, 9,22-$C_{31:2}$, 6,9,25-$C_{31:3}$) were discovered for the first time.

Conclusions: A highly efficient DMDS derivatization approach is developed to yield abundant mono-DMDS adducts of polyunsaturated alkyl alkenes for elucidating double-bond positions using GC/MS.

1 | INTRODUCTION

Elucidation of double-bond positions on the alkyl chain in unsaturated lipids is fundamental in natural product and synthetic organic chemistry.[1,2] Iodine (I_2)-catalyzed addition reaction between double bonds and dimethyl disulfide (DMDS) represents a convenient one-pot derivatization for unsaturated compounds for locating the double-bond positions on the alkyl chain.[3–8] The resulting adducts yield highly diagnostic ions caused by the cleavage of single bonds between carbon atoms adducted with CH_3S groups in electron ionization (EI) mass spectrometry. The DMDS reaction has been used to determine double-bond positions in various fatty

acids[7,9–11] and alkenes.[5,12] Traditionally, the reaction between DMDS and unsaturated compounds was performed under heated conditions for an extended period of time (e.g., 40°C for 4 h or overnight) to ensure all double bonds are fully derivatized.[3–7,13–15] For polyunsaturated molecules, however, such reaction conditions lead to the formation of poly- (and/or cyclized) DMDS adducts.[13,16] The significantly increased molecular weights of poly-DMDS adducts greatly increase the boiling points of the derivatized products, making them less amenable for gas chromatographic (GC) analysis, especially for relatively high molecular weight target compounds. More importantly, mass spectra of the corresponding cyclized or poly-DMDS adducts are often hard to interpret in terms of double-bond positions due to the low abundance of diagnostic ions and numerous fragmentation possibilities.[17] Therefore, the application of the DMDS reaction has been largely limited to mono-unsaturated compounds.[3,4,7,15]

Recently, we demonstrated that by reducing reaction time, temperature and I_2 catalyst-to-sample ratios, desirable amounts of mono-DMDS adducts can be obtained for polyunsaturated long-chain (C_{37} to C_{42}) ketones with up to five double bonds.[1,17–20] Such mono-DMDS adducts have significantly lower molecular weights than the corresponding cyclized or poly-DMDS adducts and are thus well suitable for GC/MS analysis. They also provide highly specific diagnostic ions in mass spectra that allow unambiguous identification of all double-bond positions. With such method, we have successfully identified double-bond positions in a series of novel methyl-branched long-chain unsaturated ketones (e.g., penta-unsaturated long-chain ketones with double bonds located at Δ^4, Δ^7, Δ^{14}, Δ^{21} and Δ^{28}, numbering from the carbonyl group),[18] as well as novel C_{36} shorter-chain unsaturated ketones from *Emiliania huxleyi* algal cultures.[1]

In this paper, we demonstrate the efficacy of forming mono-DMDS adducts on a series of polyunsaturated alkenes with two to six double bonds for the determination of double-bond positions on the alkyl chains. As an example of application to natural product organic chemistry, we also determined the double-bond positions in long-chain alkenes produced by *Isochrysis litoralis* (*I. litoralis*, a species in the order Isochrysidales of the Haptophyte algae) for the first time.

2 | MATERIALS AND METHODS

2.1 | Chemicals

All solvents were HPLC grade from Fisher Scientific (USA). Diethyl ether (>99%), dimethyl disulfide (>98%), iodine (>99.8%), sodium thiosulfate (>99%) and sodium sulfate (anhydrous, >99%) were purchased from Sigma Aldrich (USA). 7(*Z*),11(*Z*)-Nonacosadiene ($C_{29}H_{56}$) (>98%) was purchased from Cayman Chemical (USA). 1,5,9,13-Tetradecatetraene ($C_{14}H_{22}$) (>95%, containing *cis*/*trans*-isomers) was purchased from Alfa Aesar (USA). Squalene ($C_{30}H_{50}$) (>98%) was purchased from MP Biomedicals (France).

2.2 | Culture experiments and extraction of alkenes

I. litoralis AC18 was obtained from the Algobank-Caen. Culture growth conditions and harvesting procedure followed those reported by Liao et al.[21,22] *I. litoralis* was acclimatized for 2 weeks before the start of the corresponding culture experiments with f/2 medium at 15°C.[23] The f/2 medium was prepared from seawater collected from Vineyard Sound, Woods Hole, MA, USA, at a salinity of 32 ppt (filtered using a 0.2 μm Whatman nylon membrane filter and then autoclaved). The culture experiment was performed under a light:dark cycle set at 16:8. The light intensity was 140 μE m^{-2} s^{-1}. The culture was harvested at the early stationary phase (monitored using hemocytometer counts; Hausser Scientific, PA, USA) by filtering onto 0.7 μm glass fiber filters (Merck Millipore, MA, USA). All filters were wrapped with aluminum foil and immediately frozen at −20°C.[21]

Filters of culture samples were freeze-dried overnight and then sonicated three times with dichloromethane (DCM, 3 × 30 min, 20 mL each time) for lipid extractions. Total extracts were divided into three fractions using silica gel (230–400 mesh, 40–63 μm) in glass pipettes, and eluted with 4 mL hexane, 4 mL DCM and 4 mL methanol. Alkenes were in the hexane fraction.

