Structural Characterization of Triacylglycerols Using Electrospray Ionization-MS^n Ion-Trap MS

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ABSTRACT: Structural analysis of mixtures of TAG requires the determination of the M.W. of each compound, the characterization of the structure(s) of the individual FA substituents, and identification of the relative disposition of the acyl groups on the glycerol backbone (regiospecificity). In this study we demonstrated that ion-trap MS in combination with electrospray ionization provides an easy and detailed characterization of the TAG structure. We showed that electrospray mass spectra are characterized by intense molecular ion adducts and that the acyl group disposition on the TAG backbone can be determined on the basis of relatively well-defined quantitative differences of fragment ions produced from MS^2 data. Moreover, additional spectral data can be generated by MS^3 experiments on moieties containing individual acyl ion fragments. When hyphenated with chromatographic separations, ion-trap electrospray MS might become a routine and powerful way to analyze TAG mixtures.

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Natural fats are a complex mixture of triacylglycerols (TAG). Structural analysis of TAG has typically required their isolation from the matrix, saponification, and isolation of the fatty acyl groups followed by derivatization of the released FA to methyl esters in order to increase their volatility. Separation and detection are then accomplished by GC (1). However, if FA positional information is required, a number of laborious enzymatic procedures are involved (1).

Several GC and HPLC techniques have been described for the determination of the TAG composition of food samples. These methods enable determination of the carbon number of each constituent of the mixture, but no real structural identification is possible. Moreover, limitations of the GC methods include baseline instability, rapid column aging because of the need for elevated oven temperatures, and possible thermal degradation of the samples. HPLC techniques may suffer from mobile phase and detector incompatibilities.

MS provides a direct and rapid method for the structural characterization of TAG (2,3). As a result, various ionization methods have been reported for TAG analysis including electron ionization (EI) (4), chemical ionization (CI) (5), desorption chemical ionization (6), field desorption (7), and plasma desorption (8). The first MS studies of TAG utilized EI and provided information such as M.W., carbon number, and degree of saturation of a TAG (4,9). Although this method cannot distinguish between FA substituents at the primary (*sn*-1 and *sn*-3) and secondary (*sn*-2) positions, DAG ions $[M - RCO_2CH_2]^+$ were only formed by cleavage from the primary positions (4).

Softer ionization methods such as CI have the advantage of producing less fragmentation than EI. Methane (10), ammonia (11), and isobutane (12) have been successfully utilized as CI reagent gases for TAG analysis. CI showed minimal fragmentation and produced primarily DAG $[M - RCOO]^+$ ions and protonated molecular $[M + H]^+$ ions when a mixture of TAG separated by RP-HPLC was analyzed (13). However, only DAG ions were detected when TAG containing saturated FA. In addition, Myher *et al.* (14) were able to distinguish *sn*-1/3 fatty acyl groups from *sn*-2 fatty acyl groups based on the yields of the DAG fragment ions. They found that under CI conditions, cleavage of fatty acyl groups was four times greater if the groups were located in the primary positions. Conflicting findings have been presented that cite fatty acyl chain length affecting $[M - RCO_2]^+$ ion yields rather than *sn* position (15).

Preferential cleavage of the fatty acyl group from the primary (*sn*-1/3) positions has also shown increased formation of the $[M - H - RCO_2H - 100]^-$ ion using negative ion chemical ionization (NICI) (16). The abundance of this ion was used to distinguish between primary and secondary positions. More recently, Stroobant *et al.* (17) discussed the fragmentation pathways of TAG using NICI. The main pathway, led by the formation of a ketone enolate, is sensitive to the positions of fatty acyl residues (*sn*-1/3 vs. *sn*-2) and explains the presence of the $[M - H - RCO_2H - 100]^-$ ion.

A unique approach for the determination of isomeric TAG has been proposed by Kallio and Rua (18). The method is based on the use of a mathematical model that incorporates data from tandem MS (MS/MS) analyses of the TAG and the FA. The data are then used to interpret the distribution of FA between the *sn*-2 and *sn*-1/3 positions in order to finally reconstruct the TAG composition of the mixture. The model was based on the study of 24 major M.W. groups of TAG. Although interesting and powerful, the method is indirect.

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Perhaps the most detailed study of the effectiveness of MS for complete TAG characterization was published by Cheng *et al.* (19). Molecular ion adducts (e.g. $[M + H]^+$, $[M + Na]^+$, $[M + NH_4]^+$) were produced by either electrospray or fast atom bombardment and then subjected to collisionally activated dissociation (CAD). Fragment ions reflecting the FA structure (including the position of double bonds and the degree of unsaturation) and the complete structure of the TAG were produced by a combination of high-energy collisions and/or metastable decay. Although the results were impressive, implementation of such methodology into routine analysis would be problematic. For cost reasons, the availability of sector instruments to conduct high-energy CAD is difficult. Moreover, the spectral differences are often minor, and in the absence of chromatographic separation the difficulties of making structural assignments are expected to be further exacerbated.

Use of atmospheric pressure chemical ionization (APCI) for the structural characterization of TAG has been drawing increased interest and showing considerable potential. For example, Mottram *et al.* (20) identified positional isomers of TAG using DAG $[M - RCO_2]^+$ ion ratios present in their APCI–MS spectra. Mottram and Evershed (21,22) used positive-ion APCI–MS coupled to HPLC for the analysis of TAG mixtures from various sources. The propensity of terminal (*sn*-1/3) acyl groups to undergo more facile elimination than do groups located in the β-position (*sn*-2) was demonstrated and proposed as a means for distinguishing between AAB and ABA isomers. Although these fragmentation differences were well defined, the relative intensities of the protonated molecules were generally low, thus raising the potential of problems with regard to M.W. determination. Moreover, the absence of MS^n data made further characterization of individual FA impossible. Finally, the chromatographic conditions employed did not allow separation of positional isomers such as LLO vs. LOL or POL vs. OPL, etc., where L = linoleic, O = oleic, and P = palmitic acids. On the other hand, partial resolution of such isomers was achieved by Laakso and Voutilainen (23) using silver ion HPLC coupled to APCI. Molecular ion peaks were better defined, and the data further confirmed the more favorable loss of *sn*-1/3 rather than *sn*-2 R-COO groups. Silver columns, however, are somewhat cumbersome and difficult to handle and do not lend themselves to routine use. In addition, use of a single-stage quadrupole MS prevented generation of information about the individual FA.

