
Electrospray Ionization Multiple-Stage Linear Ion-trap Mass Spectrometry for Structural Elucidation of Triacylglycerols: Assignment of Fatty Acyl Groups on the Glycerol Backbone and Location of Double Bonds

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Linear ion-trap multiple-stage mass spectrometric approach (MS^n) towards nearly complete structural elucidation of triacylglycerol (TAG) including (1) assignment the fatty acid substituents on the glycerol backbone and (2) location of the double bond(s) on the unsaturated fatty acyl groups is reported. The characterization is established by the findings that MS^2 on the $[M + Li]^+$ ions of TAG yields more abundant ions reflecting losses of the outer fatty acid substituents either as free acids (i.e., $[M + Li - R_1CO_2H]^+$ and $[M + Li - R_3CO_2H]^+$ ions) or as lithium salts (i.e., $[M + Li - R_1CO_2Li]^+$ and $[M + Li - R_3CO_2Li]^+$ ions) than the ions reflecting the similar losses of the inner fatty acid substituent (i.e., $[M + Li - R_2CO_2Li]^+$ and $[M + Li - R_2CO_2H]^+$ ions). Further dissociation (MS^3 of $[M + Li - R_nCO_2H]^+$ ($n = 1, 2, \text{ or } 3$) gives rise to the ion series locating the double bonds along the fatty acid chain. These ions arise from charge-remote fragmentations involving β -cleavage with γ -H shift, analogous to those seen for the unsaturated long-chain fatty acids characterized as initiated ions. Significant differences in abundances in the ion pairs reflecting the additional losses of the fatty acid moieties, respectively, were also seen in the MS^3 spectra of the $[M + Li - R_nCO_2H]^+$ and $[M + Li - R_nCO_2Li]^+$ ions, leading to confirmation of the fatty acid substituents on the glycerol backbone. MS^n on the $[M + Na]^+$ and $[M + NH_4]^+$ adduct ions also affords location of fatty acid substituents on the glycerol backbone, but not the position of the double bond(s) along the fatty acid chain. Unique ions from internal losses of the glycerol residues were seen in the MS^3 spectra of $[M + Alk - R_nCO_2H]^+$ ($n = 1, 2, 3$) and of $[M + Alk - R_nCO_2Alk]^+$ ($Alk = Li, Na, NH_4; n = 1, 3$). They are signature ions for glycerides and the pathways leading to their formation may involve rearrangements. (J Am Soc Mass Spectrom 2010, 21, 657–669) © 2010 American Society for Mass Spectrometry

High-energy CAD with a tandem sector instrument permits structural characterization of triacylglycerol (or triacylglyceride) (TAG) as sodiated adducts desorbed by FAB, leading to identify each fatty acid substituent and positions of fatty acid substituents on the glycerol backbone. However, the sensitivity is poor and the application in elucidation of the location of double bond(s) for the unsaturated fatty acid substituents has not been demonstrated [1]. High-energy CAD product-ion spectra of the sodiated adducts of TAG obtained with tandem sector instrument coupled with ESI were unsatisfactory for structural characterization, but those from the $[M + NH_4]^+$ ions identified the fatty acid substituents, nevertheless, are not informative for assignment of the fatty acid sub-

stituents on the glycerol backbone [1]. Similarly, tandem quadrupole mass spectra of the $[M + Na]^+$ ions of TAG did not contain structurally informative fragment ions, but those from $[M + NH_4]^+$ ions contained fragment ions that identify the fatty acid groups [2]. Neither the positions of the fatty acid substituents on the glycerol backbone nor the locations of double bonds in unsaturated fatty acid chains could be determined from these spectra [2]. Recently, Pittenauer and Allmaier demonstrated high-energy CAD using MALDI-TOF/RTOF-MS for characterization of TAGs as alkali adduct ions. The product ions from $[M + Na]^+$ ions of TAG afford determination of the position of fatty acid substituents on the glycerol backbone and in some favorable cases the location of double bonds or hydroxy groups. However, the method is limited by the insufficient precursor ion gating after MS^1 (gating window of 4 Da), and ion species differed by 2 Da cannot be separated [3].

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By contrast, tandem quadrupole [4] and quadrupole ion-trap [5] product-ion spectra from the $[M + Li]^+$ ions of TAG is readily applicable for identification of the fatty acid substituents and location their position on the glycerol backbone. Skimmer CAD on the lithiated adduct ion of TAG, followed by tandem quadrupole mass spectrometry on the dilithiated adduct ions of fatty acid substituents generated by skimmer CAD are applicable for location of double bonds. But the method for reliable location of the unsaturated sites for the fatty acid chain in TAG mixtures remains untested [4]. The employment of ozone electrospray ionization and ozone induced dissociation mass spectrometry to unveil the double bond position of triecosatetraenoin and trioleoryglycerol standards was recently reported by Thomas et al., but assignment of the fatty acid substituents on the glycerol backbone has not been demonstrated [6–8]. The method also required a special set-up to generate harmful ozone gas to be emitted to an ion-trap instrument. Herein, we report a multiple-stage (MS^n) ($n = 2, 3, 4$) mass spectrometric approach with an LIT instrument for near-complete characterization of TAG as the $[M + Li]^+$ ions, including identification of fatty acid substituents, the site of double bond of fatty acyl substituents and their positions on the glycerol backbone. The application of this LIT MS^n method on the $[M + Na]^+$ and $[M + NH_4]^+$ ions of TAG for structural characterization and the mechanism(s) underlying the fragmentation processes leading to ion formation are also discussed.

Experimental

Material and Chemicals

Triacylglycerol standards of rac-glyceryl-1,3-dipalmitate-2-oleate [(16:0/ Δ^9 18:1/16:0)-TAG], rac-glyceryl-2,3-dipalmitate-1-oleate [(Δ^9 18:1/16:0/16:0)-TAG], rac-glyceryl-1-palmitate-2-oleate-3-stearate [(16:0/ Δ^9 18:1/18:0)-TAG], rac-glyceryl-1-palmitate-2-stearate-3-oleate [(16:0/18:0/ Δ^9 18:1)-TAG], rac-glyceryl-1,3-distearate-2-oleate [(18:0/ Δ^9 18:1/18:0)-TAG], rac-glyceryl-2,3-distearate-1-oleate [(Δ^9 18:1/18:0/18:0)-TAG], and tripalmitin [(16:0/16:0/16:0)-TAG] were purchased from Matreya (Pleasant Gap, PA). Synthetic triarachidonin [($\Delta^{5,8,11,14}$ 20:4/ $\Delta^{5,8,11,14}$ 20:4/ $\Delta^{5,8,11,14}$ 20:4)-TAG] and trilinolein [($\Delta^{9,12}$ 18:2/ $\Delta^{9,12}$ 18:2/ $\Delta^{9,12}$ 18:2)-TAG] standards were purchased from NuChek Prep (Elysian, MN). 1,3(d₅)-Diheptadecanoyl-2-(10Z-heptadecenoyl)-glycerol [d₅-(17:0/ Δ^{10} 17:1/17:0)-TAG] was purchased from Avanti Polar lipid (Alabaster, AL). All TAG standards are >98% purity. Solvents and other chemicals were obtained from Fisher Chemical (Suwanee, GA, USA).

Mass Spectrometry

Low-energy CAD tandem mass spectrometry experiments were conducted on a Finnigan (San Jose, CA) linear ion-trap (LIT) mass spectrometer (MS) with Xcali-

bur operating system. Triacylglycerols were dissolved in chloroform/methanol (1/4) at final concentration of 10 pmol/ μ L and lithium hydroxide was added to achieve a final $[Li^+]$ concentration of 1 mM before infusion (2 μ L/min) to the ESI source, where the skimmer was set at ground potential, the electrospray needle was set at 4.5 kV, and temperature of the heated capillary was 350 °C. The automatic gain control of the ion trap was set to 5×10^4 , with a maximum injection time of 200 ms. Helium was used as the buffer and collision gas at a pressure of 1×10^{-3} mbar (0.75 mTorr). The MS^n experiments were carried out with an optimized relative collision energy ranging from 16% to 25% and with an activation q value at 0.25, and the activation time at 30–100 ms to leave a minimal residual abundance of precursor ion (around 20%). The isolation window for the precursor ions were set at 1 Da. Mass spectra were accumulated in the profile mode, typically for 3–10 min for MS^n ($n = 2, 3, \text{ and } 4$) spectra. The mass resolution of the instrument was tuned to 0.6 Da at half peak height.

Results and Discussion

TAG molecules that differ only with respect to the fatty acyl groups at *sn*-1 and *sn*-3, such as (A/B/C)-TAG versus (C/B/A)-TAG (where A, B, and C are distinct fatty acid residues) are enantiomers and cannot be distinguished by the present mass spectrometric approach. In this premise for assignment of the fatty acid substituents on the glycerol backbone as described in the text, only the fatty acid substituent assigned to *sn*-2 is specific, and the fatty acid substituents assigned to *sn*-1 and at *sn*-3 is exchangeable (i.e., A/B/C)-TAG and (C/B/A)-TAG are not distinguishable). No effort was made to determine the chirality of TAGs in this study.

Structural Determination of the TAG Species that Possesses Different Fatty Acid Substituents (A/B/C-TAG; $A \neq B \neq C$)

Upon resonance excitation in an ion-trap, the $[M + Li]^+$ ions of TAG yielded abundant fragment ions corresponding to $[M + Li - R_nCO_2H]^+$ and $[M + Li - R_nCO_2Li]^+$ (where n denotes the glycerol carbon to which the fatty acid is esterified), which identify the fatty acid substituents. The differential formation of these ions also leads to the location of the fatty acid substituents on the glycerol backbone, similar to those previously observed with a tandem quadrupole instrument [4]. As shown in Figure 1a, the LIT MS^2 spectrum of the $[M + Li]^+$ ion of (16:0/18:1/18:0)-TAG at m/z 867 contain ions at m/z 611, 583, and 585, reflecting neutral losses of palmitic acid (16:0), stearic acid (18:0), and oleic acid (18:1), respectively. Ions at m/z 605, 577, and 579 reflect neutral losses of these fatty acids as the lithium salts.