2.3 | DMDS derivatization reaction

Mono-dimethyl disulfide (DMDS) adducts of alkenes were prepared following our procedure reported previously by Richter et al[17] and Liao et al,[18] but with two important modifications: (1) we set the reaction temperature at 25°C (i.e., close to room temperature) in order to further simplify the derivatization method; and (2) we tested four different reaction durations (20, 40, 80, 160 min) for two compounds, (7(*Z*),11(*Z*)-nonacosadiene and 1,5,9,13-tetradecatetraene), at 25°C, in order to understand how yields of mono-DMDS adducts change. About 100 μg alkenes were dried and then dissolved in 50 μL hexanes. Then 40 μL iodine solution (60 mg I_2 in 1 mL Et_2O) and 200 μL DMDS were added to initiate the reaction. The reaction was performed at 25°C (room temperature) for 20 min for all alkenes studied in this work. Additional DMDS reactions at 25°C for 40, 80 and 160 min were performed for 7,11-nonacosadiene and 1,5,9,13-tetradecatetraene. The reaction was then 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 the mixture was allowed to sit for 30 s. The average yield for mono-DMDS adducts was ~19% when the reaction was performed at 25°C for 20 min. The resulting hexane solutions were separated and immediately analyzed by GC/flame ionization detection (FID) and GC/MS.

Cyclized and poly-DMDS adducts of alkenes produced by *I. litoralis* were prepared by reacting DMDS with alkenes at 50°C for 20 min. The following cleanup procedures were the same as the procedure mentioned above.

2.4 | GC analysis

Mono-DMDS adducts of derivatized alkenes were analyzed by GC-FID (Agilent 7890B) and GC/EI-MS (Agilent 6890 interfaced to 5973 MSD) equipped with mid-polarity poly(trifluoropropylmethylsiloxane) RTX-200 columns (105 m × 250 μm × 0.25 μm).[21] For the analysis by GC-FID, the carrier gas was hydrogen. Samples were injected in pulsed splitless mode at 320°C. The initial pulse pressure was 35 psi for the first 1 min. Then the purge flow to split vent was 35.0 mL/min at 1.1 min. The flow rate (constant flow mode) was 1.5 mL/min. The initial oven temperature was 45°C for 1 min, then increased to 255°C at 20°C/min, then increased to 300°C at 1°C/min, then increased to 320°C at 10°C/min, and held for 120 min. For the analysis by GC/EI-MS, samples were injected in pulsed splitless mode at 315°C. The carrier gas used for GC/EI-MS was helium. The initial pulse pressure was 35 psi for the first 1.0 min. The purge flow to split vent was 50 mL/min at 1.1 min. The flow rate (constant flow mode) was 1.5 mL/min. The initial oven temperature was 40°C for 1 min, then increased to 255°C at 20°C/min, then increased to 300°C at 1°C/min, then increased to 320°C at 10°C/min, and held for 120 min. Samples were analyzed in full-scan mode (*m/z* 50–700).

3 | RESULTS AND DISCUSSION

3.1 | Reaction conditions to obtain mono-DMDS adducts of polyunsaturated alkenes

Mono-DMDS adducts are transient products during the adduction between DMDS and double bonds in polyunsaturated compounds. Thus, with prolonged heating and relatively high reagent-to-sample ratios, mono-DMDS products initially formed could further react with more DMDS to form cyclized, poly-DMDS or even intermolecular polymerized adducts.[17] Therefore, in order to obtain relatively large quantities of mono-DMDS adducts for elucidating double-bond positions in polyunsaturated compounds, it is important to use appropriate reaction conditions that would not cause over-reaction and inadvertently diminish yields of transient mono-DMDS products.

We have previously investigated the experimental parameters for the production of mono-DMDS adducts in C_{37} to C_{42} long-chain unsaturated alkyl ketones containing up to five double bonds.[17,18] Due to the excessively high molecular weights of our target compounds, only mono-DMDS adducts of these unsaturated ketones would be amenable for GC/MS analysis (poly-DMDS adducts would have too high molecular weights to pass through GC columns). The main finding from our previous experiment is that the DMDS reaction rate decreases with decreasing I_2 amount and reaction temperature.[17] In order to preferentially form mono-DMDS adducts, it is important to reduce, rather than increase the reaction rate, so that the reaction can be stopped at the desired point when mono-DMDS adducts, which are transient products in nature, are formed in high abundance for elucidating double-bond positions. The majority of the previous DMDS derivatization procedures, in contrast, have aimed (in our opinion, mistakenly) to derivatize all double bonds, and therefore adopted excessive heating (e.g., 40°C or higher temperatures), prolonged reaction time (e.g., overnight) and with a relatively large amount of I_2 catalyst (up to ~20 mg/mL, with I_2/substrate mole ratio up to ~8000).[5,7,15] When we applied similar reaction conditions for long-chain unsaturated ketones, our reaction products were almost entirely composed of poly-DMDS adducts that did not elute through the conventional GC columns at all.[17,18] In contrast, we successfully obtained sufficient amounts of mono-DMDS adducts of long-chain alkenones by significantly reducing reaction temperature, time and iodine concentration. The highest yield was obtained at room temperature for 160 min (23%) or 0°C for 250 min (22.6%) with I_2 concentration at ~8 mg/mL (I_2/substrate mole ratio at ~150).[17]

In this study, we further tested the impact of different reaction durations on the yield of mono-DMDS adducts for di-unsaturated 7,11-nonacosadiene and tetra-unsaturated 1,5,9,13-tetradecatetraene at 25°C (room temperature), using the same reagent ratios as reported previously (Figure S1, supporting information).[17,18] Our primary goal is to identify a convenient and efficient reaction condition to yield sufficient mono-DMDS adducts for structural elucidation using GC/MS. A temperature of 25°C was selected as it is close to room temperature in most chemical laboratories; hence no heating or cooling is required. Under this temperature, we did not observe significant formation of cyclized or poly-DMDS adducts for alkenes studied in this work.

