Lipid Methodology - Chromatography and Beyond. Part I. GC/MS and LC/MS of Glycerolipids

A. KUKSIS, J.J. MYHER and L. MARAI, Banting and Best Department of Medical Research, University of Toronto, Toronto, Ontario, Canada

ABSTRACT

This paper reviews recent examples of the application of combined high temperature gas-liquid chromatography (GLC) and reversed phase high pressure liquid chromatography (HPLC) with electron impact and chemical ionization mass spectrometry for structural studies of natural diacyl and triacylglycerols. It was concluded that the combination of reversed phase HPLC with direct liquid inlet chemical ionization mass spectrometry provides the most complete resolution and most reliable identification of natural acylglycerols, far exceeding the capabilities of either technique alone. The LC/MS method is suitable for quantitative analysis following appropriate calibration of the total or fragment ion response.

INTRODUCTION

Natural triacylglycerols and glycerophospholipids are made up of complex mixtures of fatty acids, and the experimental determination of their detailed structure remains the most formidable problem in lipid chemistry, continuing to hamper the understanding of glycerolipid metabolism and its role in cell structure and function. High temperature GLC of triacylglycerols (1) and of the diacylglycerol moieties of glycerophospholipids (2) provides a good indication of the molecular association of the acyl groups in the acylglycerol molecules, but gives little indication of the positional distribution of the fatty acids. In contrast, the stereospecific analysis of Brockerhoff (3) and of Lands et al. (4) allows a differentiation between the sn-1- and sn-3-positions to compliment the composition of the sn-2-position (5,6), making a structural analysis of the triacylglycerol molecule possible. Likewise, the hydrolysis of natural glycerophospholipids with phospholipase A_2 allows a differentiation between the acyl groups in the sn-1- and sn-2-positions, but the molecular pairing of the fatty acids remains uncertain (7). A determination of the complete structure of the acylglycerol molecules requires knowledge of both positional distribution and molecular association of the fatty acids. Hammond (8) has proposed a hypothetical scheme for obtaining this knowledge based on prefractionation of the acylglycerol molecules to effect a complete chromatographic resolution, which is then followed by stereospecific analysis of individual molecular species or small groups of species. This method of analysis is too complex for a practical assessment of the structure of even very simple mixtures of triacylglycerols.

In the following we wish to review the present limits of chromatographic resolution of glycerolipids and to call attention to progress made in extending these limits by combining chromatography with other, complementary analytical methods. In Part I of this series we consider the use of combinations of GLC and HPLC with mass spectrometry. In Part II we point out the advantages of the further combination of chromatography and mass spectrometry with specific enzymic hydrolyses. This series of reviews concludes with Part III, which discusses the use of all the above techniques together to distinguish between natural and deuterium labelled molecules as a means of gaining new insights into the metabolism of glycerolipids and its role in the maintenance of the structure and function of cell membranes.

Structural Analysis of Triacylglycerols

Ryhage and Stenhagen (9) and Barber et al. (10) have shown that good mass spectra of triacylglycerols can be obtained by introducing the sample directly into the ion source. Molecular ions, $[M]^+$, and ions due to the loss of 18 atomic mass units (amu) from $[M]^+$, $[M\text{-}18]^+$, are observed along with other major peaks. Hites (11) has shown that these ions can be used to obtain the molecular weight distribution of triacylglycerols in a mixture from its mass spectrum. There are no intense peaks within at least 200 amu of the molecular ion to act as interference. Because molecular weights give only the number of carbon atoms and double bonds in the triacylglycerol, it is not possible to distinguish between positional isomers PSO, POS and OPS or between double bond isomers, such as OOS and SSL, where P, O, S and L represent palmitic, oleic, stearic and linoleic acids, respectively. This limitation reduces the number of molecular species that can be recognized. The problem can be overcome by an appropriate preliminary chromatographic resolution of the triacylglycerol mixture. The method, however, cannot distinguish between enantiomeric triacylglycerols, such as OPS and SPO.

