Characterization of Castor Bean Neutral Lipids by Mass Spectrometry/Mass Spectrometry

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A method has been developed for the characterization of intact neutral lipids isolated from castor bean (*Ricinus communis* L.) by mass spectrometry/mass spectrometry (MS/MS). The molecular weights of the trimethylsilyl (TMS) derivatives of the neutral lipids are determined by using both electron impact and chemical ionization (ammonia). Collision-induced dissociation daughter spectra of the $(M\text{-}CH_3)^+$ ions yield fragment ions that allow easy determination of the acyloxy groups present. The chain-length and degree of unsaturation for each acyloxy group are indicated by R in the ion represented by the general formula $(RCO + 74)^+$. Other ions of diagnostic value include $(M-RCOO)^+$, $(M-RCOOH)^+$, $[(M-CH_3-RCOOH]^+$ and $[(M-RCOOH)-16]^+$. The presence of a TMS group in any of these fragments results in the formation of ions representing the loss of OTMSH. Prior to MS/MS analysis, partial fractionation by high-performance liquid chromatography (according to degree of unsaturation in the neutral lipids) is useful because daughter spectra are generated free of any isotopic contamination, and minor components are concentrated in single fractions, which aids their characterization. By using this method, 11 neutral lipids were characterized in castor bean.

KEY WORDS: Castor bean, daughter ion, diacylglycerol, MS, MS/MS, neutral lipid, ricinoleic acid, *Ricinus communis* L., triacylglycerol, trimethylsilyl derivative.

Angiosperm plants contain complex mixtures of neutral lipids, primarily triacylglycerols, which have uses as edible and industrial oils. As part of a seed oil modification program at this Institute, it was necessary to characterize the neutral lipids in castor bean (*Ricinus communis* L.). Currently, the acyl composition of storage lipids is usually extrapolated from fatty acid profiles obtained from transmethylation experiments (1), but these experiments reveal very little about the structures of the intact neutral lipids.

Several methods have been used for the analysis of intact neutral lipids. Liquid chromatography is useful (2), although given the complexity of these mixtures, complete separation of the components is often not possible and characterization is limited by the number of standards available. However, separation according to degree of unsaturation can be achieved, which is extremely important for this method of analysis, as will be discussed later. Electron ionization mass spectrometry (MS), with the solids probe used for sample introduction, has been shown to be useful for characterizing the structures of pure triacylglycerols (3). However, MS analyses of complex endogenous plant lipids is more difficult as specific diagnostic fragment ions cannot be assigned to any particular molecular ion without prior fractionation. Hites (4) has described a mass spectrometric method for determining the molecular weight distribution of triacylglycerols (TAGs) in a number of vegetable oils. This method does not confirm the presence of specific acyloxy groups and therefore cannot distinguish between positional isomers or isologs. Although gas chromatography/mass spectromety (GC/MS) has been used for intact neutral lipid mixtures with some success (5), preliminary experiments conducted in this laboratory showed that this technique is not useful for castor bean lipids, due to the involatility of the hydroxy-containing fatty acyl components (Hogge, L.R., E.W. Underhill and D.C. Taylor, unpublished results).

Since tandem mass spectrometry (MS/MS) has been shown to be the method of choice for the analysis of other complex mixtures (6,7), we have investigated its usefulness for the characterization of these lipids. MS/MS offers the advantage of providing structural information from which the specific acyloxy groups present in each individual lipid can be deduced. This report includes a description of the partial separation of these lipids by liquid chromatography, the mass spectrometric method used for the characterization of the trimethylsilyl (TMS) derivatives of these lipids, the collision-induced dissociation (CID) spectra obtained for the individual components of the mixture, and the characteristic CID ions used for structural assignment.

MATERIALS AND METHODS

A total lipid extract was prepared from seeds (minus seed coats) of castor bean (*Ricinus communis* L. cv B296) according to the method of Hara and Radin (8), with the following modification—the hexane/isopropanol extract was backwashed once with one-half volume of 1 M KCl in 0.2 M H_3PO_4, the hexane-rich upper phase containing the total seed lipids was removed and the solvent evaporated under a stream of nitrogen at 35°C.