The yield of mono-DMDS adducts for 7,11-nonacosadiene increased from 28% to 54% with increasing reaction times of 20, 40, 80 and 160 min (Figure S1a, supporting information). On the other hand, the highest yield of mono-DMDS adducts for 1,5,9,13-tetradecatetraene was obtained when the reaction was performed for 80 min (32%). A further increase in the reaction time to 160 min decreased the yield to 7.9% (Figure S1b, supporting information). Our results suggest that reaction times to achieve maximum yields of mono-DMDS adducts are dependent on the target molecules. A shorter reaction time is likely favorable for obtaining higher yields of mono-DMDS adducts for alkenes with more double bonds. However, for analytical purposes, we argue that it is not critical to always achieve the highest yields for every compound, since mono-DMDS adducts are well resolved chromatographically from unreacted compounds and poly-DMDS adducts by GC. Thus, obtaining high-quality mass spectra of mono-DMDS adducts does not always require obtaining the highest yield of mono-DMDS adducts. Rather, our goal is to identify an efficient and convenient reaction condition (e.g., room temperature, relatively fast derivatization) that will allow us to obtain sufficiently large amounts of mono-DMDS adducts for structural elucidation using GC/MS. It is possible that higher yields of mono-DMDS adducts can be obtained at different temperatures/reaction time/I_2 amount, but we did not carry out additional experiments to identify (nor do we think it necessary) the condition that would maximize the production of mono-DMDS adducts for all compounds studied. Nevertheless, 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.

3.2 | Mono-DMDS adducts of di-unsaturated 7,11-nonacosadiene

3.2.1 | Derivatization of 7,11-nonacosadiene

Carlson et al investigated the DMDS adduction of 7(*Z*),11(*Z*)-nonacosadiene at 40°C for 4 h, with an I_2/substrate mole ratio of ~2000.[5] The primary product formed under such reaction conditions was a cyclized DMDS adduct, the intended product of derivatization. However, mass spectra of the cyclized adducts are generally difficult to interpret in terms of double-bond positions due to the involvement of multiple fragmentation patterns and relatively low abundances of diagnostic ions. Production of cyclized DMDS adducts of polyunsaturated alkenes may also lead to complex chromatograms even for a single target compound, because of the formation of cyclized adducts with different ring sizes.[24,25] Interestingly, Carlson et al mentioned that mono-DMDS adducts of 7(*Z*),11(*Z*)-nonacosadiene were also obtained as the by-products of the derivatization procedure, presumably undesirable ones.[5] Unfortunately, the reaction yields and chromatographic resolution of the mono-DMDS adducts were not reported, and the potential for using the mono-DMDS adducts to elucidate double-bond positions through GC/MS analyses was not explored.

Through reacting at 25°C for 20 min, we achieved a relatively good yield (28%) for mono-DMDS adducts of 7,11-nonacosadiene, which allows us to obtain high-quality mass spectra to study their fragmentation patterns. We identified four peaks in the chromatogram, with a partial co-elution between peaks 3 and 4 (Figure 1b). Peak 1and peak 4 share a similar fragmentation pattern, corresponding to the adduct at the C-7 position, while mass spectra of peak 2 and peak 3 share a similar fragmentation pattern, corresponding to the adduct at the C-11 position (Figure S3, supporting information). The existence of additional mono-DMDS adducts at peak 3 and peak 4 is related to the impurities of the 7(*Z*),11(*Z*)-nonacosadiene standard. When we analyzed the 7(*Z*),11(*Z*)-nonacosadiene standard, we found two peaks that eluted slightly earlier than the dominant 7(*Z*),11(*Z*)-nonacosadiene (Figure 1a). The similarity of their mass spectra to that of 7(*Z*),11(*Z*)-nonacosadiene suggests that these two peaks are geometric isomers of 7,11-nonacosadiene containing *trans*-double bonds (Figure S2, supporting information). Our measured purity of 7(*Z*),11(*Z*)-nonacosadiene based on our GC-FID analysis is ~91%, lower than the manufacturer-stated purity of 98%. Based on our data, we assign peak 1 and peak 2 to mono-DMDS adducts of 7(*Z*),11(*Z*)-nonacosadiene, while peak 3 and peak 4 correspond to mono-DMDS adducts of 7,11-nonacosadiene containing *trans*-double bonds (Figure S3, supporting information).

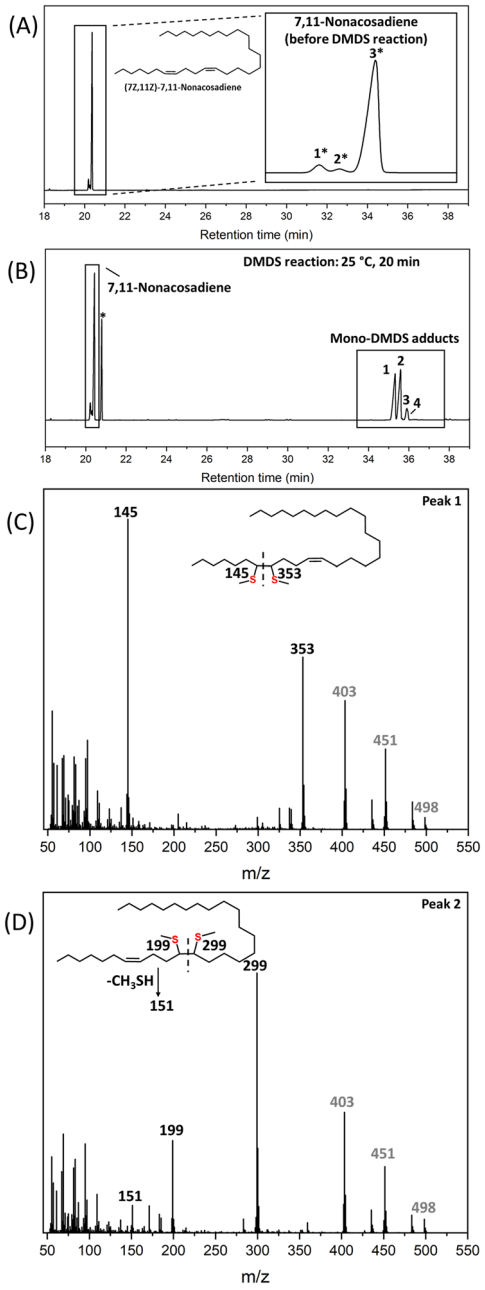

FIGURE 1 Gas chromatograms showing the distribution of compounds a, before and b, after DMDS reaction of 7,11-nonacosadiene. The DMDS reaction was performed at 25°C for 20 min. The peak labelled with * is a phthalate contaminant. Mass spectra of mono-DMDS adducts of c, peak 1 and d, peak 2 for 7,11-nonacosadiene. Ion fragments related to double-bond positions are highlighted