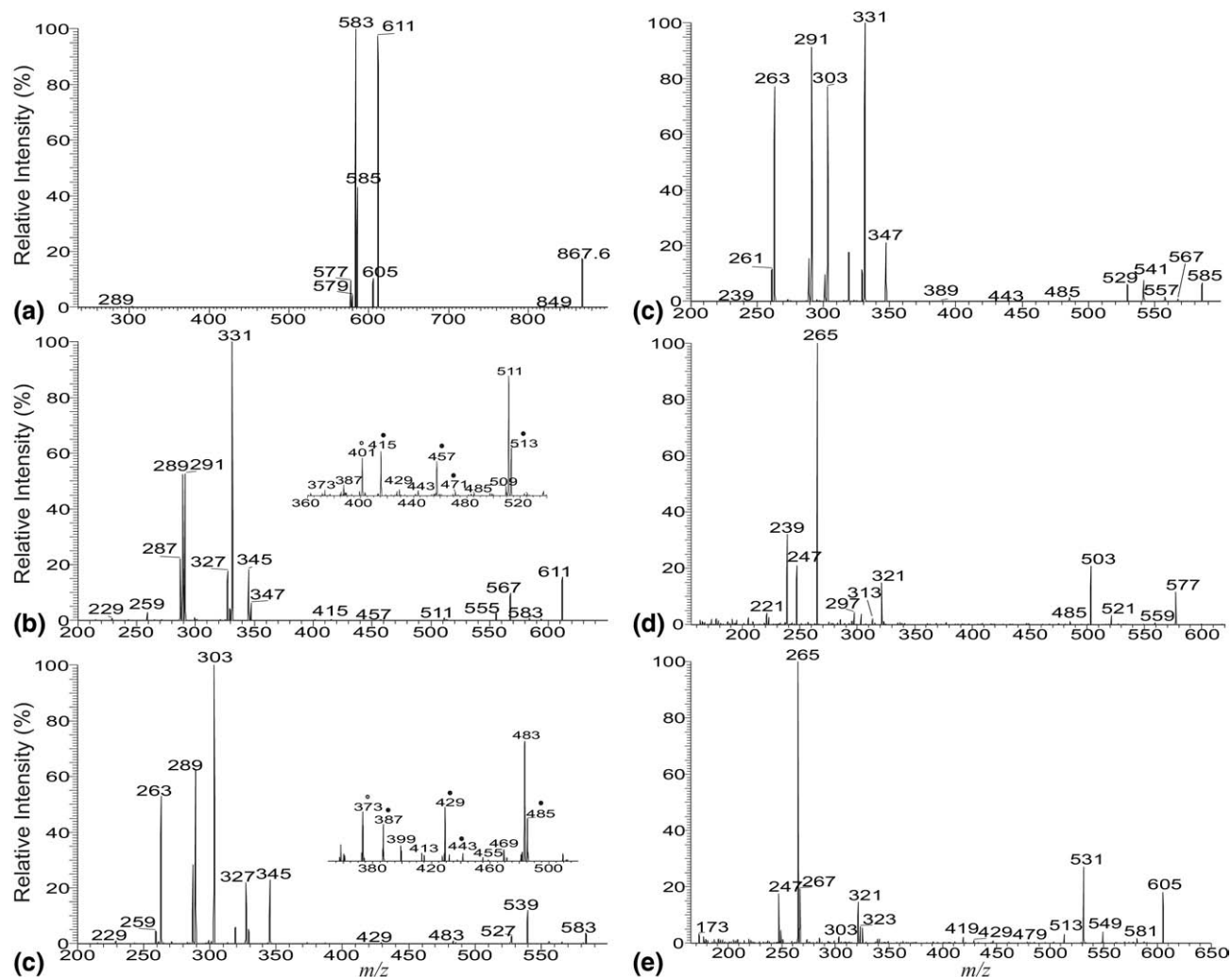
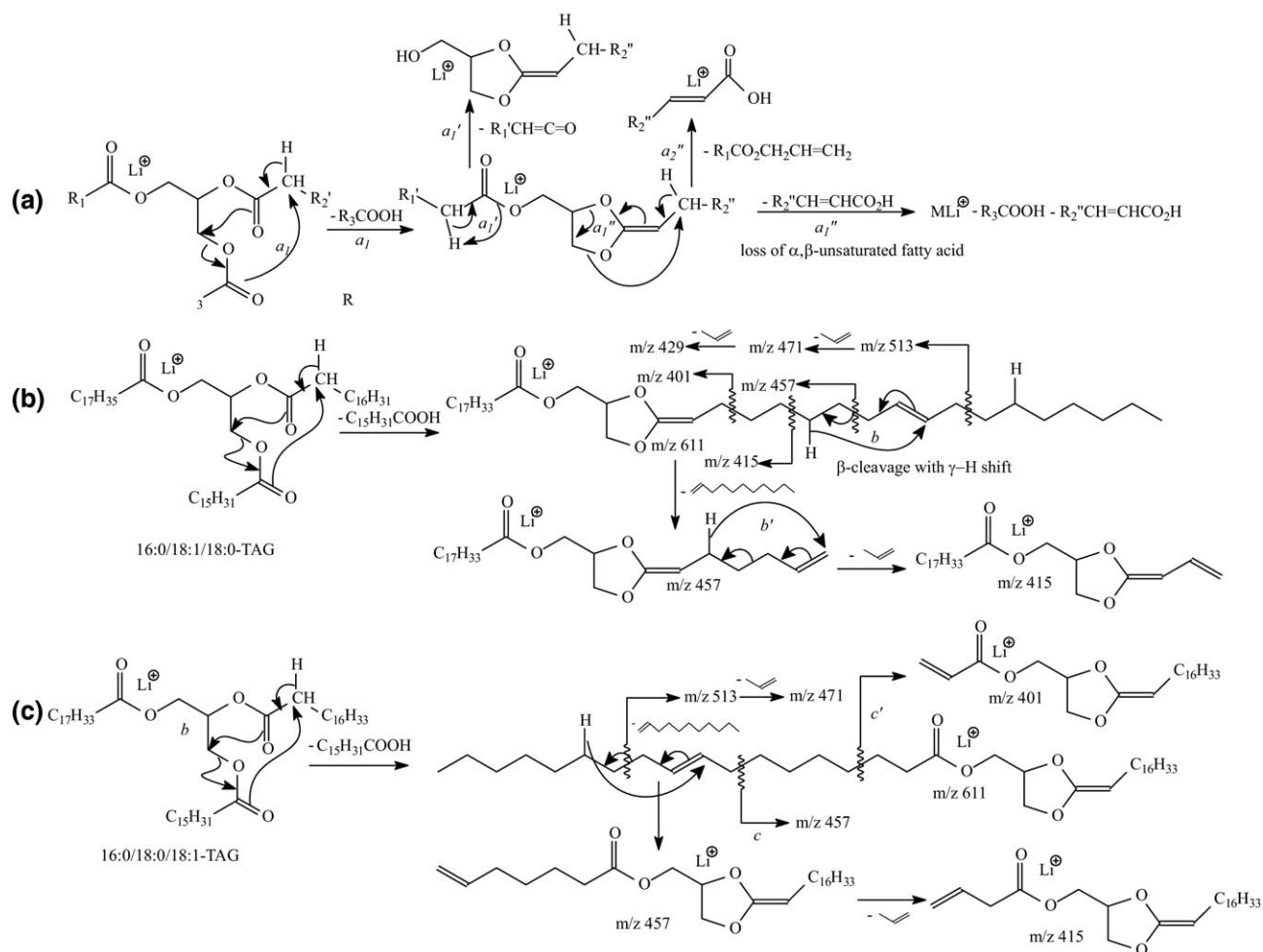


Figure 1. The LIT MS² spectrum of the $[M + Li]^+$ ion of the 16:0/18:1/18:0-TAG at m/z 867 (a), its MS³ spectra of the ions at m/z 611 ($867 \rightarrow 611$) (b), at m/z 583 ($867 \rightarrow 583$) (c), at m/z 585 ($867 \rightarrow 585$) (d), at m/z 577 ($867 \rightarrow 577$) (e), and at m/z 605 ($867 \rightarrow 605$) (f). In the subsets (panel b and c), the ions labeled with filled circle identified the position of double bond(s).

The ions at m/z 583 and 611 are of similar abundance, but are significantly more abundant than the ion at m/z 585; the ions at m/z 605 and 577 are also of similar abundance but are more abundant than the ion at m/z 579. The results indicate that neutral loss of the *sn*-2 fatty acid substituent either as a free fatty acid or as a lithium salt was less abundant than the corresponding losses of either the *sn*-1 or the *sn*-3 fatty acid substituent, resulting in assignment of the fatty acid substituents and their position on the glycerol backbone. These results are consistent with the notion that the α -hydrogen of the fatty acid substituent at *sn*-2 that participates in the formation of the ions at m/z 611 and 583 via elimination of the 16:0-fatty acid at *sn*-1 and of the 18:0-fatty acid at *sn*-3 (Scheme 1a) is more labile than that located at *sn*-1 or *sn*-3, which involves the loss of the adjacent 18:1-fatty acid at *sn*-2 (i.e., the m/z 585 ion), leading to a greater abundance of the ions at m/z 583 and 611 than the m/z 585 ion [9, 10].