The mass spectra of triacylglycerols contain other characteristic peaks, the origin of which can be readily recognized. One of these major peaks results from the loss of an acyloxy group from the parent molecular ion, and in the case of mixed acid triacylglycerols, peaks corresponding to the loss of each acyloxy group are obtained (9,10). Lauer et al. (12) have recognized an additional type of ion $(RCO + 115)^+$ and a homologous series of ions $(RCO + 128 + 14\,n)^+$ of which $(RCO + 128)^+$ is the simplest member. This series of ions provides evidence of the location of unsaturation or substitution in the mass spectra of the triacylglycerols and is best observed for pure triacylglycerols or for small groups of them, such as those obtained by chromatographic prefractionation.

High temperature GLC provides a separation of natural triacylglycerols according to carbon number but does not readily distinguish between saturated and unsaturated species within a carbon number, and thus cannot identify very many molecular species. Therefore, mass spectrometry in combination with high temperature GLC provides an excellent means of increasing the resolution of natural triacylglycerols beyond the limit obtained by either technique alone. Murata and Takahashi (13) have demonstrated the general suitability of this system for the identification of triacylglycerols with carbon numbers 28-54 in coconut oil and of triacylglycerols with carbon numbers 46-54 in various animal fats. The double bond distribution of the fatty acids combined with the glycerol was obtained from the molecular weight distribution. The compositions of isologs (16:0 18:1 20:1 vs. 18:0 18:1 18:1 vs. 18:0 18:0 18:2) and of positional isomers (16:0 18:0 14:0 vs. 18:0 16:0 14:0 vs. 18:0 14:0 16:0) were not determined. Nevertheless, the combined GC/MS method allowed the identification and quantitation of 3 to 8 different triacylglycerol species within each carbon number.

The GC/MS method, however, is not satisfactory for several reasons. In case of triacylglycerols of carbon number larger than 54 (e.g. 56, 58, 60 and 62) the method does not provide a sufficient number of molecular ions for sensitive detection. In order to identify the higher molecular weight triacylglycerols, Murata (14) employed GLC in combination with ammonia chemical ionization mass spectrometry.

The characteristic ions were retrieved by a computer from the total ion spectra to provide appropriate mass chromatograms of both low and high molecular weight species. In the ammonia chemical ionization mass spectra of C_{58}-C_{62} triacylglycerols the $[MH\text{-}RCO_2H]^+$ ion is base peak. This ion was used to determine the fatty acid composition of triacylglycerol groups ranging from 50 to 62 acyl carbon atoms and to determine the distribution of the triacylglycerol groups and the triacylglycerol types in peanut oil, rapeseed oil and in mustard seed oil. The quasi-molecular ion $[M+NH_4]^+$ is intense enough to be used for structural studies. Using this ion, 4 to 8 different triacylglycerol groups could be recognized for each GLC peak. Therefore, GLC in combination with ammonia chemical ionization mass spectrometry can provide more information than GLC with electron impact mass spectrometry. By monitoring the quasi-molecular ion it was possible to demonstrate that the retention times of triacylglycerols within the same carbon number depended on the degree of unsaturation of the C_{18} fatty acids. The range of retention times for species within any given carbon number was found to increase with the number of C_{18} acyl chains per molecule. Thus, the range for C_{54} triacylglycerols, which have three C_{18} acyl chains per molecule, was wider compared to C_{52}, C_{50} and C_{48} triacylglycerols, which have fewer C_{18} acyl chains per molecule.

In the GLC runs recorded on the short packed columns, the mass spectrometer may be used to recognize the presence of odd carbon number species, which are normally obscured by adjacent even carbon number triacylglycerols (14). This is not a problem in capillary GLC of triacylglycerols even when relatively short capillary columns are used (15). However, there have been no reports of the combination of capillary GLC with either electron impact or chemical ionization mass spectrometry for triacylglycerol analysis.

The combination of GLC of triacylglycerols with chemical ionization mass spectrometry did not provide more information about the positional isomer and isolog composition than did the combination of GLC with electron impact mass spectrometry. Clearly, there remains a need for further chromatographic resolution, and this need has been met by reversed phase HPLC, which can be combined with chemical ionization mass spectrometry via direct liquid inlet interfaces.