The neutral lipids were isolated from the total lipid extract in a 20 cm × 2.5 cm silica gel chromatography column (grade 60, Merck, Darmstadt, Germany). The column was eluted with 50% hexane in diethyl ether. The solvent was removed from the eluted neutral lipid fraction by rotary evaporation under reduced pressure.

Transmethylation of the neutral lipids to produce fatty acid methyl esters (FAMEs) was conducted by the following procedure (1): 2 mL of 1 N methanolic HCl (3 N methanolic HCl from Supelco Canada Ltd., Oakville, Ontario; diluted with Omni-Solv methanol) was added to the neutral lipid sample and allowed to stand at 80°C for 1 hr. The reaction mixture was cooled on ice, 2 mL of 0.9% (w/v) NaCl was added and the mixture was extracted three times with 2 mL of hexane. The combined hexane extracts containing the FAMEs were taken to dryness on a cooling block at −10°C under a stream of nitrogen.

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Partial separation of the intact neutral lipids was achieved by high-performance liquid chromatography (HPLC) with a 1 × 50 cm Whatman Partisil M9 10/50 ODS-2 (Whatman, Clifton, NJ) reverse-phase column connected to a Spectra Physics SP8700 solvent delivery system (San Jose, CA). Samples of up to 100 μL (neat material) were injected with a Rheodyne 7125 injector fitted with a 200-μL loop and eluted from the column by a linear gradient of 100% acetonitrile to 100% acetone in 60 min at a flow rate of 3 mL/min. The effluent was directed through a splitter, which delivered 97% for manual fraction collection and 3% for peak detection in an applied chromatography system model 750/14 mass detector (Macclesfield, Cheshire, U.K.). Fractions were collected corresponding to the recorded chromatographic peaks.

The HPLC mobile phase was removed under nitrogen flow and the neat material was derivatized by adding an excess of N,O-*bis* (trimethylsilyl) acetamide (BSA) at room temperature. The derivatization reaction was complete in 15 min and the product remained stable for several days.

Mass spectral analyses were carried out using a VG 70-250 SEQ hybrid mass spectrometer (Fisons Instruments, Manchester, England). Samples were introduced into the mass spectrometer by means of the solids probe (330°C) and analyzed either in the electron-ionization (EI) or chemical-ionization (CI) (ammonia) mode. Full (magnetic) scan data were acquired by scanning the mass range of 1300–100 daltons every 10 seconds with a 0.5 second settling time. Daughter spectra (RF-only quadrupole collision cell) were acquired by allowing only the desired parent ion to be transmitted through the double-focusing analyzer to the collision cell containing argon at an indicated pressure of 5×10^{-5} Torr and scanning the quadrupole mass filter over the range of 1300–100 daltons in 7.5 seconds. The collision energy was 14 volts (laboratory reference frame).

RESULTS AND DISCUSSION

Transmethylation experiments showed that the castor bean total neutral lipid fraction contained approximately 90% ricinoleic acid (9-*cis*-12 hydroxy octadecenoic acid). Providing that these neutral lipids consist primarily of glycerol-derived components, triricinoleoyl glycerol would be expected as a major component. However, mass spectrometry of this underivatized fraction gives no indication of triricinoleoyl glycerol (*m/z* 933) or any other hydroxy-containing TAG, but instead shows the major TAG as trilinoleoyl glycerol (*m/z* 879, data not shown). These data indicate that the hydroxy-containing TAGs must undergo a rearrangement and loss of water in the ion source of the mass spectrometer. Preparation of the TMS derivative improves the volatility of the analyte, but not sufficiently enough to facilitate GC/MS as the interface temperature of over 400°C that is necessary to keep the sample volatilized, cannot be obtained with existing equipment.