Further dissociation of the ion at m/z 611 ($867 \rightarrow 611$, Figure 1b) gives rise to m/z 331, arising from loss of the 18:1-fatty acid at *sn*-2 as an α,β -unsaturated fatty acid (Scheme 1a, route a_1''). This is in agreement with the presence of the ion at m/z 287, corresponding to a lithiated 18:2-fatty acid (Scheme 1a, route a_2''). The results support the mechanism that the primary loss of the fatty acid substituents involves the participation of the α -hydrogen of the neighboring fatty acid substituent and the 5-member intermediate has been formed [4, 11]. The spectrum also contains the ions at m/z 345 and 327 arising from losses of the 18:0-fatty acid at *sn*-1 as a ketene and as an acid, respectively, along with the ions at m/z 289 and 291, corresponding to the lithiated ions of 18:1- and 18:0-fatty acids, respectively. This loss of the fatty acid substituent at *sn*-1 (or *sn*-3), followed by further elimination of the *sn*-2 fatty acid as an α,β -unsaturated fatty acid is further evidenced by the IT MS³ spectrum of the ion at m/z 583 ($867 \rightarrow 583$, Figure 1c), in



Scheme 1. The mechanisms proposed for fragmentation of TAG (a), the β cleavage with γ -hydrogen shift mechanisms leading to location of double bond(s) for 16:0/18:1/18:0-TAG (b), and for 16:0/18:0/18:1-TAG (c). *All the schemes as shown do not imply that the fragmentation is a one-step process.

which the ion at m/z 303, arising from loss of the 18:1-fatty acid at *sn*-2 as an α,β -unsaturated fatty acid (loss as 18:2) is prominent, while the ions at m/z 345 and 327 represent further loss of the 16:0-fatty acid at *sn*-1 as a ketene and as a fatty acid, respectively. The observation of these ions is also consistent with the presence of the ions at m/z 289, 287, and 263 that respectively represent a lithiated 18:1-, 18:2, and a 16:0-fatty acid ions.

The ions at m/z 513 and 457 (Figure 1b, inset) may arise from charge-remote fragmentation (CRF) that involves β -cleavage with rearrangement of γ -H (Scheme 1b, route *b*); whereas the ions at m/z 415 (457–propylene), 471 (513–propylene), and 429 (471–propylene) arise from consecutive β -cleavage with McLafferty rearrangement (Scheme 1b, route *b'*) [11]. These ions are analogous to the ions at m/z 197, 141, 99, 155, 113 seen in the MS^2 spectrum of the dilithiated ion of Δ^9 18:1 [12], suggesting that the double bond of the 18:1-fatty acid substituent at *sn*-2 is located at C(9). Similarly, the MS^3 spectrum of m/z 583 (Figure 1c, inset) also contains the ions at m/z

485, 429, 387, 443, 401, which are 28 Da lighter than the analogous ions at m/z 513, 457, 415, 471, and 429 seen in Figure 1b. The results also show that the double bond of the 18:1-fatty acid moiety is located at C(9). The combined results from LIT MS^n ($n = 2,3$) on the $[M + Li]^+$ ions of TAG readily afford near-complete structural characterization of the major regioisomer, including assignment of its fatty acid substituents on the glycerol backbone, and location the double bonds along the unsaturated fatty acid chain.

The MS^3 spectrum of the ion at m/z 585 ($867 \rightarrow 585$, Figure 1d) is dominated by the ion at m/z 331 and 303, arising from losses of the 16:0-fatty acid (loss as 16:1-FA) at *sn*-1 and 18:0-fatty acid (loss as 18:1-FA) at *sn*-3 as an α,β -unsaturated fatty acids, respectively. The ions at m/z 567 (585–18), 557 (585–28), 541 (585–44) may arise from losses of H_2O , CO , CO_2 , respectively; while the m/z 529 (585–56) ion may arise from cleavage of glycerol residue (loss of [glycerol – $2H_2O$]) involving rearrangements (Discussion later). The ion of m/z 529 gives rise to m/z 485 (529–44) by loss of CO_2 as previously reported

[11]. The analogous ions arising from the similar losses were also seen in Figure 1b and c.

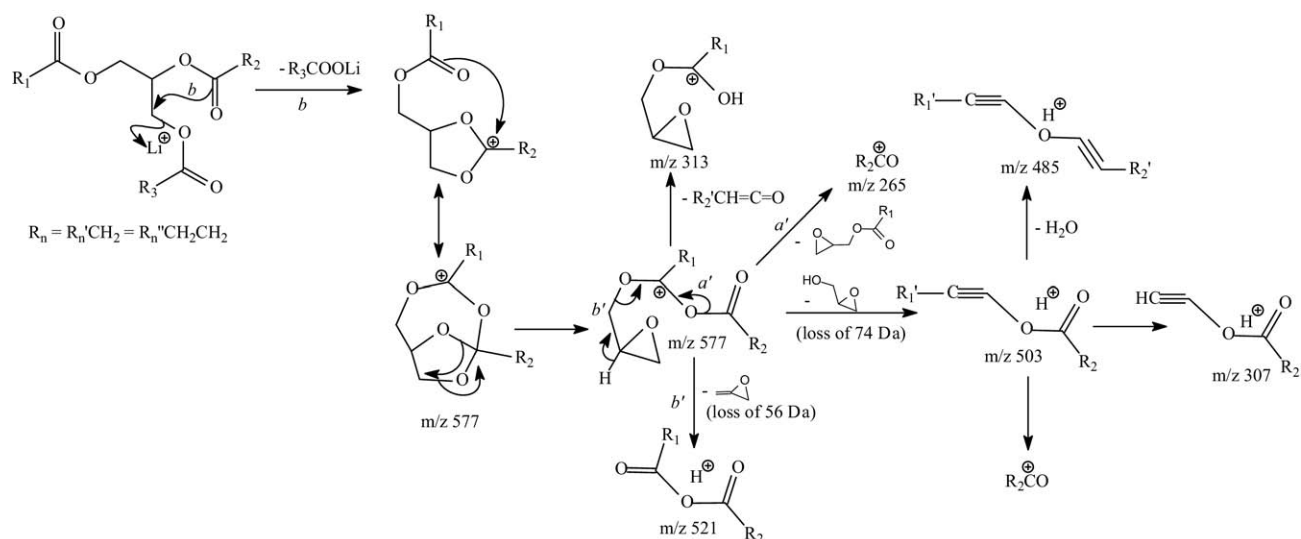
Further dissociation of the ions at m/z 577 ($867 \rightarrow 577$, Figure 1e) gives rise to ions at m/z 265 and 239, representing the 18:1- and 16:0-acylium ion, respectively, along with the ions at m/z 247 ($265 - \text{H}_2\text{O}$) and 221 ($239 - \text{H}_2\text{O}$). The ions at m/z 265 and 247 are more abundant than the ions at m/z 239 and 221, respectively, reflecting that the 18:1- and 16:0-fatty acid substituents are located at sn -2 and sn -1 of the glycerol backbone [13]. This is also consistent with findings that the ions at m/z 265 and 247 are respectively more prominent than the ions at m/z 267 (18:0-acylium ion) and 249 ($267 - \text{H}_2\text{O}$) in the MS^3 spectrum of the ion at m/z 605 ($867 \rightarrow 605$, Figure 1f), signifying that the 18:1-fatty acid substituent is located at sn -2, while the 18:0-fatty acid substituent is located at sn -3, representing an outer fatty acid substituent. This difference in the abundances of the acylium ions in the MS^3 spectrum of $[\text{M} + \text{Li} - \text{R}_n\text{CO}_2\text{Li}]^+$ ions is readily applicable for confirmation of the fatty acid substituents on the glycerol backbone.

The ions at m/z 521 ($577-56$) and 503 ($577-74$) (Figure 1e) also arise from various cleavages of the glycerol backbone involving rearrangements [14, 15]. The proposed structure of the ion at m/z 503 is further supported by its MS^4 spectrum ($867 \rightarrow 577 \rightarrow 503$; data not shown), which contains ions at m/z 485, 307 and 265 (Scheme 2). Similar losses of the glycerol residues were also seen for the ion at m/z 605 (Figure 1f), which arises from loss of the outer fatty acid; but the analogous losses were not seen in the MS^3 spectrum of the ions of m/z 579 (data not shown), a $[\text{M} + \text{Li} - \text{R}_2\text{CO}_2\text{Li}]^+$ arising from loss of inner fatty acid. These internal losses of glycerol residues are further confirmed by observation of the fragment ions that correspond to

losses of 60 and 79 Da in the MS^3 spectrum of the analogous $[\text{M} + \text{Li} - \text{R}_n\text{CO}_2\text{Li}]^+$ ions arising from d_5 -17:0/17:1/17:0-TAG in which the glycerol hydrogen atoms are substituted by deuterium atoms (see Figure 5b and the discussion therein). The ion at m/z 321 ($577-256$) from further loss of 16:0-fatty acid substituent at sn -1 is more abundant than the ion at m/z 313 ($577-282$), arising from further loss of 18:1-fatty acid substituent at sn -2. This differential loss of the fatty acid substituents dependent on the position of the fatty acid substituents on the glycerol backbone also applicable in the confirmation of the location of fatty acid substituents.

The IT MS^2 spectrum of the $[\text{M} + \text{Li}]^+$ ion of the 16:0/18:0/18:1-TAG regioisomer at m/z 867 (Figure 2a), again, is dominated by the ions at m/z 611, 585 and 583, arising from losses of the 16:0-, 18:1- and 18:0-fatty acid substituents at sn -1, sn -3, and sn -2, respectively. The former two ions are nearly of equal abundance and are more abundant than the ion at m/z 583, suggesting that the 18:0-FA is located at sn -2. The results are consistent with the finding that the ions at m/z 605 and 579, arising from losses of the 16:0-, 18:1-FA moieties at sn -1 and sn -3 as lithium salt are more abundant than the ion at m/z 577, arising from loss of the 18:0-FA acid substituent at sn -2 as a lithium salt.