Reversed phase HPLC is now well established as an effective means of separation of natural triacylglycerol mixtures (16-18), including several "inseparable" pairs or multiplets of molecular species (19-21). However, there remain numerous species which overlap completely or partially and require a recovery of the peaks for subsequent identification by complementary analytical techniques. We have combined the HPLC separation of natural triacylglycerols with direct liquid inlet chemical ionization mass spectrometry, and have found that the mass spectrometer can serve as a sensitive universal detector of all triacylglycerol species, in addition to providing structural information for identification purposes (22,23).

Figure 1 shows the LC/MS profile of natural peanut oil triacylglycerols. The peaks have been identified by recording (every 7 seconds over the entire elution profile) of full mass spectra from which the characteristic ion intensities are subsequently extracted by mass chromatography. Figure 2 gives the full LC/MS spectra of two major peaks of the elution profile. The upper spectrum (Peak 5, Figure 1) represents 16:0 18:1 18:2 as indicated by the $[MH\text{-}RCOOH]^+$ ions at m/z 575 (16:0 18:2), 577 (16:0 18:1) and 601 (18:1 18:2) and the $[MH]^+$ parent ion at m/z 857. The $[MH]^+$ ion at m/z 883 is due to the presence of 18:2 18:1 18:1 species, as also indicated by the $[MH\text{-}RCOOH]^+$ ions at m/z 601 (18:1 18:2) and 603 (18:1 18:1). The 18:2 18:1 18:1 triacylglycerol is the major component of Peak 4 (Figure 1), and it is not completely resolved from Peak 5. The lower spectrum (Peak 8, Figure 1) represents the 16:0 18:1 18:1 triacylglycerol as indicated by the $[MH\text{-}RCOOH]^+$ ions at m/z 577 (16:0

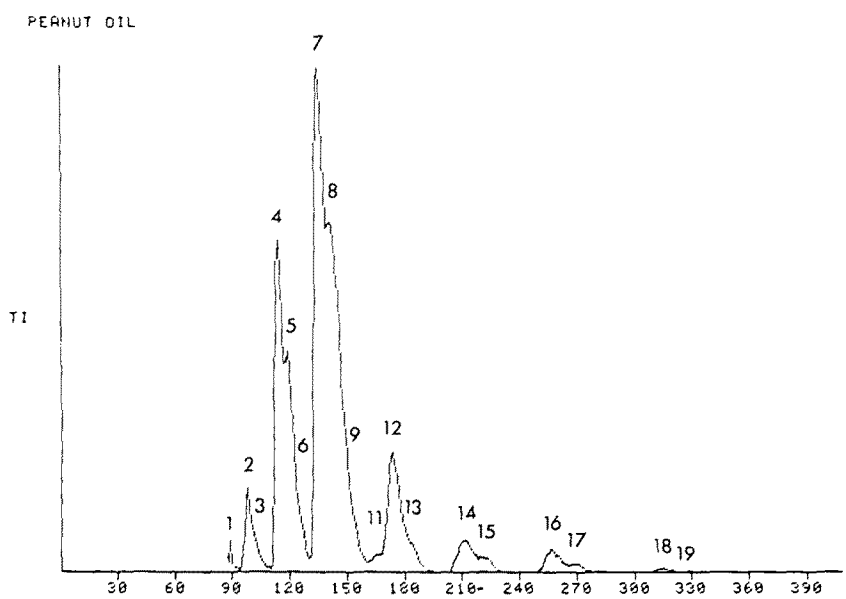

FIG. 1. Triacylglycerol profile of peanut oil as obtained by reversed phase HPLC with direct liquid inlet mass spectrometry. Peak identity: 2, 18:2 18:2 18:1; 4, 18:1 18:1 18:2; 5, 16:0 18:1 18:2; 7, 18:1 18:1 18:1; 8, 18:1 18:1 16:0; 12, 18:0 18:1 18:1; 14, 18:1 18:1 20:0; 15, 16:0 18:1 20:0; 16, 18:1 18:1 22:0. LC/MS conditions: Supelcosil LC-18 column (250 × 4.6 mm); mobile phase, acetone-acetonitrile (63.6: 36.4, isocratic); instrument, Hewlett-Packard Model 1084 Liquid Chromatograph interfaced via a direct liquid inlet to Hewlett-Packard Model 5985B quadrupole mass spectrometer. Ordinate, total chemical ionization current intensity with major peak as 100%; abscissa, mass spectrum number (scan number).