3.2.2 | Mass spectral interpretation of mono-DMDS adducts of 7,11-nonacosadiene

Mass spectra of mono-DMDS adducts exhibit low-abundance molecular ions at *m/z* 498, but with strong $[M - 47]^+$ ions at *m/z* 451 due to a loss of CH_3S and at *m/z* 403 $[M - 95]^+$ due to a further loss of a CH_3SH group (Figures 1c and 1d). Similar fragmentation

patterns have previously been observed in mono-DMDS adducts of long-chain ketones.[17] The mass spectrum of peak 1 contains characteristic ions at *m/z* 145 and 353 caused by the cleavage of the single bond between carbon atoms carrying CH_3S groups (Figure 1c). This suggests the DMDS adduct at the C-7 position. On the other hand, the mass spectrum of peak 2 contains characteristic ions at *m/z* 199 and 299, corresponding to the double bond at the C-11 position (Figure 1d). The ion at *m/z* 151 was generated through a further loss of a CH_3SH group from the ion at *m/z* 199. Such further loss of a CH_3SH group from key fragment ions has been widely reported in different types of DMDS adducts (mono-, poly- or cyclized DMDS adducts) of unsaturated compounds.[5,13,17,18] Interestingly, we notice that in the mass spectrum of the adduct at the C-7 position (Figure 1c), the ion at *m/z* 145 has higher abundance than the ion at *m/z* 353, while in the mass spectrum of the adduct at the C-11 position (Figure 1d), the ion at *m/z* 299 is higher in abundance than the ion at *m/z* 199. This suggests that the charge is more likely to be retained on the fragment with fewer double bonds during the ionization process.

3.3 | Mono-DMDS adducts of tetra-unsaturated 1,5,9,13-tetradecatetraene

3.3.1 | Derivatization of 1,5,9,13-tetradecatetraene

For polyunsaturated compounds with three or more double bonds, the traditional DMDS method that aims to derivatize all double bonds is generally considered unfeasible due to the formation of high molecular weight poly-DMDS adducts that may not permit GC/MS analysis. As one alternative solution, partial hydrogenation of samples into mono-unsaturated compounds has been applied before subsequent DMDS reaction.[9–11] This solution avoids the formation of cyclized or poly-DMDS adducts. However, the isolation and purification of mono-unsaturated compounds from a mixture of partially hydrogenated products require use of a chromatography column packed with silver nitrate silica gel, which is often cumbersome and can greatly increase the sample preparation time. Additionally, due to the difference in reduction rates for double bonds at different locations (e.g., terminal double bond is the easiest to reduce),[26] it is challenging (i.e., likely requires repeated attempts, and success or not is hard to predict) to obtain sufficient mono-unsaturated products for all individual double bonds of highly unsaturated lipids. We have previously obtained mono-DMDS adducts for long-chain unsaturated ketones with up to five double bonds.[17,18] Here, we select the tetra-unsaturated 1,5,9,13-tetradecatetraene as an example to demonstrate the feasibility of obtaining corresponding mono-DMDS adducts in highly unsaturated alkenes as well as their efficiency in determining double-bond positions.

Because 1,5,9,13-tetradecatetraene is a symmetric molecule, two types of mono-DMDS adducts can be obtained from it (one type of mono-DMDS adduct on the C-1 or C-13 double bond, while another type of mono-DMDS adduct on the C-5 or C-9 double bond). In the chromatogram recorded after the DMDS reaction, we observe five peaks of mono-DMDS adducts (Figure 2b). Similar to the scenario for 7,11-nonacosadiene, peak 1 and peak 2 share the same fragmentation pattern, corresponding to the mono-DMDS adducts at the C-5 and C-9 position (Figure S5, supporting information). On the other hand,