The position of double bond of the 18:1-FA substituent at sn -3 is revealed by further dissociation of the ions at m/z 611 ($867 \rightarrow 611$, Figure 2b) and at m/z 583 ($867 \rightarrow 583$, Figure 2c). The former spectrum contains ions at m/z 513, 457, 471, and 415 (Figure 2b, inset), identical to those seen in Figure 1b, along with the ion at m/z 401 from β -cleavage (β to the carbonyl group) (Scheme 1c) indicating that the double bond is located at C(9). The latter spectrum contains ions at m/z 485, 429, 443, 387,



Scheme 2. The fragmentation processes proposed for losses of various glycerol residues for the $[\text{M} + \text{Alk} - \text{R}_n\text{CO}_2\text{Alk}]^+$ ions ($\text{Alk} = \text{Li}, \text{Na}, \text{NH}_4, n = 1, 3$). The m/z values shown represent the ions seen for the $[\text{M} + \text{Li}]^+$ ions of 16:0/18:1/18:0-TAG. The pathways were supported by the analogous spectrum of d_5 -17:0/17:1/17:0-TAG in which the hydrogen atoms (C-H) of the glycerol backbone were replaced by deuterium atoms.

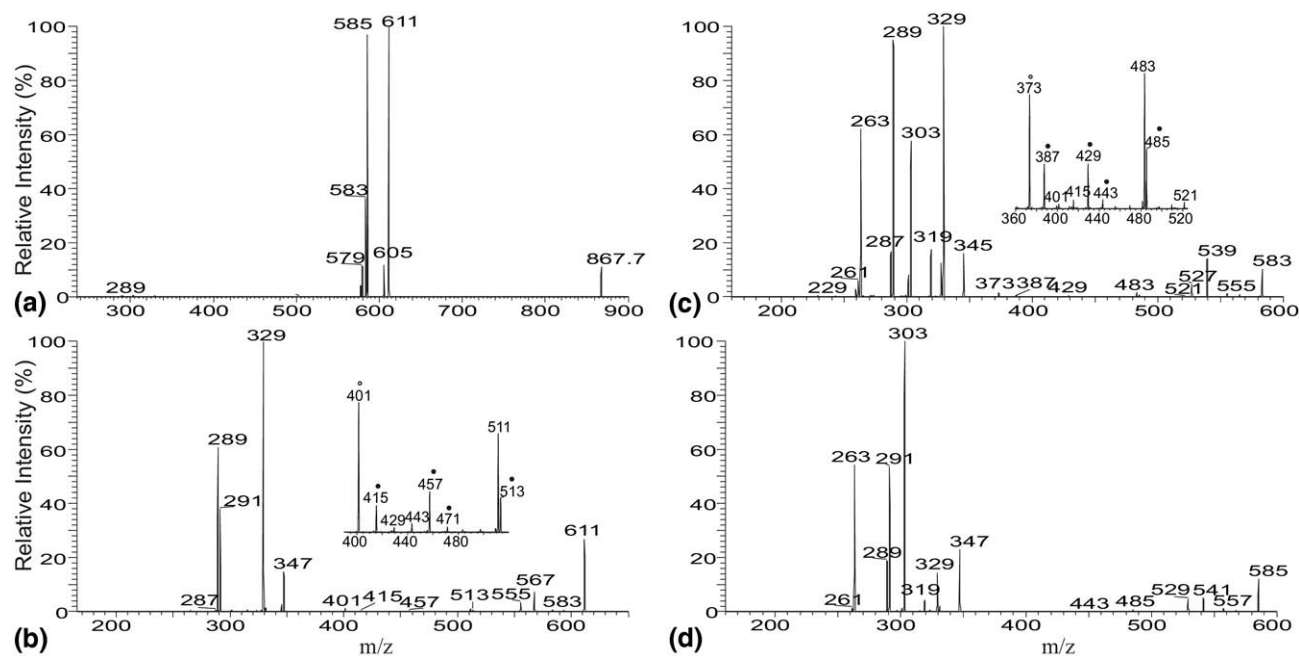


Figure 2. The LIT MS² spectrum of the [M + Li]⁺ ion of the 16:0/18:0/18:1-TAG at *m/z* 867 (a), its MS³ spectra of the ions at *m/z* 611 (867 → 611) (b), at *m/z* 583 (867 → 583) (c), and at *m/z* 585 (867 → 585) (d). In the subsets (panel b and c), the ions labeled with filled circle identified the position of double bond(s).

and 373 (Figure 2c, inset), which are identical to those seen in Figure 1c, reflecting that the double bond is located at C(9). The IT MS³ spectrum of the ion at *m/z* 585 (867 → 585, Figure 2d) is dominated by the ion at *m/z* 303, corresponding to loss of an 18:1-fatty acid residue, in agreement with the earlier finding that the 18:0-fatty acyl group at *sn*-2 was further eliminated as an α,β -unsaturated fatty acid.

Structural Characterization of A/B/A-TAG and A/A/B (or B/A/A)-TAG Regioisomers

Both the LIT MS² spectra of the lithiated ions of 16:0/18:1/16:0-TAG (Figure 3a) and 18:1/16:0/16:0-TAG (Figure 3d) at *m/z* 839 are dominated by the ion at *m/z* 583, arising from loss of 16:0-FA moiety. The ion at *m/z* 557 arising from loss of 18:1-FA is abundant in Figure 3d, but is of low abundance in Figure 3a. This is consistent with the fact that the 18:1/16:0/16:0-TAG contains a 18:1-FA at *sn*-1, while the 16:0/18:1/16:0-TAG (Figure 3a) contains a 18:1-FA at *sn*-2. The LIT MS³ spectrum of the ion at *m/z* 557 (839 → 557, Figure 3b) originated from 16:0/18:1/16:0-TAG is identical to that originated from 18:1/16:0/16:0-TAG (Figure 3e). This is consistent with the notion that the loss of the fatty acid substituents involves the participation of the α -hydrogen of the neighboring fatty acid substituent, resulting in an identical 5-member intermediate, which further eliminates the 16:0-FA as an α,β -unsaturated fatty acid (loss as 16:1-FA) to yield the prominent ion at *m/z* 303. The LIT MS³ spectrum of the ion at *m/z* 583 (839 → 583, Figure 3c) contains the prominent ion at *m/z* 303 arising

from elimination of the 18:1-FA at *sn*-2 as an α,β -unsaturated fatty acid (loss as 18:2-FA); while the LIT MS³ spectrum of the ion at *m/z* 557 (839 → 557, Figure 3e) originated from 18:1/16:0/16:0-TAG isomer is dominated by the ion at *m/z* 303, arising from loss of the 16:0-fatty acid at *sn*-2 as an α,β -unsaturated fatty acid (loss as 16:1). The results confirm that the former molecule contains 18:1-fatty acid at *sn*-2; whereas the latter TAG consists of a 16:0-fatty acid substituent at *sn*-2. In Figure 3c (subset), the ions at *m/z* 485, 429, 443, 387, and 373 that identify the position of double bond of the 18:1-fatty acid residue are also present, leading to assignment of the double bond at C(9). The set of ions of *m/z* 485, 429, 443, 387, and 373 were also seen in Figure 3f (subset), revealing that the double bond of the 18:1-FA substituent is also located at C(9).

The profiles of the LIT MS² spectrum of 18:1/18:0/18:0-TAG (see supplemental material, which can be found in the electronic version of this article, Figure S1a) and of the MS³ spectra of the ions at *m/z* 613 (Figure S1b) and at *m/z* 611 (Figure S1c) are identical to those seen in Figure 3d, e, and f, respectively, in agreement with the notion that both the 18:1/16:0/16:0-TAG and 18:1/18:0/18:0-TAG belong to the B/A/A (or A/A/B) class. The profiles of the MS^{*n*} (*n* = 2, 3) spectra from 18:0/18:1/18:0-TAG (data not shown) are also identical to those seen for 16:0/18:1/16:0-TAG (Figure 3a–c), indicating that they all belong to the A/B/A subclass. The distinction in the MS^{*n*} spectra (Figure 3a versus Figure 3d; Figure 3c versus 3f) between the TAG isomers of A/B/A and A/A/B classes are substantial, leading to differentiation of isomers by mass spectrometry.