To our knowledge, similar information on the regiospecific distribution of fatty acyl residues has not been achieved with the softer ionization method of electrospray ionization (ESI). A previous report showed that ESI-tandem mass spectrometric analysis (ESI-MS/MS) of TAG can determine the FA compositions of individual acylglycerol species after they had been separated according to their mass-to-charge ratio (24). With ESI on a triple quadrupole MS, product ions resulting from the loss of fatty acyl groups were found in a proportion similar to their presence on the TAG. However, no positional information on fatty acyl groups was obtained. This method of ionization would be beneficial to the analysis of TAG not only because of its ability to form intact molecular ions but also because of its suitability to LC. In this paper, we report on the use of ESI with an ion-trap mass spectrometer (ITMS) to distinguish TAG regiospecific isomers.

EXPERIMENTAL PROCEDURES

Reagents. The following standards of TAG were purchased from Sigma (Buchs, Switzerland): 1-myristoyl-2-oleyl-3-palmitoyl glycerol (MOP); 1,3-distearoyl-2-oleoyl-glycerol (SOS); 1,2-distearoyl-3-oleoyl-*rac*-glycerol (SSO); 1,2-dioleoyl-3-stearoyl-*rac*-glycerol (OOS); 1,3-dioleoyl-2-stearoyl-glycerol (OSO); 1,2-dioleoyl-3-palmitoyl-*rac*-glycerol (OOP); and 1,3-dioleoyl-2-palmitoyl-glycerol (OPO) and were 99% pure. All solvents were from Merck (Dietikon, Switzerland).

Instrumentation. The mass spectrometer used was a Finnigan LCQ (Finnigan, San Jose, CA) ITMS equipped with a Finnigan electrospray ion source. The system was operated in the positive ion mode with a spray voltage maintained at 4.0 kV. The capillary temperature was maintained at 200°C, and a nitrogen sheath gas was utilized at a flow rate of 20 arbitrary units. The TAG standards were dissolved in 70:30 acetonitrile/methanol with ammonium acetate added at a concentration of 10 mM. This sample solution was infused at a rate of 2 μL min^{-1} using a liquid methanol sheath at 8 μL min^{-1}. Solutions of the TAG were approximately 50 μM. Twenty scans were typically summed for each spectrum. The ammoniated precursor ion $[M + NH_4]^+$ was selected for collision (MS/MS) in each case, using helium as the collision gas and a collision energy of 30 arbitrary collision units.

RESULTS AND DISCUSSION

Mass spectrum of SOS. The ammoniated TAG ion $[M + NH_4]^+$, was generated in high relative abundance for each standard TAG by the addition of ammonium acetate to the sample solution. Figure 1 shows the CAD spectrum of ammoniated SOS at *m/z* 906.7. This type of TAG will be designated ABA. In this collision spectrum, the loss of NH_3 is evident, producing the protonated TAG ion $[M + H]^+$ at *m/z* 889.8. In addition, product ions at *m/z* 607.7 and 605.7 were formed, indicating the loss of NH_3 and, in the form of the neutral carboxylic acid RCOOH, of the oleate (O) FA from the *sn*-2 position and the stearate (S) FA from *sn*-1/3 position, respectively. The resulting DAG fragment ions $[M + NH_4 - NH_3 - RCO_2H]^+$ will be designated SO^+ and SS^+.

For SOS, the relative intensity of the $[SO]^+$ DAG ion at *m/z* 605.7 was approximately five times higher than that of the $[SS]^+$ fragment ion at *m/z* 607.7 (Table 1). This indicated that the loss of the fatty acyl group from the secondary (*sn*-2) position was energetically less favored than that from the primary positions (*sn*-1/3). A similar conclusion was drawn by Mottram *et al.* (20,25) from APCI mass spectra of TAG