TMS derivatization does, however, overcome the problem of dehydration in the source. Figure 1 shows the ions in the higher mass region for the respective EI and CI mass spectra obtained from the TMS-derivatized total neutral lipid fraction. Although the expected molecular ion, *m/z* 1149, for the TMS derivative of triricinoleoyl glycerol is not observed in the EI spectrum, an ion 15 mass units lower at *m/z* 1134 is the most intense ion (see Fig. 1A) and is postulated to correspond to the $(M-CH_3)^+$ ion. The CI spectrum (Fig. 1B) supports this mass assignment, as the most intense ion occurs 33 mass units higher at *m/z* 1167 and indicates the $(M+NH_4)^+$ adduct ion for the triricinoleoyl glycerol TMS derivative. Figure 1A also shows ions at *m/z* 1020, 1044, 1046, 1060, 1074 and 1162 that have corresponding ions 33 mass units higher in the CI spectrum (Fig. 1B), indicating acylglycerols of differing composition. The ion at *m/z* 1035 (Fig. 1A) does not have a corresponding ion 33 mass units higher in the CI spectrum, indicating that its structure differs from the other ions in this higher mass range. The ion at *m/z* 1060 (Fig. 1A) likely represents the loss of OTMSH from the protonated triricinoleoyl TMS derivative.

Although MS/MS analyses could be done on the total neutral lipid fraction, problems can occur. Since the M+2 isotopic ion is of significant intensity (>50% of M) for these higher molecular weight lipids, isotopic contamination is likely to occur in MS/MS analysis when TAGs varying by only one double bond are present in the mixture. In addition, minor components may not be present in sufficient quantities to allow MS/MS analysis. These problems were overcome by reverse-phase HPLC fractionation. Although the HPLC method achieves only a partial separation of the neutral lipids, it does separate the components by degree of unsaturation. For example, the cluster of ions from *m/z* 1042 to 1048, as shown in Figure 1, is composed of several TAGs varying only in the number of double bonds present in the acyl groups. The HPLC method separated these components, allowing daughter spectra to be obtained which are free of any M+2 isotopic contamination from the C, Si and O natural isotopes from similar components having one additional double bond. Secondly, the HPLC separation concentrates minor components in a particular fraction, thus yielding a sufficient sample size to permit the acquisition of useful daughter ion spectra.

Confirmation of the structural assignment of the ion of *m/z* 1134, (EI) was obtained by studying its collisionally induced dissociation (CID) daughter ion spectrum. Figure 2 shows the CID spectrum with the proposed structures indicated for several ions of diagnostic value. The regiospecific distribution of the acyl groups on the glycerol backbone is not known, as is the situation for all structural formulae reported here. Many of the ions observed in the CID spectra are of the same general formula as those observed in the EI spectra of TAGs (3). The general formulae proposed for all of these ions are shown in Table 1 and have each been assigned a number for easy reference. The masses and the intensities for each of these ions are also listed in Table 1. The ion at *m/z* 427, which has been observed in the EI spectra of other acyl glycerols, is proposed to have the general formula $(RCO + 74)^+$ (3) (see Table 1, #9), where R represents the aliphatic chain of the acyl group. The presence of a TMS function is indicated by the loss of OTMSH (90 mass units) from this ion yielding *m/z* 337, #10 $[(RCO+74)-OTMSH]^+$. The ion at *m/z* 263 is the result of the loss of OTMSH from the ion of general formula RCO, also observed in the EI spectra of TAGs (3). As this ion is not significantly intense in many of the spectra studied in this report, it is not included in Table 1. Fragment ions containing two acyl groups (or fragments thereof) on the glycerol backbone