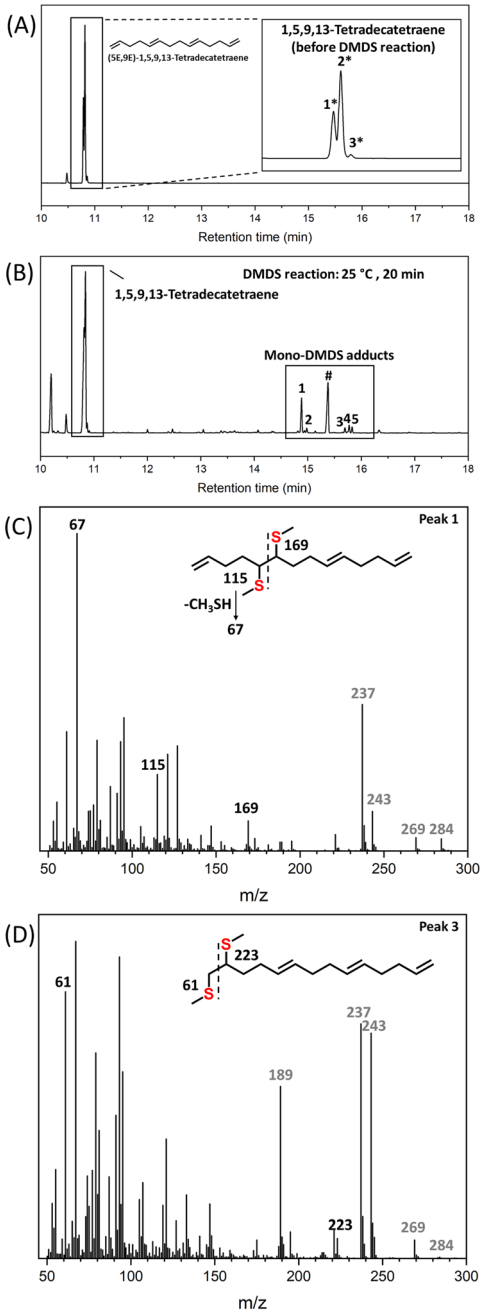

FIGURE 2 Gas chromatograms showing the distribution of compounds a, before and b, after DMDS reaction of 1,5,9,13-tetradecatetraene. The DMDS reaction was performed at 25°C for 20 min. The peak labelled with # is *N*1-(4-methoxyphenyl) benzene-1,4-diamine contaminant. Mass spectra of c, peak 1 and d, peak 3 for mono-DMDS adducts of 1,5,9,13-tetradecatetraene. Ion fragments related to double-bond positions are highlighted

peak 3, peak 4 and peak 5 correspond to mono-DMDS adducts at terminal double bonds (Figure S5, supporting information). The existence of additional peaks for mono-DMDS adducts is most likely because our 1,5,9,13-tetradecatetraene standard is actually a mixture of *cis*/*trans*-isomers at the C-5 and C-9 positions (Figure 2a, Figure S4, supporting information).

3.3.2 | Mass spectral interpretation of mono-DMDS adducts of 1,5,9,13-tetradecatetraene

Mass spectra of peak 1 and peak 2 show molecular ions at *m/z* 284 (Figure 2c). Ions at *m/z* 269 $[M-15]^+$, 243 $[M-41]^+$ and 237 $[M-47]^+$ are produced through a loss of CH_3, α-cleavage of the terminal double bond (C_3H_5) and loss of a CH_3S group, respectively (Figure 2c). Diagnostic ions related to double bonds at the C-5 and C-9 positions at *m/z* 67, 115 and 169 can be easily identified from the mass spectra (Figure 2c). Compared with the mass spectra of mono-DMDS adducts at the C-5 and C-9 positions, mass spectra of peak 3, peak 4 and peak5 with adducts at terminal double bonds contain high-abundance ions at *m/z* 189 due to a further loss of a CH_3SH group from the ion at *m/z* 237 (Figure 2d). Diagnostic ions at *m/z* 61 and 223 suggest the adducts at terminal double bonds. Similar to the mass spectra in 7,11-nonacosadiene (Figures 1c and 1d), for both types of mono-DMDS adducts of 1,5,9,13-tetradecatetraene (Figures 2c and 2d), the diagnostic ions containing fewer double bonds are present in higher abundance than those of the counterpart fragments containing more double bonds (e.g., the abundance of the ion at *m/z* 115 is higher than that of the ion at *m/z* 169 for adducts at the C-5 or C-9 position, the abundance of the ion at *m/z* 61 is higher than that of the ion at *m/z* 223 for adducts at terminal double bonds).

3.4 | Mono-DMDS adducts of hexa-unsaturated squalene

3.4.1 | Derivatization of squalene

The DMDS reaction has only so far been used to determine double-bond positions in unsaturated compounds with up to five double bonds (either through partial hydrogenation and then being derivatized with DMDS[9,10] or directly being derivatized with DMDS under mild condition to obtain mono-DMDS adducts[18]). The feasibility of using DMDS for locating double-bond positions in compounds with even more double bonds has never been tested. Here, we performed a further test on a branched (isoprenoid) alkene, squalene, that contains six double bonds.

The gas chromatogram of mono-DMDS adducts of squalene contains three peaks with each peak corresponding to DMDS adducts at one pair of double bonds (C-2 and C-22, C-6 and C-18, C-10 and C-14) (Figure 3b). The yield for mono-DMDS adducts is 2.5% relative to the amount of squalene added into the reaction solution

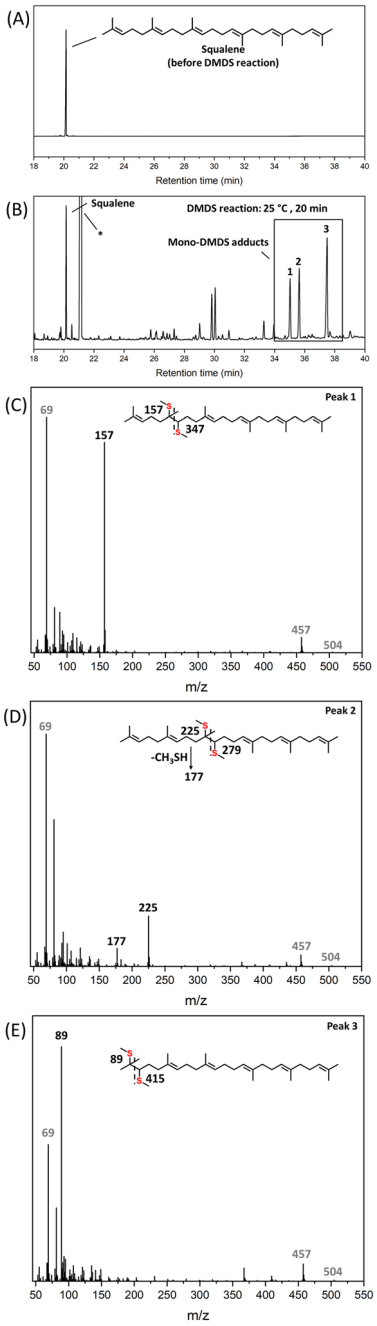

FIGURE 3 Gas chromatograms showing the distribution of compounds a, before and b, after DMDS reaction of squalene. The DMDS reaction was performed at 25°C for 20 min. The peak labelled with * is a phthalate contaminant. Mass spectra of c, peak 1; d, peak 2; and e, peak 3 for mono-DMDS adducts of squalene. Ion fragments related to double-bond positions are highlighted