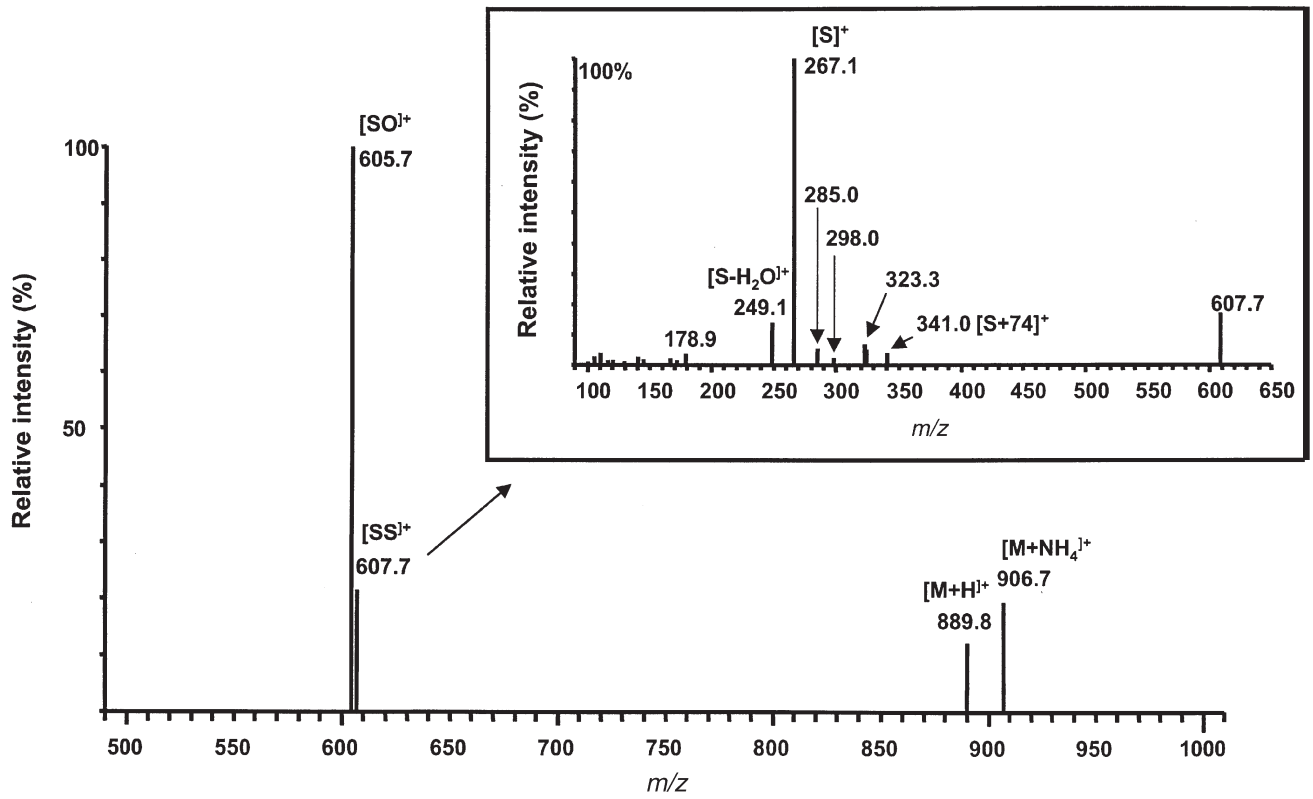


FIG. 1. Electrospray ionization-tandem MS/MS (ESI-MS/MS) spectrum of ammoniated 1,3-distearoyl-2-oleoyl glycerol (SOS) at *m/z* 906.7. Inset shows the MS^3 spectrum of the $[SS]^+$ fragment at *m/z* 607.7.

(25,26) and more recently by Hvattum (26) on a triple quadrupole instrument after CAD of $[M + NH_4]^+$ precursor ions.

The inset spectrum in Figure 1 presents the MS^3 spectrum of the *m/z* 607.7 $[SS]^+$ ion resulting from SOS. This spectrum exhibits an intense $[S]^+$ acylium ion at *m/z* 267.1 and its dehydration product at *m/z* 249.1 (loss of 18 Da). Additional minor fragments are found at *m/z* 341.0 $[S + 74]^+$, *m/z* 323.3 $[S + 74 - H_2O]^+$, 285.0 $[C_{17}H_{35} - C(OH)_2]^+$, and 298.0, an ion containing the elements of methyl stearate $[C_{17}H_{35}–CO–OCH_3]^{+\bullet}$. The formation of $[RCO + 74]^+$ product ions has earlier been proposed to arise by neutral loss of R–CH=C=O from the DAG fragment ion (19). The same types of ions have been previously reported in the $CI(NH_3)$ spectra of diacyl-PC (27), the EI spectra of TAG (15), and the CAD spectra of related types of parent ions generated by thermospray ionization of various glycerolipids (28). In the above series of ions, the mass of 74 Da represents the backbone of the TAG molecule in the form of the elements of $C_3H_6O_2$.

TABLE 1
Relative Abundance Ratios of DAG Ions in Various Triacylglycerols[a]

TAG	$[AB]+/[AA]^+$ ratio	SD
SOS	5.2:1.0	0.84 (n = 5)
SSO	1.1:1.0	0.13 (n = 4)
OSO	5.9:1.0	0.00 (n = 2)
OOS	2.0:1.0	0.25 (n = 3)
OPO	5.5:1.0	1.00 (n = 3)
OOP	2.0:1.0	0.40 (n = 3)

[a]O, oleate; S, stearate; O, oleate; S, stearate; SOS, 1,3-distearoyl-2-oleoyl-glycerol; OOS, 1,2-dioleoyl-3-stearoyl- glycerol; OSO,1,3-dioleoyl-2-stearoyl-glycerol, SSO: 1,2-distearoyl-3-oleoyl- glycerol; OPO, 1,3-dioleoyl-2-palmitoyl-glycerol; OOP, 1,2-dioleoyl-3-palmitoyl-glycerol; $[AB]^+$, diacylglycerol ion with two different FA; $[AA]^+$, diacylglycerol ion with the same FA in the two positions of the glycerol backbone.

Mass spectrum of SSO. Figure 2 depicts the CAD spectrum of the positional isomer, ammoniated SSO ion at *m/z* 906.7. Despite the statistically anticipated 2:1 ratio, the TAG ion, designated as an AAB-type TAG, fragments with a DAG product ion intensity ratio ($[SO]^+/[SS]^+$) of approximately 1:1. The actually observed 1:1 ratio is, at least in part, influenced by the less favorable cleavage of the stearyl (S) group on the 2-position. Thus, both the SOS (Fig. 1) and SSO (Fig. 2) isomers have distinct DAG ratios in their CAD spectra, and, as a result, one can use these DAG ratio differences ($[SO]^+/[SS]^+$) to distinguish between the two positional isomers.