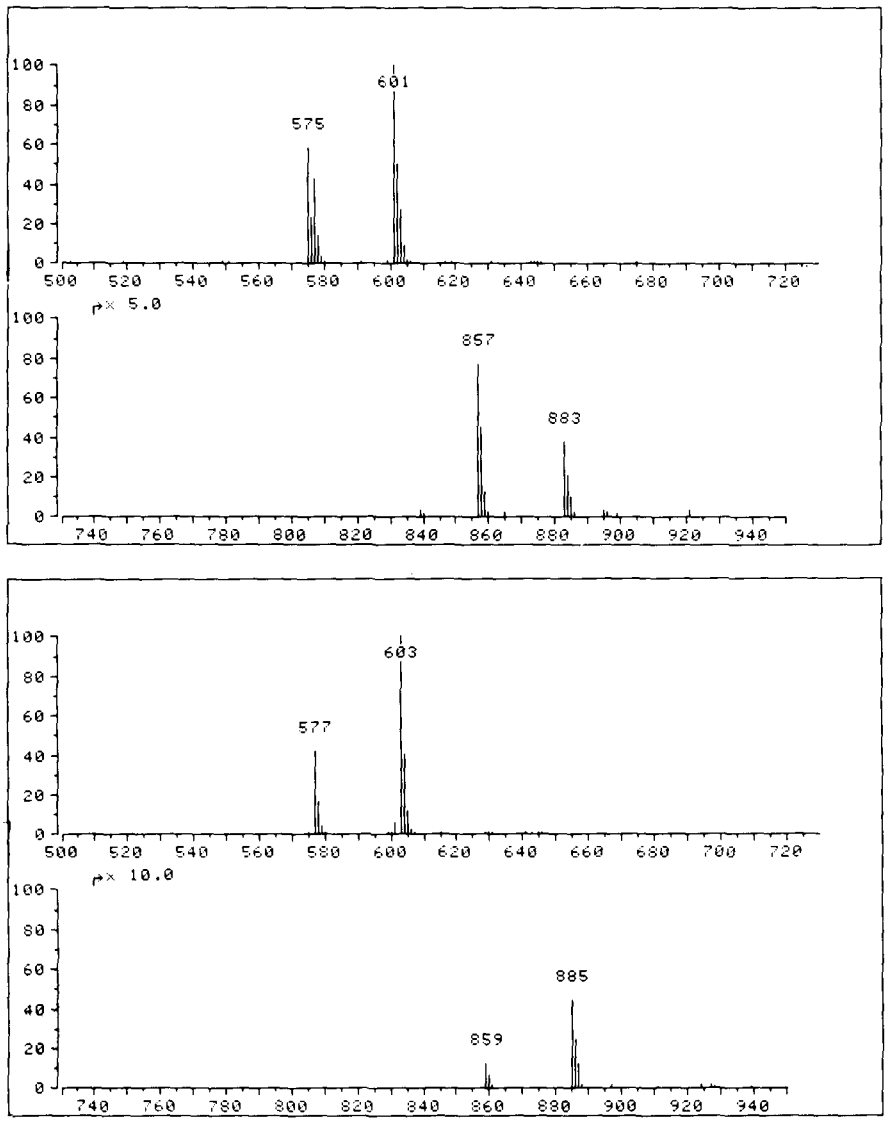

FIG. 2. LC/MS spectra of two major peaks in the peanut oil triacylglycerol profile (Peaks 5 and 7, Figure 1). Upper spectrum, 16:0 18:1 18:2 plus 18:2 18:1 18:1; Lower spectrum, 18:1 18:1 16:0 plus 18:1 18:1 18:1. LC/MS conditions as given in Figure 1.

18:1) and 603 (18:1 18:1) and the m/z 859. This spectrum also shows contamination from 18:1 18:1 18:1 (Peak 7), as indicated by the $[MH]^+$ ion at m/z 885 and the $[MH\text{-}RCOOH]^+$ ion at m/z 603 (18:1 18:1).