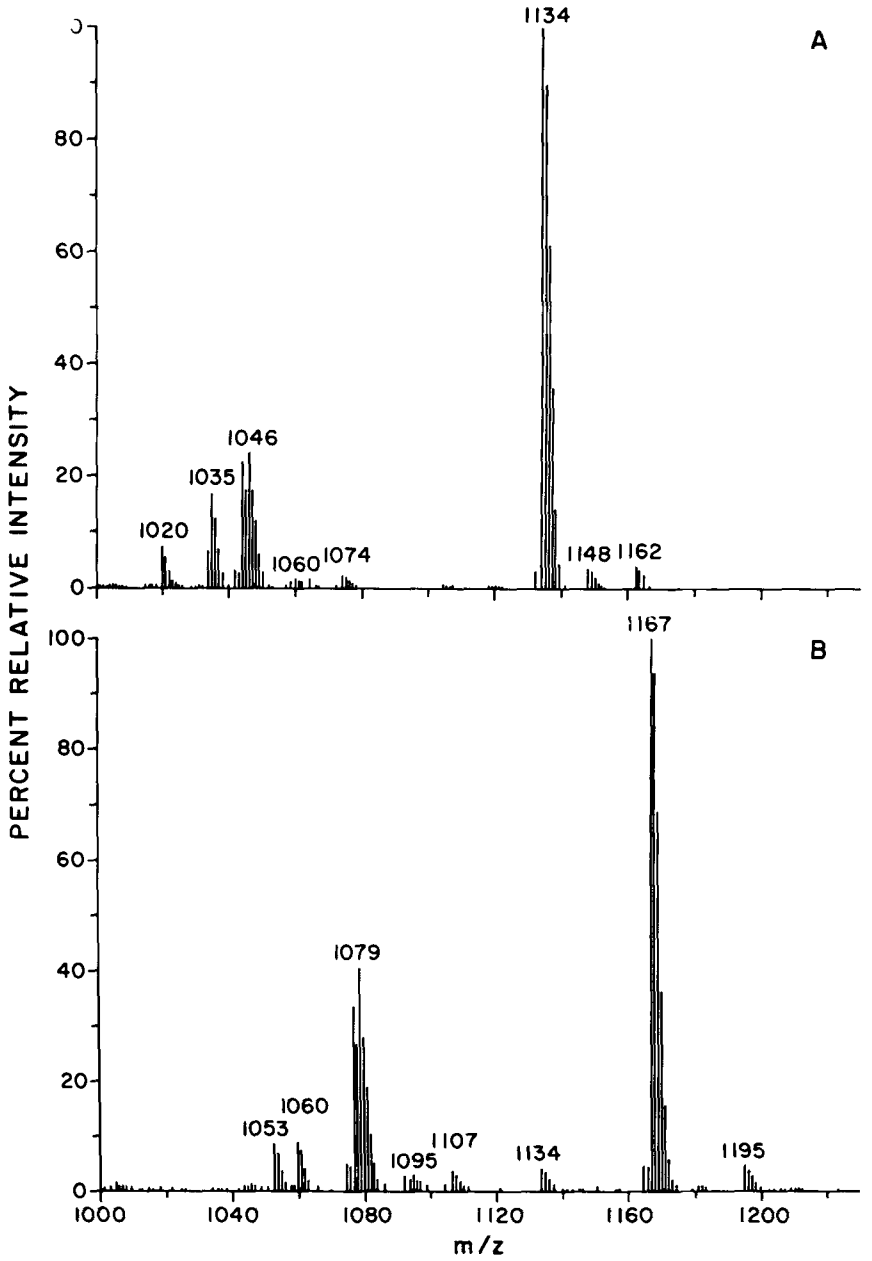


FIG. 1. Partial electron impact (A) and ammonia chemical ionization (B) mass spectrum of castor bean neutral lipid fraction.

are also observed at *m/z* 780, 764, 689 and 599 (see Fig. 2). The ion at *m/z* 780 is postulated to represent the structure resulting from the loss of the ricinoleoyl acyloxy group that has previously lost a methyl radical during the electron ionization process and is designated as #3 $(M-RCOO)^+$ (Table 1). The ion at *m/z* 764 appears to represent a loss of ricinoleic acid from the $(M-CH_3)^+$ parent, #5 $[(M-CH_3)-RCOOH]^+$. While an ion resulting from the loss of ricinoleic acid from the molecular ion is not observed in this spectrum, such an ion is observed in the spectra of TAGs containing an acyl group without a TMS function present (see Table 1). However, the ions at *m/z* 689 and 599 are likely formed from successive losses of OTMSH from $(M-RCOOH)^+$, resulting in ions #7 and #8. The ion at *m/z* 1044 (#2), which results from the loss of OTMSH from *m/z* 1134, also indicates that there are at least two TMS functions present in the parent glycerol, providing *m/z* 1134 is derived from the loss of a TMS methyl as postulated.