More structural information about SOS was sought using the multiple stages of collision (MS^n) made possible with the ITMS. When the $[SO]^+$ DAG fragment ion at *m/z* 605.5 was selected and collided further (MS^3), the presence of both the $[S]^+$ and $[O]^+$ acylium ions at *m/z* 267.1 and 265.3, respectively, was confirmed (Fig. 2, inset). In addition, product ions at *m/z* 249.3 and 247.3 show a difference of 18 Da from the $[S]^+$ and $[O]^+$ acylium ions and represent the loss of water. Analogous to $[SS]^+$ (Fig. 1, inset), the MS/MS spectrum of

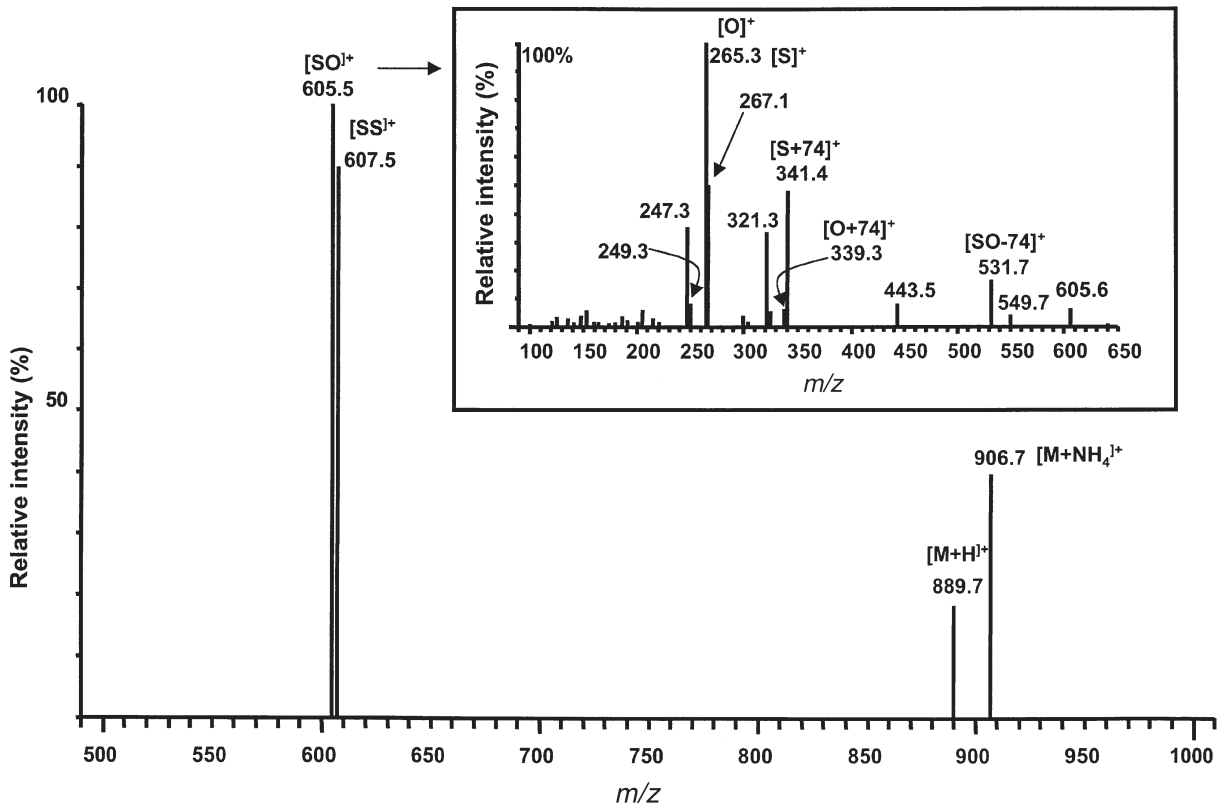


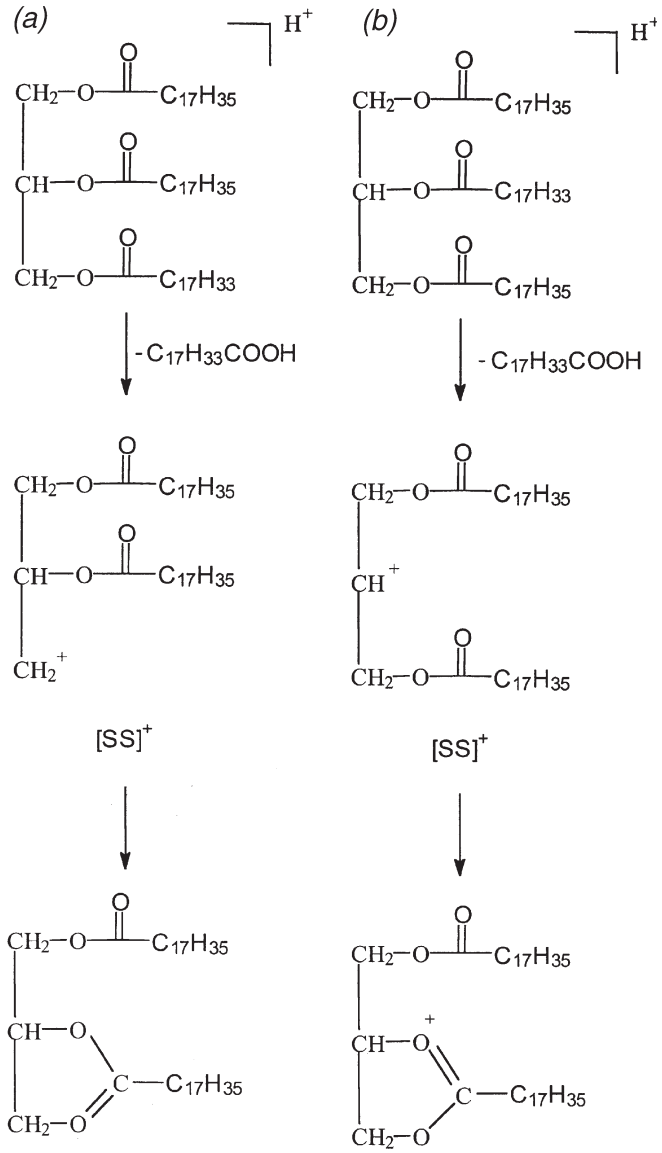
FIG. 2. ESI-MS/MS spectrum of 1,2-distearoyl-3-oleoyl glycerol (SSO) at *m/z* 906.7. Inset shows the MS^3 spectrum of the $[SO]^+$ ion at *m/z* 605.5 from SSO. For other abbreviation, see Figure 1.