The other triacylglycerol peaks gave comparable chemical ionization spectra. The overall LC/MS elution profile of the peanut oil triacylglycerols shown in Figure 1 is similar to that reported by Bezard and Ouedraogo (18). These workers, however, did not resolve the critical pairs of the triacylglycerols and could identify unequivocally only some of the triacylglycerols species, which had unique fatty acid composition. We have determined all the major peanut oil triacylglycerols and have confirmed the identities advanced by Bezard and Ouedraogo (18) on the basis of fatty acid composition of the isolated peaks, as well as others.

Using the LC/MS system with acetone-acetonitrile 63.6:36.4 as solvent, we have also identified the major and minor molecular species of corn oil triacylglycerols. The LC/MS elution pattern of the corn oil triacylglycerols was identical to that obtained by El Hamdy and Perkins (21), who employed a similar reversed phase column and eluting solvent system along with a refractive index detector. The major peak identities, as established by El Hamdy and Perkins (21) on the basis of retention times, are identical to those derived here by chemical ionization mass spectrometry. Exceptions, however, are provided by some minor triacylglycerols: 18:0 18:1 18:2, which was actually 18:1 18:1 18:1; and 18:0 16:0 18:2, which was shown by mass spectrometry to be largely 16:0 18:1 18:1, with minor amounts of 18:0 18:1 18:2, as shown in Figure 3. The upper spectrum shows a prominent $[MH\text{-}RCOOH]^+$ ion at m/z 603 and small $[MH]^+$ ion at m/z 885. There are no other significant ions. The lower spectrum represents mainly 16:0 18:1 18:1 as indicated by the $[MH\text{-}RCOOH]^+$ ions at m/z 577 (16:0 18:1) and at m/z 603 (18:1 of 18:1), and an $[MH]^+$ ion at m/z 859. The presence of smaller amounts of 18:0 18:1 18:2 is indicated by the lower intensities for the $[MH\text{-}RCOOH]^+$ ions at m/z 601 (18:1 18:2), m/z 605 (18:0 18:1) and at m/z 603 (18:1 18:1), as well as of the $[MH]^+$ ion at m/z 885. The last peak in this corn oil triacylglycerol elution pattern was identified as 16:0 16:0 18:1, as evidenced from the $[MH\text{-}RCOOH]^+$ ions at m/z 551 (loss of 18:1) and m/z 577 (loss of 16:0), as well as a minor ion for $[MH]^+$ at m/z 833. In addition, a minor peak emerging between 16:0 18:1 18:2 and 18:1 18:1 18:1 was identified as 16:0 16:0 18:2. Since the fatty