(Figure 3b). The low yield of the mono-DMDS adducts may partially result from steric hindrance of methyl branches on squalene. In addition, the residual (unreacted) squalene only accounts for 0.6% of the original squalene used before the DMDS reaction (Figure 3b). This low recovery may be explained by the polymerization via intermolecular coupling during DMDS derivatization of the poly-unsaturated squalene.

3.4.2 | Mass spectral interpretation of mono-DMDS adducts of squalene

Mass spectra of mono-DMDS adducts exhibit low-abundance molecular ions at *m/z* 504, and $[M-47]^+$ ions at *m/z* 457 due to a loss of CH_3S groups. A characteristic ion at *m/z* 69 $[C_5H_9]^+$ was observed for all mono-DMDS adducts (Figures 3c–3e), which was produced through α-cleavage of the double bond at the C-2 or C-22 position. For DMDS adducts at C-6 and C-18 double bonds, a diagnostic ion at *m/z* 157 was observed (Figure 3c). For DMDS adducts at the C-10 and C-14 double bonds, we identified the diagnostic ion at *m/z* 225 caused by the cleavage of a single bond between carbon atoms carrying CH_3S groups. The ion at *m/z* 177 was produced by a further loss of a CH_3SH group from the ion at *m/z* 225 (Figure 3d). For DMDS adducts at C-2 and C-22 double bonds, the diagnostic ion at *m/z* 89 shows exceptionally high abundance and exists as the base peak (Figure 3e).

Similar to the fragmentation pattern observed in mono-DMDS adducts of 7,11-nonacosadiene (Figures 1c and 1d) and 1,5,9,13-tetradecatetraene (Figures 2c and 2d), the abundance of the diagnostic ion fragment with fewer double bonds is higher than the complementary ion fragment for mono-DMDS adducts of squalene (Figures 3c–3e). In fact, we did not observe the corresponding fragments with more double bonds in our mass spectra (e.g., the ion at *m/z* 415 was not observed for DMDS adducts at the C-2 position, Figure 3e). Similar fragmentation and charge retention pattern could also be observed in mono-DMDS adducts for polyunsaturated long-chain ketones.[17,18]

Though we observe only one diagnostic ion for each mono-DMDS adduct in squalene, the corresponding mass spectrum is still straightforward to interpret and sufficient for determining double-bond positions due to the high abundance and uniqueness of such diagnostic ions. Other derivatization methods to determine double-bond positions in highly unsaturated lipids (e.g., Paternò-Büchi Reaction[27] and formation of acetonitrile covalent adducts[28]) have been attempted before, but the corresponding mass spectra of the resulting products contain generally low abundance of diagnostic ions for locating the double-bond positions. In addition, these derivatization reactions tend to have low yields and products are difficult to predict. Collectively, our data suggest the formation of mono-DMDS adducts represents a convenient method for determining double-bond positions in molecules containing poly-unsaturated alkyl chains, including alkyl chains with methyl branch substitutions.

3.5 | Mono-DMDS adducts of novel long-chain alkenes produced by *I. litoralis*

I. litoralis is one species of Isochrysidales (an order of haptophyte algae) that features the production of polyunsaturated long-chain unsaturated ketones (also called alkenones).[21,29] Due to the linear response between degrees of unsaturation of alkenones and

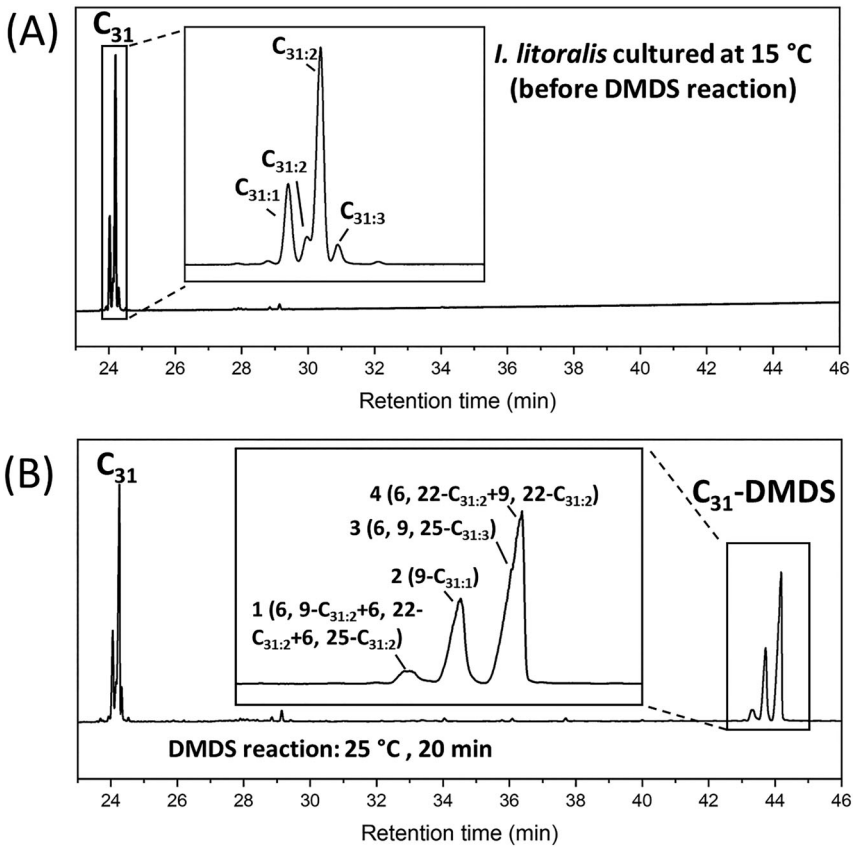

FIGURE 4 Gas chromatograms showing the distribution of compounds a, before and b, after DMDS reaction of alkenes produced by *I. litoralis* cultured at 15°C. The DMDS reaction was performed at 25°C for 20 min