$[SO]^+$ generated from fragmentation of SSO (Fig. 2, inset) exhibits the corresponding fragment ions of *m/z* 341.4 $[S + 74]^+$, 323.3 $[S + 74 - H_2O]^+$, 339.3 $[O + 74]^+$, and 321.3 $[O + 74 - H_2O]^+$, where 74 Da represents part of the glycerol backbone. Although providing no additional information on fatty acyl position, these spectra further confirmed the identity of these substituents. Thus, for the isomeric pair SOS and SSO, we found that CAD of the ammoniated TAG molecular ion was sufficient to identify the fatty acyl groups and to determine the position (*sn*-2 vs. *sn*-1/3) of the FA moieties on the glycerol backbone.

It has been postulated (15) that the DAG ions $[SS]^+$, $[SO]^+$, etc., may form stable intramolecular five- and six-membered rings with carbonyl groups in the *sn*-2 or *sn*-1 position. If the charge in these fragments remained localized at the site of initial acyl group cleavage, one would expect the formation of two distinctly different $[SS]^+$ ions from the isomeric SSO and SOS TAG. However, identical MS^3 spectra were obtained in the collision of $[SS]^+$ derived from either SSO or SOS, which suggests that the two fragment ions (Scheme 1, *a* and *b*) had effectively isomerized prior to the MS^3 ionization stage, thereby losing their original structural identities.

Unique to the CAD spectra (MS^3) of the $[SO]^+$ ions in both the SOS and the SSO isomers is the presence of a fragment at *m/z* 531.7, formed by loss of 74 Da from the $[SO]^+$ (Fig. 2, inset). It is reasonable to attribute the formation of this ion to a rearrangement process that involves the coupling of the two acyl groups with simultaneous transfer of a hydrogen to eliminate the backbone of the TAG system as a stable neutral. The same loss of 74 Da was observed in the MS^3 spectra of both $[PO]^+$ and $[OO]^+$ ions produced from OPO and OOP TAG (Figs. 3, 4, insets). On the other hand, no analogous $(SS - 74)^+$ fragment is found in the MS/MS spectra of the $[SS]^+$ (*m/z* 607.7) parent from either SOS or SSO, where both acyl groups consist of saturated alkyl chains. The same general trend (i.e., a requirement for the presence of an unsaturated site on the alkyl chain in order to induce the loss of 74 Da from DAG ions) is also observed in the MS^3 spectra of the MOP TAG (Fig. 5). Note, for example, that MS/MS of the $[MO]^+$ and $[OP]^+$ ions in this ABC type TAG produces similar fragments at *m/z* 475.6 ($[MO - 74]^+$) and 503.5 ($[OP - 74]^+$), respectively. On the other hand, MS^3 of the $[MP]^+$ ion, where both M and P consist of saturated alkyl chains, shows no detectable $[MP - 74]^+$ fragment. The apparent requirement for an unsaturated alkyl chain may be explained by invoking the transfer of an allylic hydrogen to form a stable fragment ion with the positive charge localized α to a double bond as shown in Scheme 2. This type of remote charge-induced hydrogen transfer is analogous to that observed in oxazoline and other related derivatives of FA that contain a functional group of low ionization potential (29,30).

Mass spectra of OSO and OOS. The propensity for more favorable loss of an *sn*-1/3 as opposed to an *sn*-2 acyl group is further illustrated by the CAD spectra of the ammoniated molecular ions of the two isomeric OSO and OOS TAG (Table 1;



SCHEME 1

spectra not shown). The [AB]/[AA] ratio of 5.9:1 in the spectrum of OSO is in line with that of 5.2:1 for SOS, indicating a more facile loss of the terminal acyl group upon CAD of the ammoniated molecule. Although the [AB]/[AA] ratio of 2:1 in the spectrum of the OOS isomer is distinctly higher than that of 1.1:1.0 observed in the spectrum of SSO, it is in general agreement with the expected trend. It is possible that any discrepancies may be associated with the additional unsaturation present in OOS as opposed to SSO species.

Mass spectra of OPO and OOP. To ascertain if consistent findings would be observed with other similar-length isomeric TAG, we analyzed the isomeric pair OPO and OOP. Collision of the ammoniated OPO ion at *m/z* 876.9 resulted in the protonated $[M + H]^+$ ion at *m/z* 859.7 and two DAG ions at *m/z* 577.4 $[OP]^+$ and 603.5 $[OO]^+$, as shown in Figure 3. These latter ions not only represent the loss of oleoyl and palmitoyl (P) fatty acyl groups, respectively, but also appear in an approximate relative DAG ratio of 5:1. This ratio is consistent with that found in the previous example of SOS, an ABA TAG, confirming that the FA group in the *sn*-2 position is five times less likely to be lost upon collision. Figure 4 displays the CAD spectrum of ammoniated OOP at *m/z* 876.9. Upon collision, the OOP isomer produced a relative DAG ratio $[OP]^+/[OO]^+$ (*m/z* 577.5:*m/z* 603.5) of approximately 2:1. These ratios are fully in line with the behavior of the OSO and OOS pair (Table 1), further verifying the validity of the previous observations.