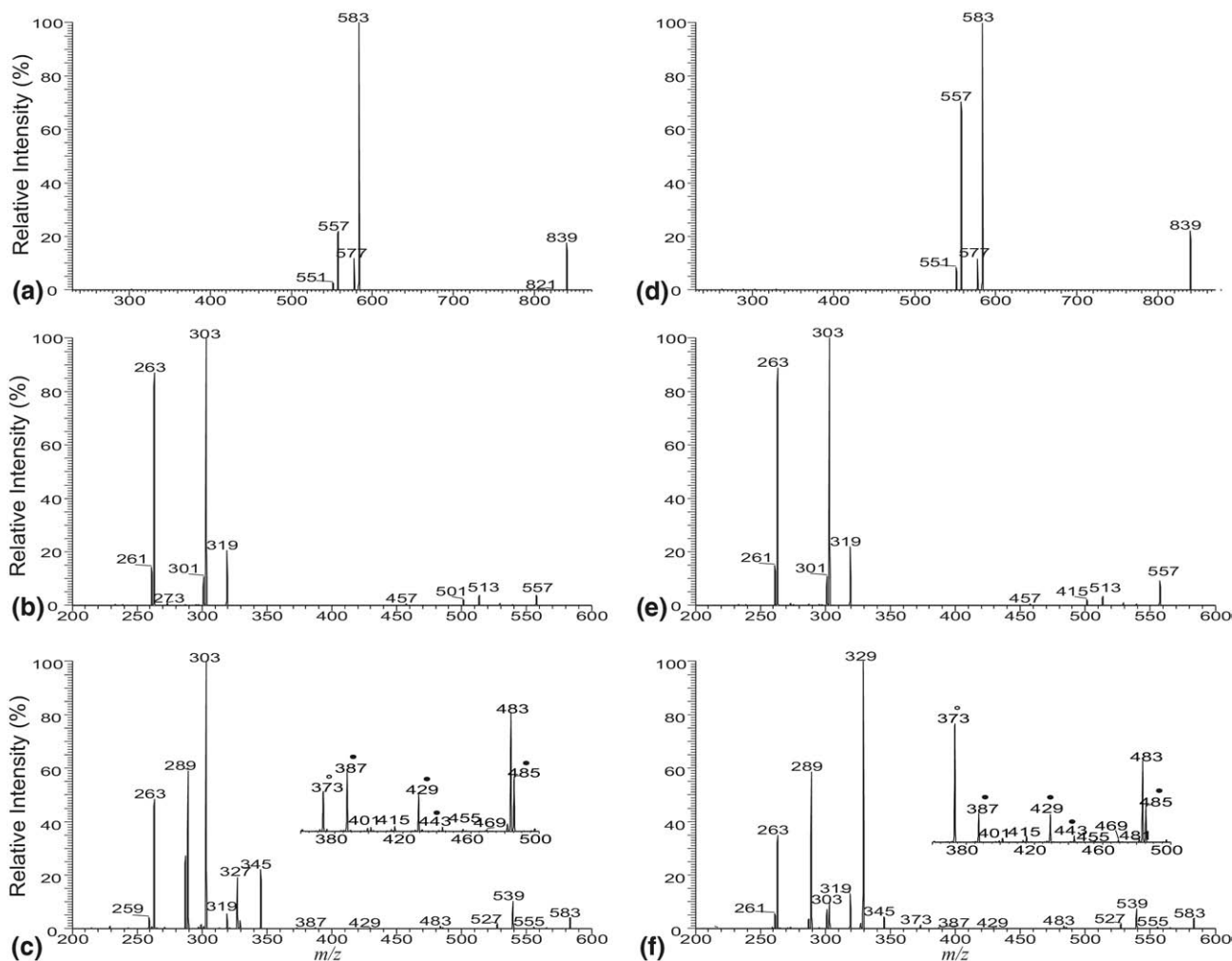


Figure 3. The LIT MS² spectrum of the [M + Li]⁺ ions of 16:0/18:1/16:0-TAG at *m/z* 839 (a), its MS³ spectra of the ions at *m/z* 557 (839 → 557) (b), and at *m/z* 583 (839 → 583) (c), and the LIT MS² spectrum of the [M + Li]⁺ ions of 18:1/16:0/16:0-TAG at *m/z* 839 (d), its MS³ spectra of the ions at *m/z* 557 (839 → 557) (e), and at *m/z* 583 (839 → 583) (f). In the subsets (panel c and f), the ions labeled with filled circle identified the position of double bond(s).

Characterization of TAG Consisting of Polyunsaturated Fatty Acid Moieties

The MS² spectrum of the [M + Li]⁺ ion of 20:4/20:4/20:4-TAG at *m/z* 957 (Figure 4a) is dominated by the ion at *m/z* 653, arising from loss of the 20:4-fatty acid substituent. By contrast, the ion at *m/z* 647 arising from loss of the 20:4-fatty acid as a lithium salt is of low abundance. Further dissociation of the ion at *m/z* 653 (957 → 653, Figure 4b) gives rise to the ion at *m/z* 351, by elimination of the 20:4-fatty acid moiety as an α,β -unsaturated fatty acid (loss as 20:5 fatty acid) as described earlier. The spectrum also contains two ion series at *m/z* 555, 515, and 475 and at *m/z* 503 and 463, indicating the presence of the homoconjugated double bonds at C(5), C(8), C(11), and C(14) (Scheme 3a). These ions are analogous to the ions at *m/z* 219, 179, 139, 167, and 127 that were previously observed for locating the double bonds for $\Delta^{5,8,11,14}$ -20:4 fatty acid using LIT MS² on the dilithiated adduct ions [12].

The ion at *m/z* 609 (Figure 4b) may arise from loss of a CO₂ residue. Further dissociation of the ion at *m/z* 609 (957 → 653 → 609, Figure 4c) also gives rise to the ion at *m/z* 553, arising from loss of a [glycerol - 2H₂O] residue as described earlier. The spectrum also contains two sets of ions at *m/z* 511, 471, 431, and at *m/z* 459 and 419. These ions are analogous to those seen in Figure 4b, and are 44 Da lighter, consistent with the fact that the precursor ion at *m/z* 609 arises from loss of a 44 Da residue from *m/z* 653. The results further support the assignment of the position of double bonds of the 20:4-fatty acid substituents. The MS⁴ spectrum of the ion at *m/z* 553 (957 → 653 → 553, data not shown) contains two ion series at *m/z* 455, 415, 375, and at *m/z* 403, 363; and the MS⁴ spectrum of the ion at *m/z* 351 (957 → 653 → 351, Figure 4d) also contains two ion series at *m/z* 253, 213, and 173, and at *m/z* 201, 161 (Scheme 3b). The observation of these ions, again, is consistent with the assignment of the position of double bonds along the 20:4-fatty acyl chain (Scheme 3).

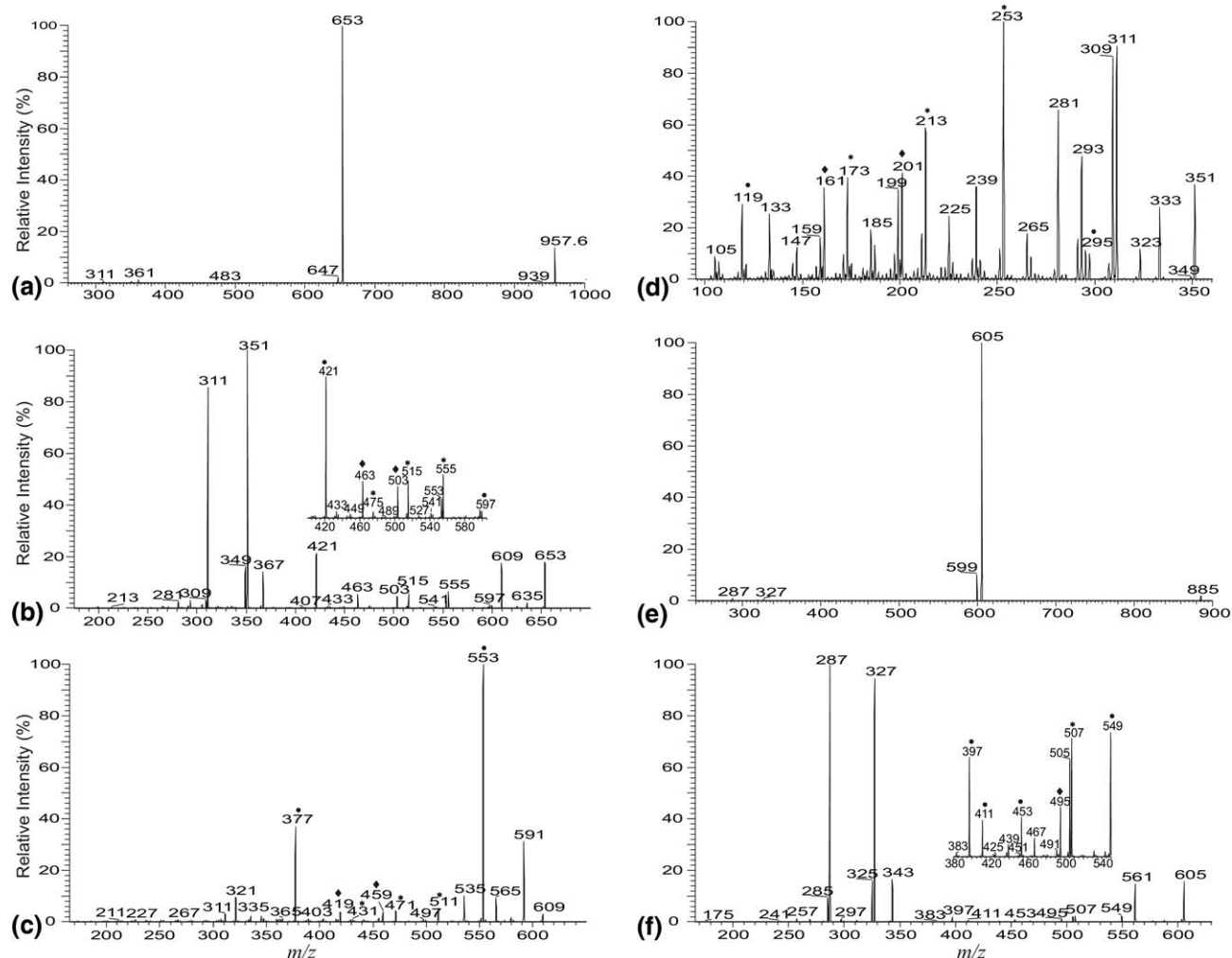


Figure 4. The LIT MS² spectrum of the [M + Li]⁺ ions of 20:4/20:4-TAG at m/z 957 (a), its MS³ spectrum of the ions at m/z 653 (957 → 653) (b), and its MS⁴ spectra of the ions at m/z 609 (957 → 653 → 609) (c), and at m/z 351 (957 → 653 → 351). The LIT MS² spectrum of the [M + Li]⁺ ions of 18:2/18:2/18:2-TAG at m/z 885 (e), and its MS³ spectrum of the ions at m/z 605 (885 → 605) (f) are also shown. In panels c and d, and in the subsets (panel b and f), the ions labeled with a filled diamond (vinyl cleavage), asterisk (allylic cleavage), and filled circle (β -cleavage) are feature ions that identify the position of the double bonds of the 20:4-fatty acid moiety (see Scheme 3).