Figure 3 shows the daughter spectrum of *m/z* 1020, postulated to be the $(M-CH_3)^+$ ion for the TAG containing one palmitoyl (16:0) and two ricinoleoyl-TMS [18:1 (OTMS)] acyl groups. Ions corresponding to the proposed general formula in Table 1 are indicated above the appropriate masses. In this case, the daughter ions are similar to those discussed in the previous paragraph

TABLE 1

Diagnostic Ions from MS/MS Analysis of Castor Bean Neutral Lipids Partially Fractionated by Reverse-Phase HPLC

	Parent ions	Proposed structures of daughter ions (percent relative intensity)								
Acylglycerol	# 1 $(M-CH_3)^+$	# 2 $[(M-CH_3)-90)]^+$	# 3 $(M-RCOO)^+$	# 4 $(M-RCOOH)^+$	# 5 $[M-15)-RCOOH]^+$	# 6 $[(M-RCOOH)-16]^+$	# 7 $[(M-RCOOH)-90]^+$	# 8 $[(M-RCOOH)-2 \times 90]^+$	# 9 (RCO+74)	# 10 $[(RCO+74)-90]^+$
16:0 18:1 (OTMS) 18:1 (OTMS)	1020(100)	930(4.6)		665(0.7)	764(0.3)	649(0.7)	575(3.3) 689(1.9)	599(1.2)	313(1.8) 427(1.1)	337(3.9)
18:3 18:1 (OTMS) 18:1 (OTMS)	1042(100)	952(4.0)		687(1.0)	764(0.6)	671(1.0)	597(3.4) 689(3.4)	599(2.1)	335(2.2) 427(1.4)	337(4.3)
18:2 18:1 (OTMS) 18:1 (OTMS)	1044(100)	954(7.1)		689(5.1)	764(0.5)	673(2.0)	599(10.9) 689(5.1)	599(10.6)	337(8.7) 427(3.1)	337(8.7)
18:1 18:1 (OTMS) 18:1 (OTMS)	1046(100)	956(6.5)		691(3.3)	764(0.2)	675(1.3)	601(4.5) 689(1.6)	599(1.1)	339(1.7) 427(1.7)	337(1.7)
18:0 18:1 (OTMS) 18:1 (OTMS)	1048(100)	958(3.7)		693(0.4)	764(0.5)	677(0.5)	603(2.9) 689(1.6)	599(0.8)	341(1.4) 427(0.8)	337(2.1)
20:2 18:1 (OTMS) 18:1 (OTMS)	1072(100)	982(4.4)		717(2.0)	764(0.8)	701(.80)	627(4.3) 689(1.3)	599(2.7)	365(6.9) 427(1.6)	337(11)
20:1 18:1 (OTMS) 18:1 (OTMS)	1074(100)	984(6.3)		719(0.8)	764(0.2)	703(0.4)	629(4.1) 689(2.3)	599(1.1)	367(2.3) 427(1.1)	337(5.1)
20.0 18:1 (OTMS) 18:1 (OTMS)	1076(100)	986(7.1)		721(0.6)	764(0.3)	705(0.5)	631(4.3) 689(1.8)	599(1.5)	369(2.1) 427(1.0)	337(2.1)
18:2 (OTMS) 18:1 (OTMS) 18:1 (OTMS)	1132(100)	1042(38.9)	778(2.2) 780(6.7)		762(2.2) 764(1.9)		687(20.4) 689(19.6)	599(3.9) 597(4.6)	425(5.1) 427(9.9)	335(6.4) 337(12.6)
18:1 (OTMS) 18:1 (OTMS) 18:1 (OTMS)	1134(100)	1044(7.6)	780(0.4)		764(0.7)		89(5.1)	599(4.0)	427(2.3)	337(4.3)
20:1 (OTMS) 18:1 (OTMS) 18:1 (OTMS)	1162(100)	1072(3.7)	780(0.4) 808(0.7)		792(0.4) 764(0.9)		717(2.9) 689(1.6)	627(1.6) 599(0.6)	455(1.2) 427(1.6)	365(2.4) 337(4.9)