MS^3 was further conducted on the $[OP]^+$ (*m/z* 577.4) from OPO TAG (Fig. 3, inset) and $[OO]^+$ (*m/z* 603.5) ions from the OPO TAG (Fig. 4, inset). In both spectra, the $[P]^+$ and $[O]^+$ ions are found at *m/z* 239.0 and 265.2, respectively. In the MS^3 spectrum of $[OP]^+$ from OPO TAG, there is a strong peak at *m/z* 313.3, presumably corresponding to $[P + 74]^+$ (Fig. 3, inset). In the MS^3 spectrum of $[OO]^+$ from OOP TAG, the corresponding ion $[O + 74]^+$ at *m/z* 339.3 is prominent, whereas its dehydration product at *m/z* 321.2 is of lesser relative abundance (Fig. 4, inset). In addition, as discussed earlier, there is a facile loss of 74 Da from the $[OP]^+$ and $[OO]^+$ ion to give a fragment at *m/z* 503.7 and 529.6, respectively.

In the MS^3 spectrum of the $[OO]^+$ ion (Fig. 4, inset), it is important to point out the typical fragments at *m/z* 265.3, $[O]^+$; 247.1, $[O - 18]^+$; 339.3, $[O + 74]^+$; and 321.2 $[O + 74 - 18]^+$, reflecting the presence of the O group.

Mass spectrum of MOP. The general consistency of the above observations was further confirmed by examining the spectrum of MOP, a "mixed" TAG of the ABC type. The CAD spectrum of the ammoniated molecular ion of *m/z* 822.6 is shown in Figure 5. As expected, the $[OP]^+$ and $[MO]^+$ product ions generated from loss of the terminal acyl groups were the dominant species, whereas the $[MP]^+$ ion was of significantly lower abundance, resulting in a ratio of [MO]/[OP]/ [MP] = 2.5:2.2:1.0. This generally strong propensity for loss of the terminal *sn*-1/3 acyl group is probably related to the facile initial attachment of the NH_4^+ reagent ion to the more exposed substituent.

The MS^3 spectra of the three combinations of DAG ions from MOP are shown in the insets of Figure 5. Fragments corresponding to the different acyl groups are found at the expected *m/z* values, and their identities are indicated in the figure. The loss of 74 Da in the CAD spectra of $[OP]^+$ and $[MO]^+$ but the absence of the same ion in the spectrum of $[MP]^+$ is significant. As discussed earlier, unsaturation in at least one of the FA side chains appears to be essential to sustain this fragmentation.

Although the data presented in this article are encouraging with regard to the potential applicability of ESI-ITMS for the analysis of isomeric TAG, it is clear that the utility of the method will be significantly enhanced if it is used in combination with HPLC, enabling analysis of TAG mixtures and oils. RP-HPLC represents one of most popular techniques for separating a maximal number of TAG prior to their identification by MS, using either APCI or ESI sources (13,20, 25,26). Nevertheless, RP-HPLC does not allow the separation between critical pairs of isomeric TAG (e.g., SSO/SOS).

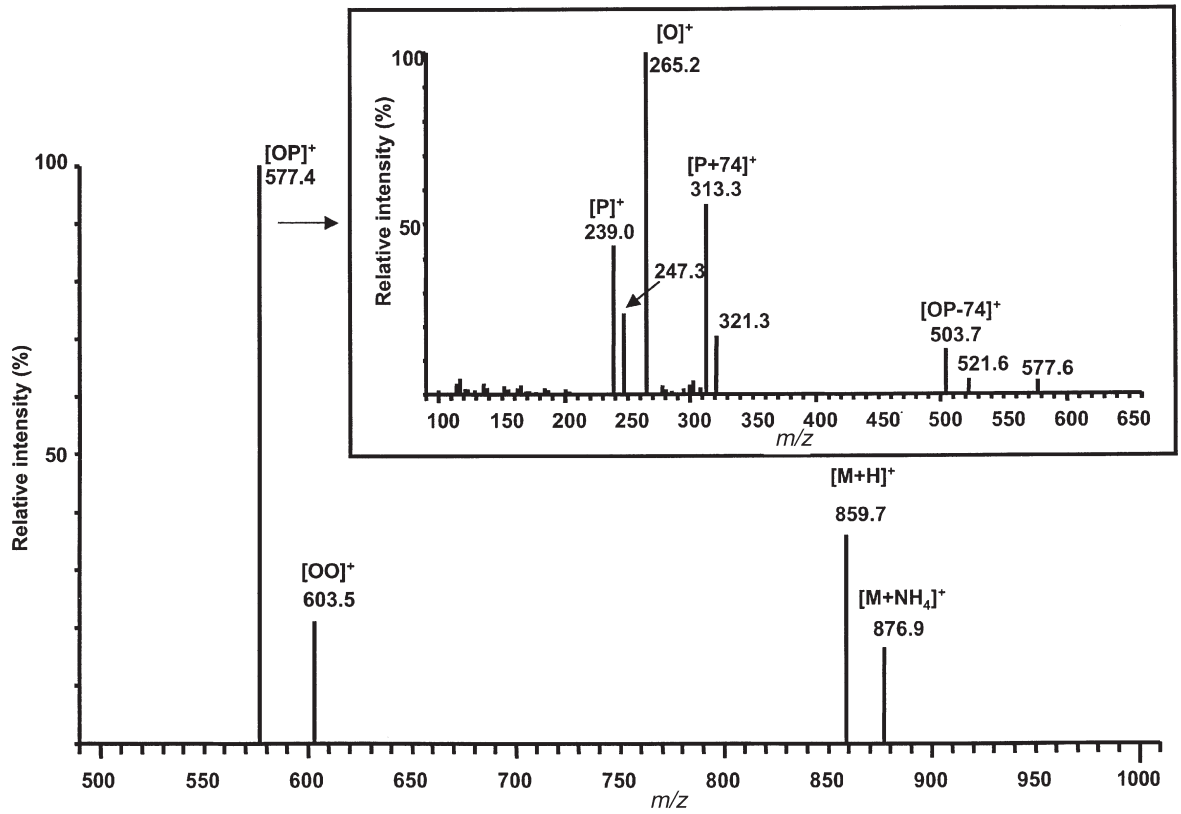


FIG. 3. ESI-MS/MS spectrum of ammoniated 1,3-dioleoyl-2-palmitoyl-glycerol (OPO). Inset shows the MS^3 spectrum of the $[OP]^+$ ion at *m/z* 577.4. For other abbreviation, see Figure 1.