The LIT MS² spectrum of the [M + Li]⁺ ion of 18:2/18:2/18:2-TAG at m/z 885 (Figure 4d) is dominated by the ion at m/z 605, arising from loss of the 18:2-fatty acid, along with the ion at m/z 599, arising from loss of 18:2-fatty acid as a lithium salt. The LIT MS³ spectrum of the ion at m/z 605 (885 → 605, Figure 4f) contains the prominent ion at m/z 327 (605–278), from further elimination of the 18:2-fatty acid as an α,β -unsaturated fatty acid (loss as 18:3 fatty acid). The position of the double bond of 18:2-fatty acid is seen by the ions at m/z 549 and 453 (Figure 4f, subset), arising from CRF cleavages of C(7)–C(8) and C(14)–C(15) bonds with McLafferty rearrangements, along with ions at m/z 507 and 495, arising from α -cleavage with allylic hydrogen shift (see supplemental material Scheme 1). These ions are analogous to those previously observed in the MS² spectrum of the dilithiated ion of $\Delta^{9,12}$ -18:2 [12], leading to locate the

double bonds of the 18:2-fatty acyl groups at C(9) and C(12).

The Fragmentation Pathways Supported by LIT MSⁿ on 1,3(d_5)-Diheptadecanoyl-2-(10Z-Heptadecenoyl)-Glycerol [d_5 -(17:0/ Δ^{10} 17:1/17:0)-TAG]

The profile of the LIT MS² spectrum of the [M + Li]⁺ ion of [d_5 -(17:0/ Δ^{10} 17:1/17:0)-TAG] at m/z 858 (Figure 5a) is similar to that shown in Figure 3a, arising from an A/B/A TAG subclass. The ions at m/z 588 (858 – C₁₆H₃₃CO₂H) and 590 (858 – C₁₆H₃₁CO₂H) arise from losses of the 17:0- and 17:1-fatty acid substituents, and the ions at m/z 582 and 584 arise from losses of the 17:0- and 17:1-fatty acid substituents as lithium salt, respectively. The observation of the losses of the C₁₆H₃₃CO₂H

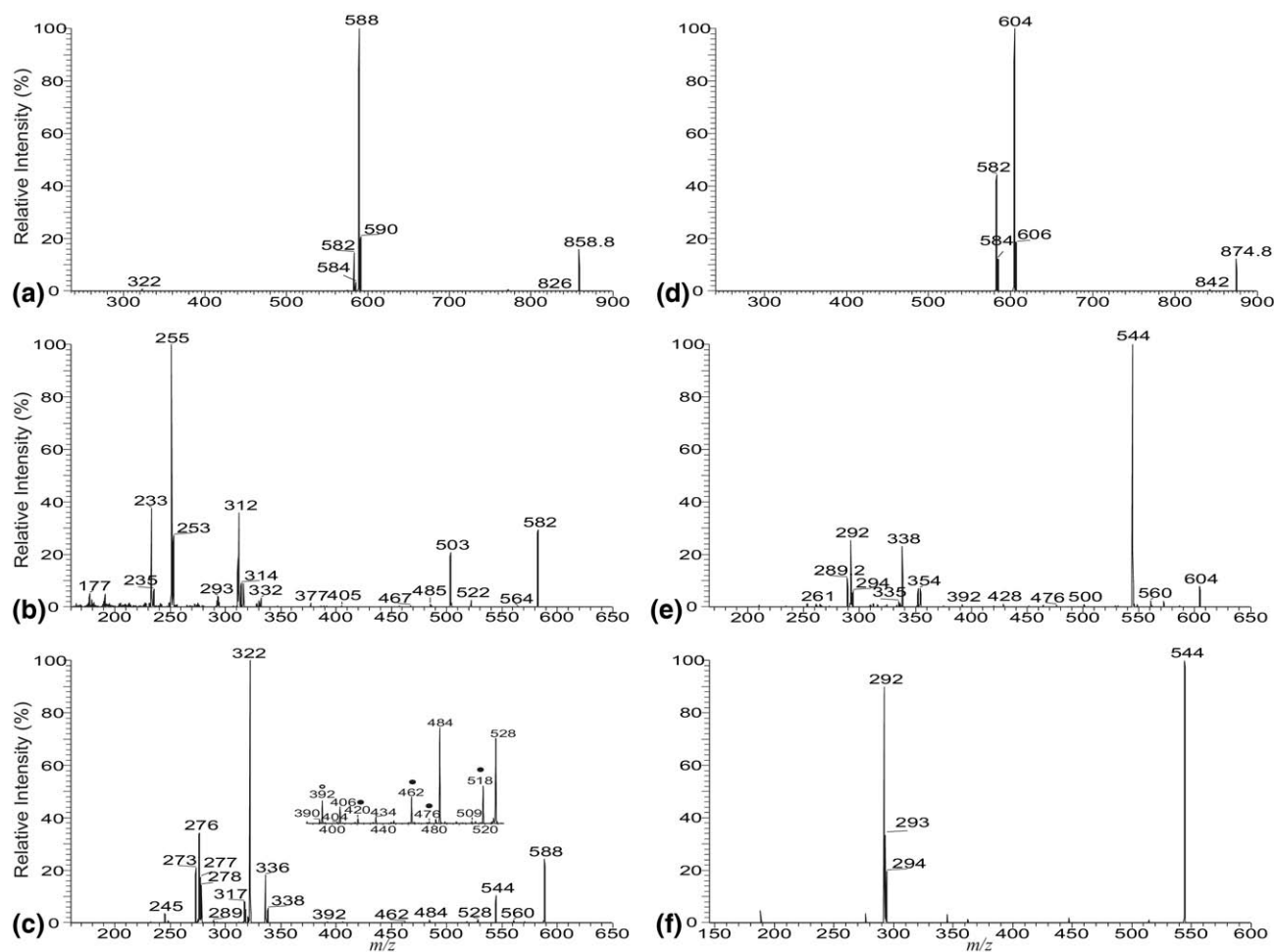


Figure 5. The LIT MS² spectrum of the [M + Li]⁺ ions of d₅-17:0/17:1/17:0-TAG at *m/z* 858 (a), and its MS³ spectra of the ions at *m/z* 582 (858 → 582) (b), and at *m/z* 588 (858 → 588) (c). The LIT MS² spectrum of the [M + Na]⁺ ions of d₅-17:0/17:1/17:0-TAG at *m/z* 874 (d), its MS³ spectrum of the ions at *m/z* 604 (874 → 604) (e) and its MS⁴ spectrum of the ion at *m/z* 544 (874 → 604 → 544) (f). In the subset (panel c), the ions labeled with filled circle identified the position of double bond(s).

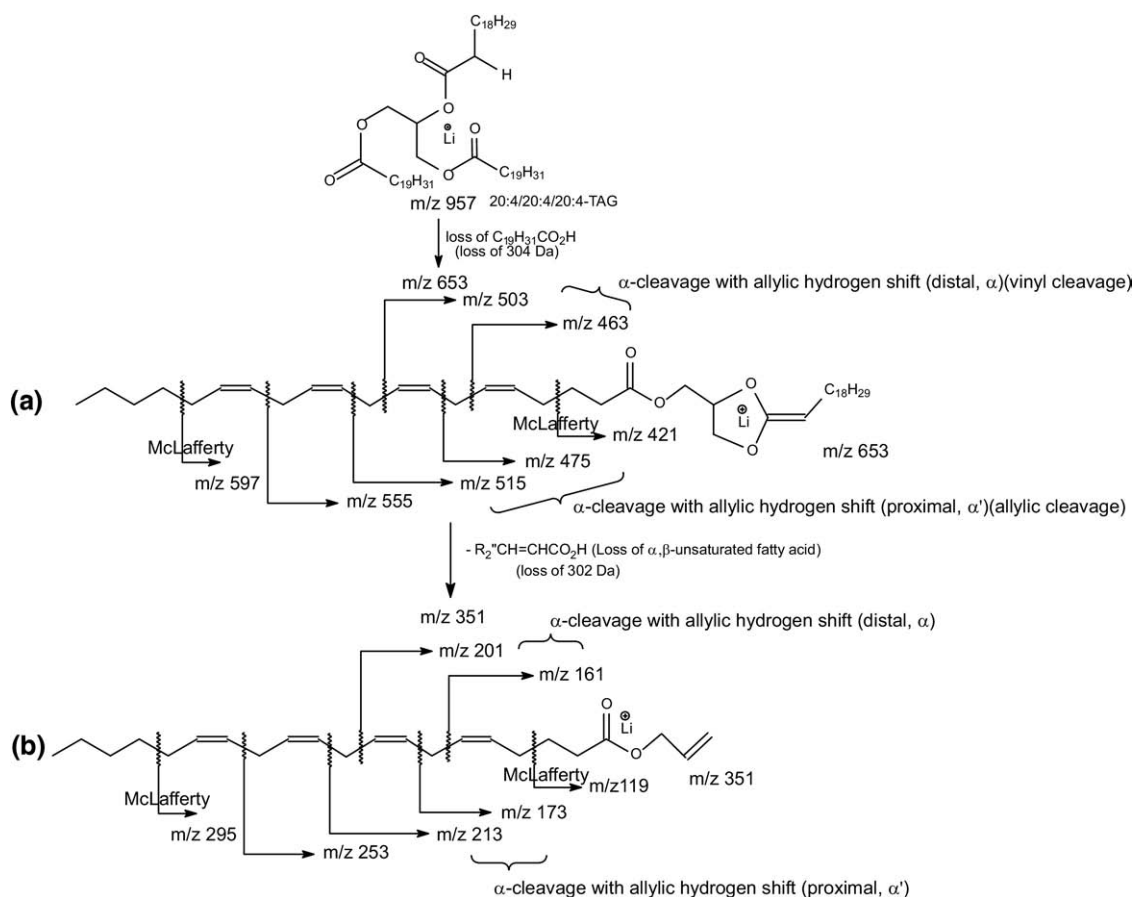
and C₁₆H₃₁CO₂H residues rather than losses of the C₁₆H₃₃CO₂D and C₁₆H₃₁CO₂D residues indicates that the elimination of the acid residues does not involve the participation of the hydrogen atom on the glycerol backbone. This is consistent with the proposed mechanisms in which the pathways leading to the fatty acid losses involve the participation of the α -hydrogen of the neighboring fatty acyl moiety (Scheme 1) [4]. The MS³ spectrum of the ion of *m/z* 582 (858 → 582, Figure 5b) contains the ions at *m/z* 522 (loss of C₃D₄O), 503 (loss of C₃HD₅O₂), and 485 (loss of C₃H₃D₅O₃), of which the mass shifts match the proposed structures (Scheme 4) and support the earlier findings of the various internal losses of the glycerol residues.