environmental temperature, alkenones have been widely used for paleo-sea surface temperature (SST) reconstructions over the past 40 years.[30,31] Different Isochrysidales species vary in their temperature calibrations and/or growth seasons.[21,32–34] Identifying alkenone producers is important for proper paleoclimate reconstructions.[32] However, identification of alkenone producers based solely on the alkenone distributions is not always possible, due to the overlapping of alkenone profiles among different groups of Isochrysidales species.[21,22,32] Structures of alkenes may provide additional chemotaxonomic differentiation among different species of Isochrysidales.[21,32,35]

Long-chain alkenes of Isochrysidales have previously been reported in culture samples of *Isochrysis galbana* (*I. galbana*) and *E. huxleyi* (*E. huxleyi*) (another two species of Isochrysidales),[12,36] but alkenes in *I. litoralis* have not been studied. Based on the previous studies,[12,36] *I. galbana* produces C_{31} and C_{33} alkenes with double bonds at the C-1, C-2, C-22 and C-24 positions, and *E. huxleyi* produces C_{31}, C_{33}, C_{37} and C_{38} alkenes, with double bonds at C-1, C-2, C-3, C-15, C-16, C-22, C-23 and C-24 positions. In comparison, our *I. litoralis* culture sample only contains mono- to tri-unsaturated C_{31} alkenes (Figure 4a). We observed four peaks of mono-DMDS adducts for these alkenes produced by *I. litoralis* (~40% yield at 25°C for 20 min, there is a partial co-elution between peak 3 and peak 4, Figure 4b). Mass spectra of peak 1 (Figure 5a) and peak 4 (Figure 5d) show molecular ions at *m/z* 526, corresponding to mono-DMDS adducts of di-unsaturated $C_{31:2}$ alkenes. In the mass spectrum of peak 1 (Figure 5a), ions at *m/z* 131 and 395 correspond to the double bond at the C-6 position with another double bond in the fragment with *m/z* 395. On the other hand, ions at *m/z* 171 and 355 correspond to the double bond at the C-9 position with another double bond in the fragment with *m/z* 171. In the mass spectrum of peak 4 (Figure 5d), ions at *m/z* 173 and 353 correspond to the double bond at the C-22 position with another double bond in the fragment with *m/z* 353. We notice the double bond at C-9 is symmetrical to the double bond at the C-22 position (i.e., the same carbon chain length starting from the nearest terminal carbon atom). Diagnostic ions observed in mass spectra of mono-DMDS adducts of $C_{31:2}$ thus suggest the presence of four di-unsaturated alkenes: 6,9-$C_{31:2}$, 6,22-$C_{31:2}$, 6,25-$C_{31:2}$ and 9,22-$C_{31:2}$. The mass spectrum of peak 2 shows a molecular ion at *m/z* 528, corresponding to the mono-