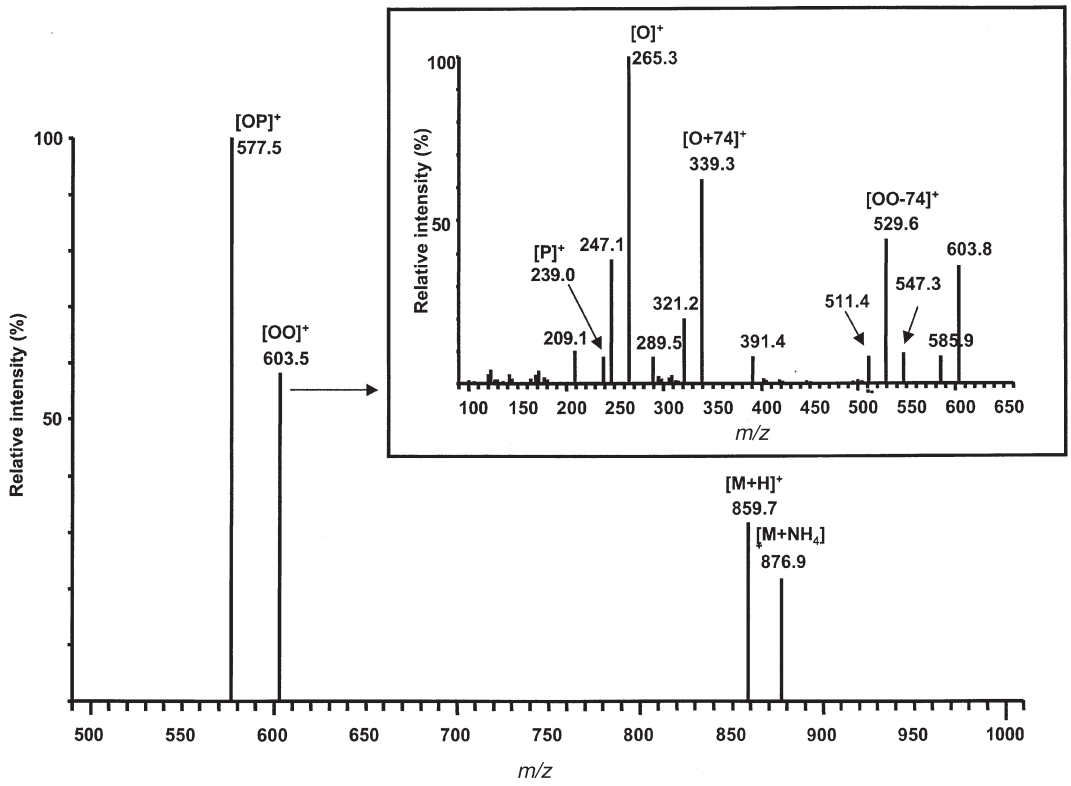


FIG. 4. ESI-MS/MS spectrum of ammoniated 1,2-dioleoyl-3-palmitoyl-glycerol (OOP). Inset shows the MS^3 spectrum of the $[OO]^+$ ion at *m/z* 603.5. For other abbreviation, see Figure 1.