Further dissociation of the ion of *m/z* 588 (858 → 588, Figure 5c) yielded ions at *m/z* 518 and 462 (Figure 5c, inset), arising from β -cleavage with γ -H shift fragmentations. These ions together with ions at *m/z* 420, 406 and 392 demonstrate that the double bond of the 17:1-fatty acid substituent at *sn*-2 is located at C(10), consistent with the notion that the molecule is a d₅-(17:

0/ Δ ¹⁰17:1/17:0)-TAG. The spectrum is dominated by the ion at *m/z* 322, arising from loss of the 17:1-fatty acyl group as an α,β -unsaturated fatty acid (loss as 17:2-fatty acid), along with ion at *m/z* 273, representing a lithiated 17:2-fatty acid cation. The observation of these ions along with the ions at *m/z* 336 arising from loss of 17:0-fatty acid as a ketene and at *m/z* 317 arising from loss of d₁-17:0-fatty acid (loss as C₁₆H₃₃CO₂D) supports the proposed mechanism depicted in Scheme 1a.

LIT MSⁿ Spectra of the [M + Na]⁺ and [M + NH₄]⁺ Ions of TAG

The LIT MS² spectrum of the [M + Na]⁺ ions of TAG is similar to that arising from the [M + Li]⁺ ions and is readily applicable for assignment of the fatty acyl substituents on the glycerol backbone. The product-ion spectrum of the sodiated (16:0/18:1/18:0)-TAG at *m/z* 883 (Figure 6a) contains the ions at *m/z* 627 and 599 arising from losses of the outer 16:0- and 18:0-fatty acids



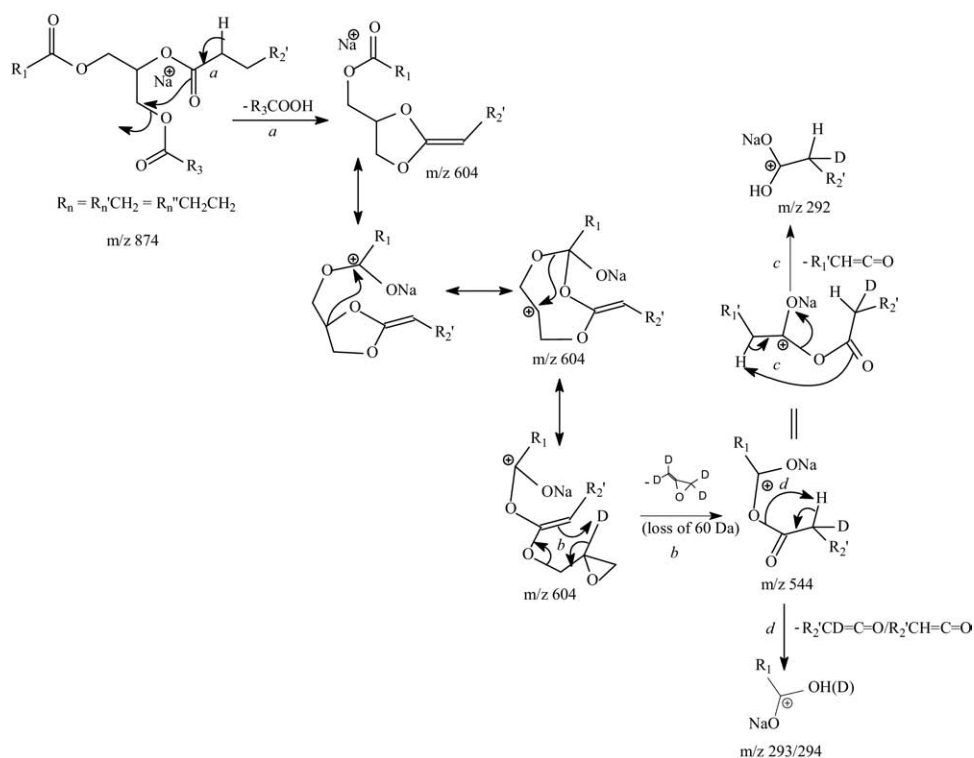
Scheme 3. The fragmentation pathways proposed for location of the double bonds along the fatty acid chain of 20:4/20:4/20:4-TAG from MS³ on the [M + Li - R₃CO₂H] ions (A), and from MS⁴ on the [M + Li - R₃CO₂H - R₂*CH=CHCO₂H] ions.

and the ion at m/z 601 arising from loss of the 18:1-fatty acid at *sn*-2. The ions at m/z 627 and 599 are more abundant than the ion at m/z 601. The ions at m/z 605 and 577 arising from losses of the outer 16:0- and 18:0-fatty acids as sodium salt (loss of R_{*n*}CO₂Na) are also more abundant than the ion at m/z 579, arising from the analogous loss of the 18:1 at *sn*-2. The results indicate that the 16:0- and 18:0-fatty acids are located at *sn*-1 and *sn*-3, respectively.

The ions at m/z 605, 577, and 579 are more prominent than those seen in Figure 1a, indicating that the losses of fatty acids as sodium salts are more facile than the same losses as lithium salts. The ions corresponding to losses of the fatty acids as potassium salts become dominant in the MS² spectrum of the corresponding [M + K]⁺ ions of TAG (data not shown). The ions at m/z 605, 577, and 579 arising from losses of the fatty acid as ammonium salt become the exclusive ions in the MS² spectrum of the [M + NH₄]⁺ ions of (16:0/18:1/18:0)-TAG at m/z 878 (Figure 6d). The results indicate that the strength of metal and NH₄⁺ ions attached to TAG is in the order of Li⁺ > Na⁺ > K⁺ > NH₄⁺ [16]. The Li⁺ is localized on the [M + Li - R_{*n*}CO₂H]⁺ ions remote from the fragmentation sites, resulting in the formation of the ion series informative for location of the double bonds; while the

Na⁺, K⁺, and NH₄⁺ on the sodiated, potassiated, and ammoniated precursor ions were lost before the CRF processes take place. The loss of metal ions is evidenced by observation of metal ions in the tandem quadrupole product-ion spectra of the [M + Na]⁺ ion at m/z 881 (see supplemental material Figure S2, panel a) and of the [M + K]⁺ ions at m/z 897 (panel b) of 16:0/18:0/18:1-TAG, respectively. The former spectrum contained the ion at m/z 23, corresponding to the Na⁺ ion, while the latter spectrum is dominated by the K⁺ ion at m/z 39. The metal ion loss becomes even more prevalent, when MS² on the [M + Cs]⁺ ions at m/z 991 with the same tandem quadrupole instrument was performed. The spectrum (see supplemental material Figure 2c), which was obtained with a much lower collision energy again, is dominated by the Cs⁺ ion at m/z 133. However, these metal ions are not detectable using a LIT because of the low-mass cut-off nature of the instrument.

The MS³ spectrum of the ion at m/z 627 (883 → 627, Figure 6b) is dominated by the ion at m/z 571 (627–56), probably representing a sodiated ion of octadecanoyl octadecenoyl anhydride (18:0/18:1 acid anhydride) after removal of the glycerol residue from the ion of m/z 627 (Scheme 4). This speculation is based on observation of the ions at m/z 307 and 305 representing the



Scheme 4. The proposed fragmentation processes leading to internal loss of [glycerol – 2H₂O] (56 Da) produced by MSⁿ on the [M + Na]⁺ ions of TAG. The *m/z* values shown in the scheme represent the observed mass seen for d₅-17:0/17:1/17:0-TAG. The observation of loss of 60 Da (Figure 5e) supports the proposed internal loss of the glycerol residue. The internal loss was further supported by MS⁴ on the ion of *m/z* 544, which gives ions at *m/z* 292, 293, and 294 (see Figure 5f), deriving from the fragmentation processes as proposed.

sodiated ions of 18:0- and 18:1-fatty acids, respectively, in the MS⁴ spectrum of the ions at *m/z* 571 (883 → 627 → 571, Figure 6c) (Scheme 4). The ion series reflecting the location of double bond of the 18:1-fatty acid substituent at *sn*-2 as seen in Figure 1b are absent in Figure 6b, attributable to the notion that the sodiated precursor ion is not stable and the internal energy of the precursor ion decomposed by CRF to yield the ions applicable for characterization is less than the energy required for releasing the Na⁺ ion [16]. The MS³ spectra of the ions at *m/z* 601, and at 599 (data not shown) are also dominated by the ions at *m/z* 545 and 543, respectively, arising from further loss of 56 Da (loss of [glycerol – 2H₂O]) and representing a 16:0/18:0 and a 16:0/18:1 fatty acid anhydride residues. This loss of 56 Da related to the glycerol backbone is supported by the MS³ spectrum of the analogous ion of *m/z* 604 (874 → 604) (Figure 5e) arising from the [M + Na]⁺ ions of d₅-(17:0/Δ¹⁰17:1/17:0)-TAG (Figure 5d). The spectrum (Figure 5e) is dominated by the ion at *m/z* 544, representing a sodiated ion of d₁-17:0/17:1 acid anhydride arising from the analogous loss of a d₄-glycerol residue (Scheme 4), along with the ions at *m/z* 338 from loss of 17:1-fatty acid at *sn*-2 as a α,β -unsaturated acid (loss as 17:2-FA) and the ion at *m/z* 289, representing a sodiated 17:2-fatty acid ion. The observation of the ions at *m/z* 292, 293, and 294 arising from further losses of 17:0-, d₁-17:1-, and

17:1-fatty acyl ketene from *m/z* 544, respectively, in the MS⁴ spectrum of the ion of *m/z* 544 (874 → 604 → 544) (Figure 5f) is consistent with the formation of the sodiated d₁-17:0/17:1 acid anhydride ion by loss of the glycerol residue (loss of 60 Da) (Scheme 4). Similar losses of the glycerol residues were also observed in the MS³ spectrum of the ion of *m/z* 606 (874 → 606), which is dominated by the ion at *m/z* 506 (data not shown).