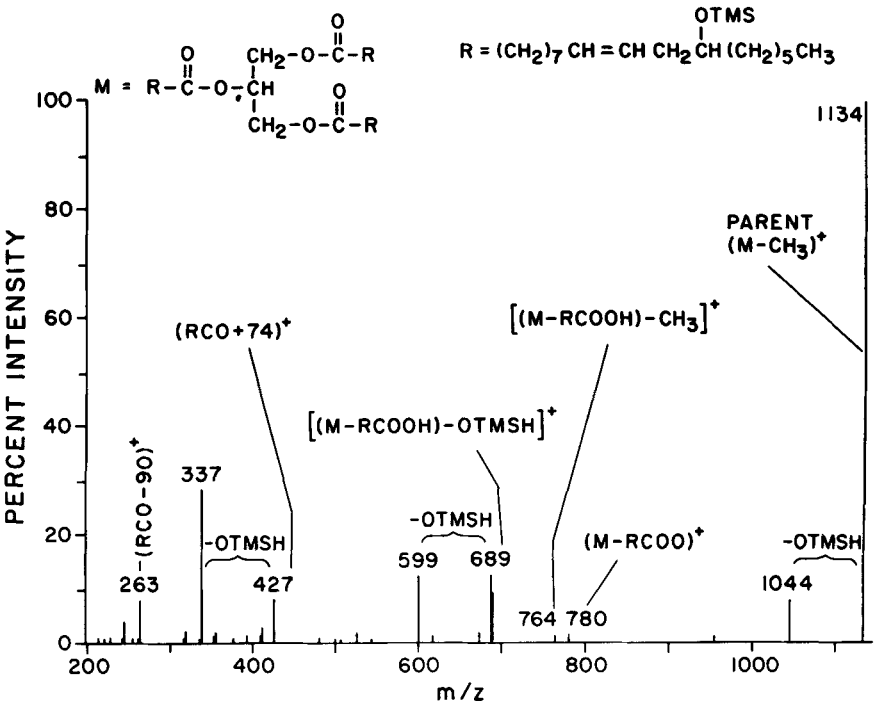


FIG. 2. CID daughter ion spectrum of *m/z* 1134 from castor bean neutral lipid fraction (TMS derivative). Acylglycerol structures shown do not imply regiochemical specificity.

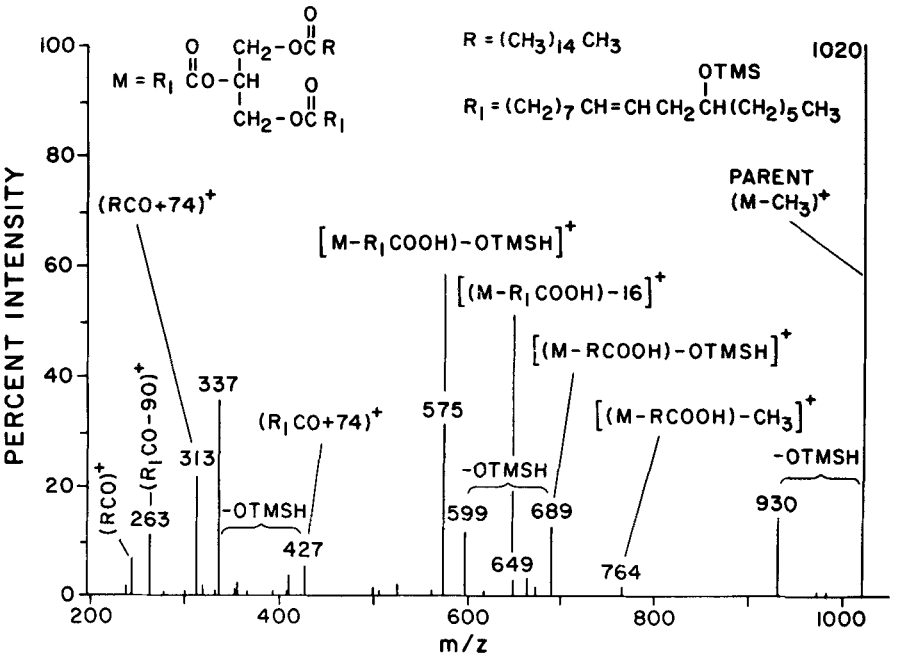
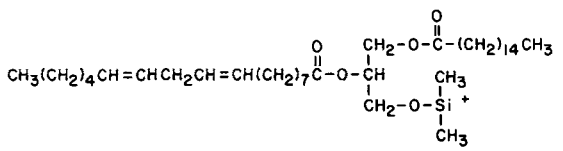