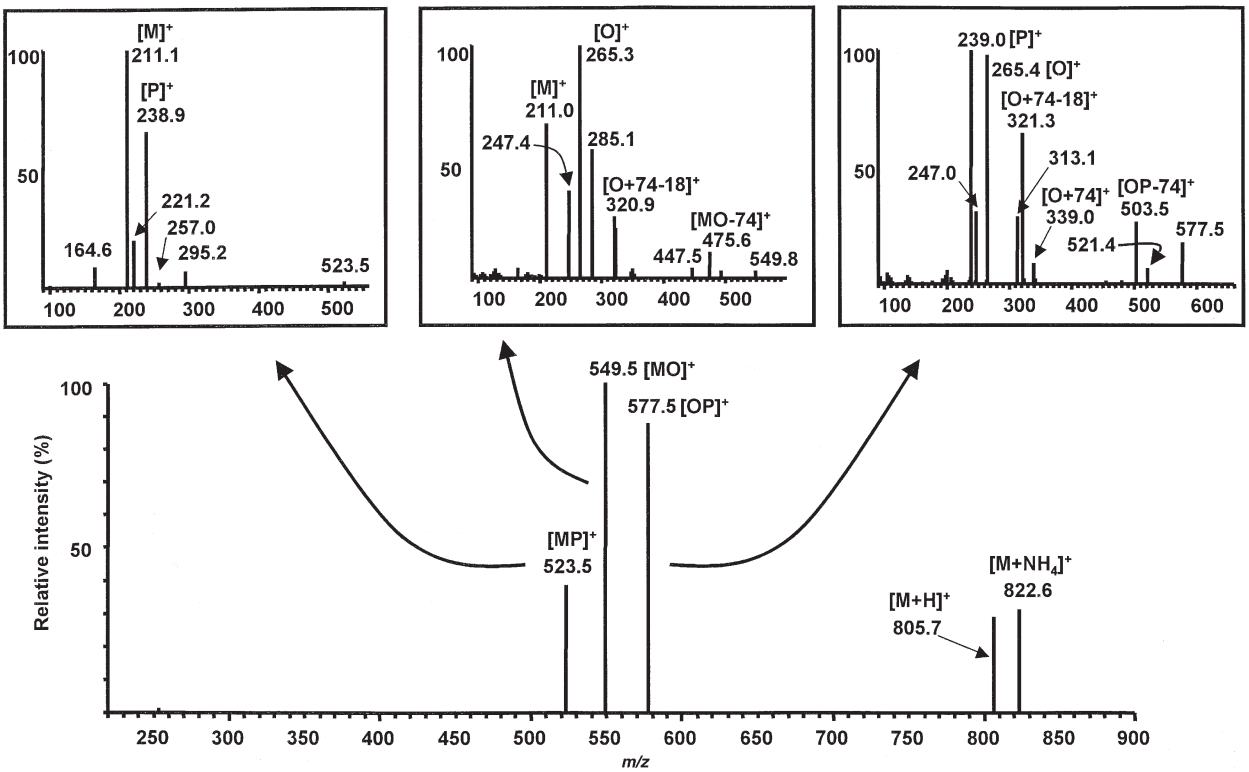
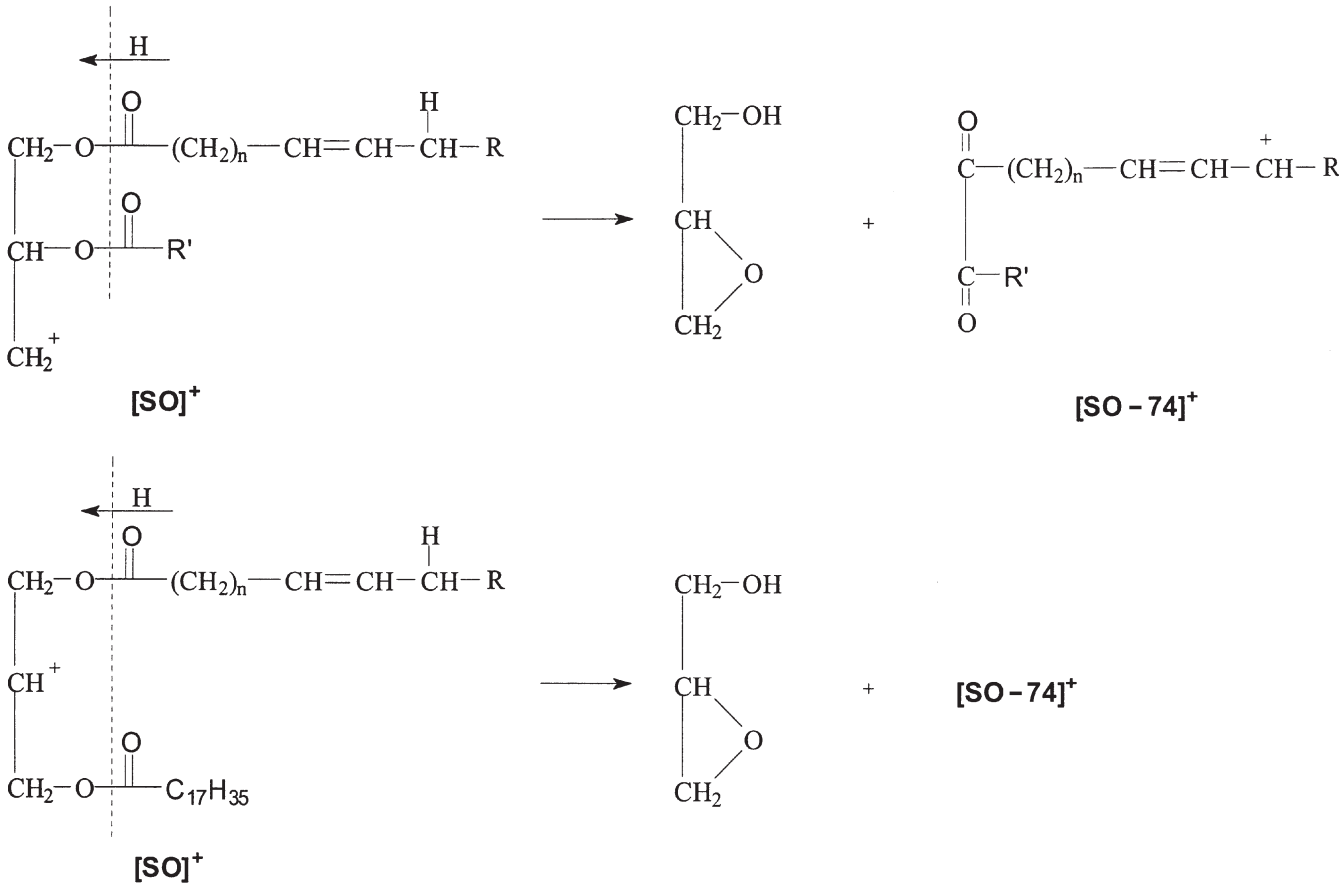


FIG. 5. ESI-MS/MS spectrum of ammoniated 1-myristoyl-2-oleoyl-3-palmitoyl glycerol (MOP) at *m/z* 822.6. Insets show the MS3 spectrum of the $[MP]^+$, $[MO]^+$, and $[OP]^+$ ions at *m/z* 523.5, 549.5, and 577.5, respectively. For other abbreviation, see Figure 1.



SCHEME 2

Some authors have reported such a separation using either silver-ion HPLC (31,32) or, more recently, chiral phase HPLC (33). These results open new ways for TAG analysis, and our future work will be focused on the hyphenation of such chromatographic separations to the ITMS.

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