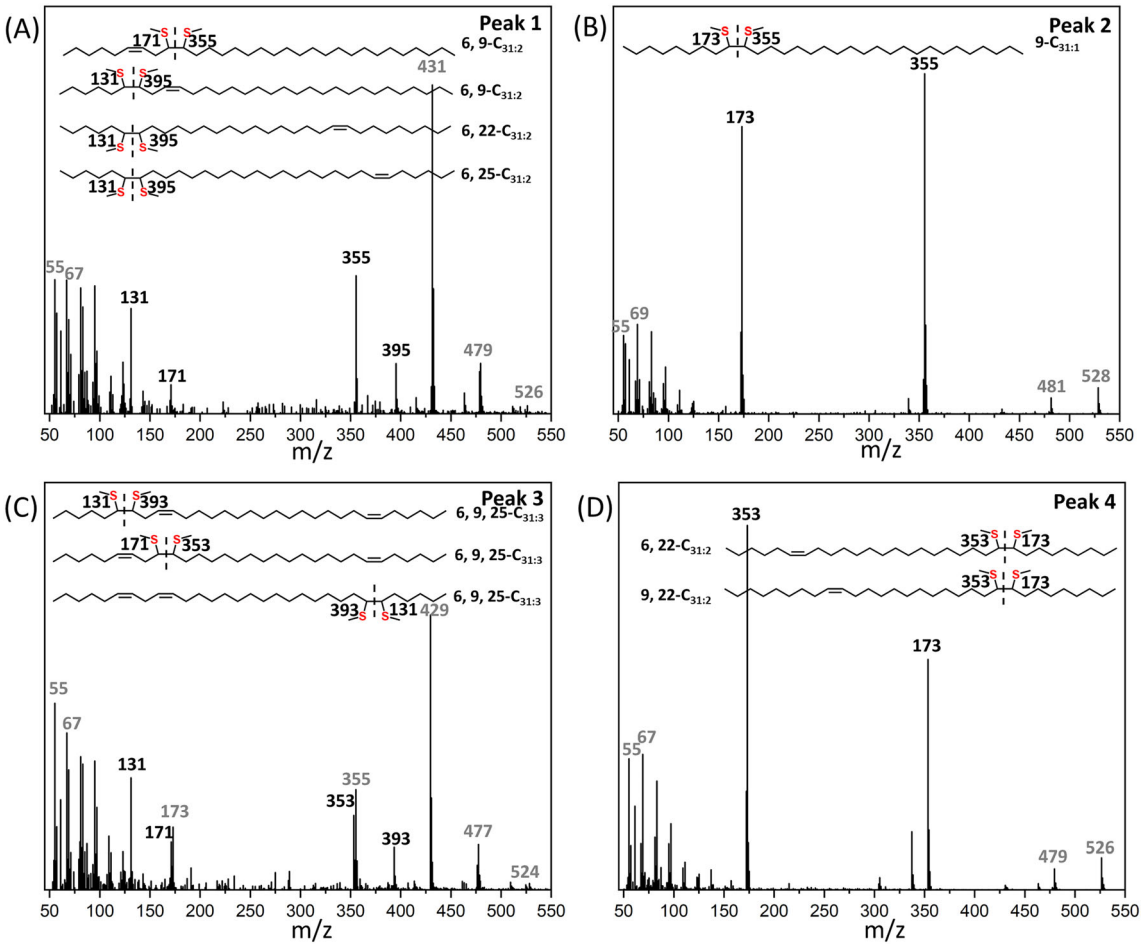

FIGURE 5 Mass spectra of DMDS adducts of long-chain alkenes at a, peak 1; b, peak 2; c, peak 3; and d, peak 4 as labelled in Figure 4b. Ion fragments related to double-bond positions are highlighted. Double bonds in alkenes were assumed to be in the *cis* configuration based on alkenes produced by other Isochrysidales species[12,36]

DMDS adduct of the $C_{31:1}$ alkene (Figure 5b). Ions at *m/z* 173 and 355 suggest the double bond at the C-9 position (i.e., 9-$C_{31:1}$). The mass spectrum of peak 3 contains a molecular ion at *m/z* 524, corresponding to mono-DMDS adducts of tri-unsaturated alkenes (Figure 5c). Ions at *m/z* 353 and 171 correspond to the double bond at the C-9 position, while ions at *m/z* 131 and 393 correspond to double bonds at the C-6 and C-25 positions (i.e., 6,9,25-$C_{31:3}$).

We also derivatized *I. litoralis* alkenes with DMDS at 50°C for 20 min, so that we could obtain some cyclized and poly-DMDS adducts for di-unsaturated $C_{31:2}$ alkenes (Figure S6, supporting information). Although the mass spectra of these cyclized and poly-DMDS adducts contain ions related to double-bond positions, interpretation of the mass spectra in terms of double-bond positions is difficult. This is because key diagnostic ions for cyclized and poly-DMDS adducts are of low abundance and multiple ions with different fragmentation mechanisms are involved in the mass spectra (Figure S6, supporting information). Nevertheless, cyclized and poly-DMDS adducts of alkenes are still helpful for further confirmation of the structural assignments based on GC/MS analyses of mono-DMDS adducts.

The chain lengths and double-bond positions of alkenes produced by *I. litoralis* are significantly different from alkenes produced by *I. galbana* and *E. huxleyi*: *I. galbana* produces 1,22-$C_{31:2}$, 1,24-$C_{31:2}$, 2,22-$C_{31:2}$ and 1,24-$C_{33:2}$; *E. huxleyi* produces 1,22-$C_{31:2}$, 2,22-$C_{31:2}$, 3,22-$C_{31:2}$, 2,24-$C_{31:2}$, 2,24-$C_{33:2}$, 15,22-$C_{37:2}$, 1,15,22-$C_{37:3}$, 15,22-$C_{38:2}$, 1,15,22-$C_{38:3}$, 1,16,23-$C_{38:3}$[12,36]; while *I. litoralis* produces 9-$C_{31:1}$, 6,9-$C_{31:2}$, 6,22-$C_{31:2}$, 6,25-$C_{31:2}$, 9,22-$C_{31:2}$ and 6,9,25-$C_{31:3}$. More studies are needed to understand the underlying biosynthetic pathways for different alkenes produced in different species of Isochrysidales. However, different alkene structures in different Isochrysidales species are important for chemotaxonomic purposes in environmental samples.[21,33]

4 | CONCLUSIONS

We report a convenient and efficient experimental approach (25°C for 20 to 160 min) to produce mono-DMDS adducts for polyunsaturated alkenes for the purpose of determining double-bond positions on the alkyl chain (including the branched alkyl chain). We show mass spectra of these mono-DMDS adducts contain high-abundance diagnostic ions that allow a full elucidation of double-bond positions in polyunsaturated alkyl alkenes with two to six double bonds. Our approach overcomes the limit of traditional DMDS derivatization methods which are generally limited to mono-unsaturated molecules.

We applied our experimental procedure to a mixture of unknown long-chain alkenes produced by a laboratory culture of *I. litoralis*, in order to determine their double-bond positions. We successfully identified six mono- to tri-unsaturated C_{31} alkenes with double-bond positions at C-6, C-9, C-22 and C-25 (9-$C_{31:1}$, 6,9-$C_{31:2}$, 6,22-$C_{31:2}$, 6,25-$C_{31:2}$, 9,22-$C_{31:2}$ and 6,9,25-$C_{31:3}$). Alkenes produced by *I. litoralis* show significant differences in chain lengths and double-bond positions compared with alkenes produced by other common Isochrysidales species such as *I. galbana* and *E. huxleyi*. Such chemotaxonomic differences could be used to differentiate different species in environmental samples. Our success in obtaining mono-DMDS adducts for an unknown and complex mixture of long-chain alkenes further validates the high efficacy of our derivatization approach for elucidating double-bond positions.

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DATA AVAILABILITY STATEMENT

Data available in article supplementary material

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SUPPORTING INFORMATION

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