By contrast, the LIT MS² spectrum of the [M + NH₄]⁺ ion of (16:0/18:1/18:0)-TAG at *m/z* 878 (Figure 6d) contained ions at *m/z* 605, 577, and 579, arising from neutral losses of 16:0-, 18:0-, and 18:1-fatty acid substituents as ammonium salt (loss as R_nCO₂NH₄), respectively (or the combined losses of the corresponding fatty acids and NH₃ [17]), and the ions arising from losses of the fatty acid substituents were absent. The ions at *m/z* 605 and 577 are significantly more abundant than the ion at *m/z* 579. These preferential losses of the outer 16:0- and 18:0-fatty acid moieties as ammonium salt than the analogous loss of inner *sn*-2 18:1-fatty acid moiety are readily applicable for assignment of fatty acid substituents on the glycerol backbone.

The LIT MS³ spectrum of the ion at *m/z* 605 (878 → 605, Figure 6e) is nearly identical to that shown in Figure 1e, consistent with the notion that the 18:1- and 18:0-fatty acyl groups are located at *sn*-2 and *sn*-3, respectively. However, the ion at *m/z* 341 from further

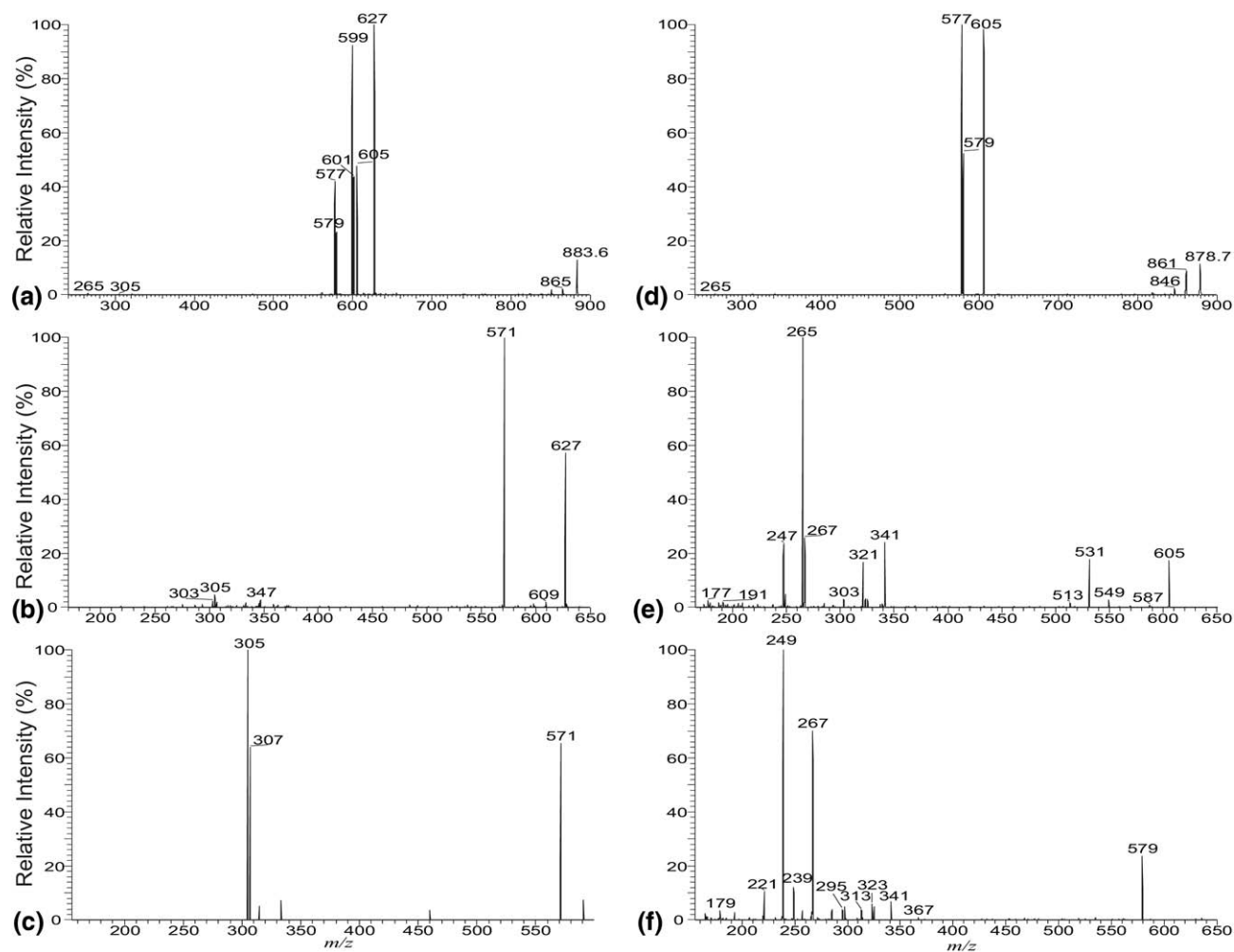


Figure 6. The LIT MS² spectrum of the $[M + Na]^+$ ions of 16:0/18:1/18:0-TAG at m/z 863.6 (a), its MS³ spectrum of the ions at m/z 627 ($863 \rightarrow 627$) (b), and its MS⁴ spectrum at m/z 571 ($863 \rightarrow 627 \rightarrow 571$) (c). The LIT MS² spectrum of the $[M + NH_4]^+$ ions of 16:0/18:1/18:0-TAG at m/z 878.7 (d), and its MS³ spectra of the ions at m/z 605 ($878 \rightarrow 605$) (e), and at m/z 579 ($878 \rightarrow 579$) (f).

loss of 18:1-fatty acyl ketene (designated as $[RCO^+ + 74]$ ion in references [1, 3, 15]) is more abundant than the similar ions seen in Figure 1, indicating that they may arise from different mechanisms [17]. The LIT MS³ spectrum of the ions at m/z 577 (data not shown) is similar to Figure 6e, consistent with the notion that both the ions of m/z 605 and 577 arise from loss of the outer fatty acid substituent. In contrast, the LIT MS³ spectrum of the ions at m/z 579 (Figure 6f), arising from loss of the fatty acid moiety at *sn*-2 is readily distinguishable from Figure 6e. The abundances of the ions at m/z 341 (loss of *sn*-1 fatty acyl ketene) and 323 (loss of *sn*-1 fatty acid) are close to those of the ions at m/z 313 (loss of *sn*-3 fatty acyl ketene) and 295 (loss of fatty acid at *sn*-3), respectively, in agree with the fact that they all retain the outer fatty acid moieties. The ions at m/z 523, 505, and 487 arising from various losses of the glycerol residues are absent in the spectrum (Figure 6f) supporting the notion that the ion at m/z 579 arises from loss of the fatty acid group at *sn*-2 as described earlier. These spectra features

dependent on the origin of precursor ions deriving from elimination of the fatty acid substituents at different positions (on the glycerol backbone) are readily applicable for confirmation of the assignment of the fatty acyl groups on the glycerol backbone.

The $[M + NH_4 - R_nCO_2H]^+$ ions analogous to the $[M + Li - R_nCO_2H]^+$ ions are absent in the MS² spectra (Figure 6d), thus, ions from MS³ on $[M + NH_4 - R_nCO_2H]^+$ for location of double bonds for the unsaturated fatty acid moieties as seen for the $[M + Li]^+$ ions (i.e., ions from MS³ on the $[M + Li - R_nCO_2H]^+$) are not available.

Conclusions

The employment of LIT MSⁿ on the $[M + Li]^+$ ions of TAG desorbed by ESI affords near-complete structural characterization of TAG molecules, including the position of double bond(s) of the unsaturated fatty acyl moieties. The feature ions informative for location of the

position of double bonds derived from further dissociation of the $[M + Li - R_nCO_2H]^+$ ions in an ion trap. However, these ions are not present in the MS^3 spectra of $[M + Na - R_nCO_2H]^+$ or $[M + NH_4 - R_nCO_2H]^+$ ions and characterization of TAG by LIT MS^n on the $[M + Na]^+$ and $[M + NH_4]^+$ ions is incomplete. Momchilova et al. demonstrated the separation of isobaric triacylglycerol positional isomers by reversed-phase high-performance liquid chromatography (RP-HPLC) [18]. Thus, this LIT MS^n with ESI approach coupled with RP-HPLC should have broad application in the characterization of TAGs, in particular, of biological origin which often consists of numerous isobaric species [17, 19] that are not separable by a mass spectrometer.

Acknowledgments

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Appendix A Supplementary Material

Supplementary material associated with this article may be found in the online version at [doi:10.1016/j.jasms.2010.10.007](https://doi.org/10.1016/j.jasms.2010.10.007)

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