FIG. 3. CID daughter spectrum of *m/z* 1020 from castor bean neutral lipid fraction (TMS derivative). Acylglycerol structures shown do not imply regiochemical specificity.

except that #3, $(M-RCOO)^+$, is not observed for either acyl group while the ion representing the loss of a ricinoleic acid, #4, $(M-RCOOH)^+$, is present. Also, there are two ions present for each of the general structures #7, $[(M-RCOOH)-90]^+$, and #9, $(RCO + 74)^+$, one representing the 16:0 acyl group and one representing the 18:1 (OTMS) group. Two ions would not be expected for the fragment of the general formula #4, $(M-RCOOH)^+$, because this ion is formed from the loss of the acyl group that has previously lost (in the electron impact ionization process) a CH_3^+ from the TMS moiety. As there are no TMS methyl groups on the palmitoyl acyl moiety, ion #4

is neither expected nor observed. However, ion #5, $[(M-CH_3)-RCOOH]^+$, is observed for the loss of the palmitic acid moiety, as expected. No ion was observed for the loss of the ricinoleic acid moiety that had not previously lost a TMS CH_3^+ radical. In the case of structure #4, only the ion resulting from a loss of a ricinoleic acid is present. The other ion possible, resulting from a loss of palmitic acid, would produce a fragment containing two TMS ricinoleoyl groups, one containing a TMS group minus a CH_3^+ radical. It is possible that the loss of either one or two silylated groups from this fragment is such a favorable process that only fragments due to these losses are observed.

As stated above, with fragment #5, only the ion resulting from the loss of the palmitic acid is observed. However, there is an ion observed 1 mass unit lower than expected at *m/z* 649 that appears to be due to the loss of a ricinoleic acid $+CH_3$, as well as an additional H, indicated in Table 1 as #6, $[(M-RCOOH)-16]^+$. This ion could result from the loss of OTMSH to form an 18:2 acyl moiety (the position of the double bonds are not confirmed) and an intramolecular transfer of a dimethylsilyl moiety within the acyl chain that has lost a TMS methyl to produce the ion shown below as Scheme 1.



SCHEME 1

As the chain is sufficiently long and flexible, the positively charged Si could be attacked by the nucleophilic oxygen, resulting in the transfer and subsequent loss of the corresponding neutral fragment (9).

Table 1 also lists the remaining triacylglycerols identified by studying their respective CID daughter spectra, and also shows the masses and intensities for each of the proposed diagnostic fragments. The spectra of TAGs containing an acyl group without an OTMS function all have an ion present represented by structure #4, $(M-RCOOH)^+$, and #6, $[(M-RCOOH)-16]^+$, but they do not have the ion #3, $(M-RCOO)^+$. For TAGs with OTMS functions on all acyl groups, fragment #3 is observed (for each acyl group present) while fragment #4 and #6 are not. The ion at *m/z* 1035 (shown in Fig. 1) does not have a similar daughter spectrum to the above TAGs, indicating that it has a much different structure. Work is continuing on the characterization of this component.

As a check on the reliability of this method, each LC fraction was transmethylated and the fatty acid methyl ester content determined by GC and GC/MS. In all cases the fatty acid content of the LC fractions was consistent with the MS/MS data obtained.

We have shown that MS/MS of the TMS derivatives of castor bean neutral lipids can overcome the problems associated with the characterization of these compounds. The advantage of this method over transmethylation experiments is that much more specific structural information can be obtained. Daughter spectra of triacylglycerols contain a number of fragment ions similar to those observed in the EI spectra of TAGs that allow easy determination of the acyl groups present.

Although MS/MS analyses can be done on crude lipid extracts, for detailed characterizations, partial separation by reverse-phase HPLC is advantageous. This method prevents any isotopic interference in the daughter spectra of components varying in structure by only a double bond. From our experience, we feel MS/MS will undoubtedly be useful for the analyses of neutral lipids in many other plants.

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