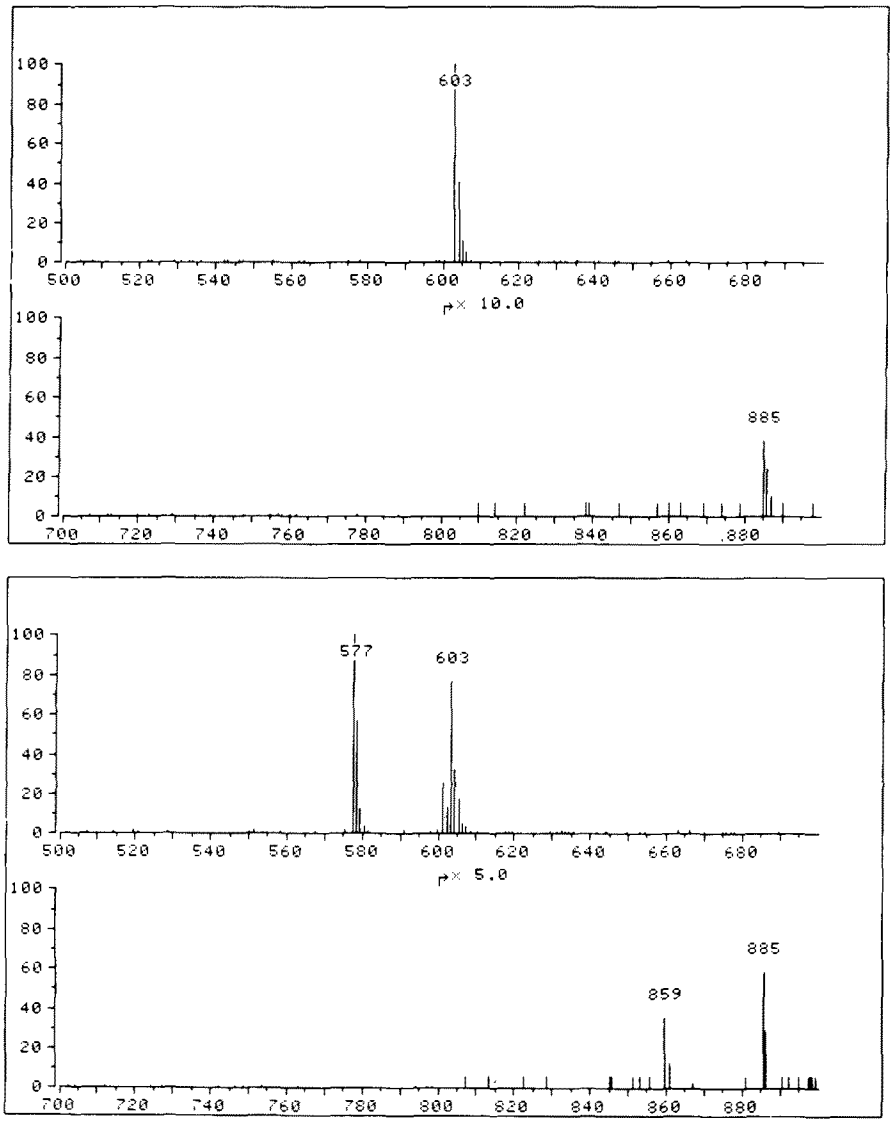

FIG. 3. LC/MS spectra of two minor peaks in the HPLC elution profile of corn oil triacylglycerols. Upper spectrum, 18:1 18:1 18:1 (Peak 7); lower spectrum, 16:0 18:1 18:1 with smaller amounts of 18:0 18:1 18:2 (Peak 8). The total ion current profile of corn oil triacylglycerols is given in Figure 4 as part of the computer plot. LC/MS conditions as given in Figure 1.

acid composition of the corn oil varies from sample to sample, the proportions of the triacylglycerol species in the various HPLC peaks also may vary. Hence, identifications based on relative retention times or other retention values must be confirmed by mass spectrometry or by peak isolation and determination of the fatty acid composition. The major peaks, however, can be identified with considerable certainty, when the fatty acid composition of the total sample is known. Figure 4 shows an LC/MS/COM plot of the composition of corn oil as obtained by displaying the mass distribution for the triacylglycerol parent ions and for the ions of the corresponding diacylglycerols in the HPLC profile. The overall distribution of the masses provides a characteristic profile, which can be compared to that of other oils in three-dimensional LC/MS. Figure 4 also demonstrates a fall off in the high mass $[MH]^+$ ions (m/z >700) relative to the $[M\text{-}RCOOH]^+$ ions (m/z 500-700) with increasing saturation (increasing scan number) of the eluted triacylglycerols.

We have since observed that a gradient of 30-90% propionitrile in acetonitrile gives a comparable resolution of the "inseparable" pairs or multiplets of triacylglycerols and comparable chemical ionization spectra (24). Propionitrile and acetonitrile mixtures previously have been used by Herslof and Pelura (25) for HPLC of triacylglycerols, while Schulte (19) used pure propionitrile for this purpose. Since the propionitrile is a better solvent than acetonitrile-acetone or tetrahydrofuran for saturated triacylglycerols, it gives higher recoveries of the latter species. Phillips et al. (26) recently have reported excellent resolutions of "inseparable" pairs of synthetic triacylglycerols by HPLC using reversed phase columns and a gradient of methylene chloride in acetone. The suitability of the latter solvent system for the direct liquid inlet LC/MS has not yet been investigated.

Using propionitrile and acetonitrile as elution solvents and reagent gases in the LC/MS system, the yields of both quasi-molecular and fragment ions were found to vary with the molecular weight, degree of unsaturation and positional distribution of the fatty acids in the triacylglycerol molecule, and appropriate calibration factors were necessary for accurate quantitation. We have determined preliminary

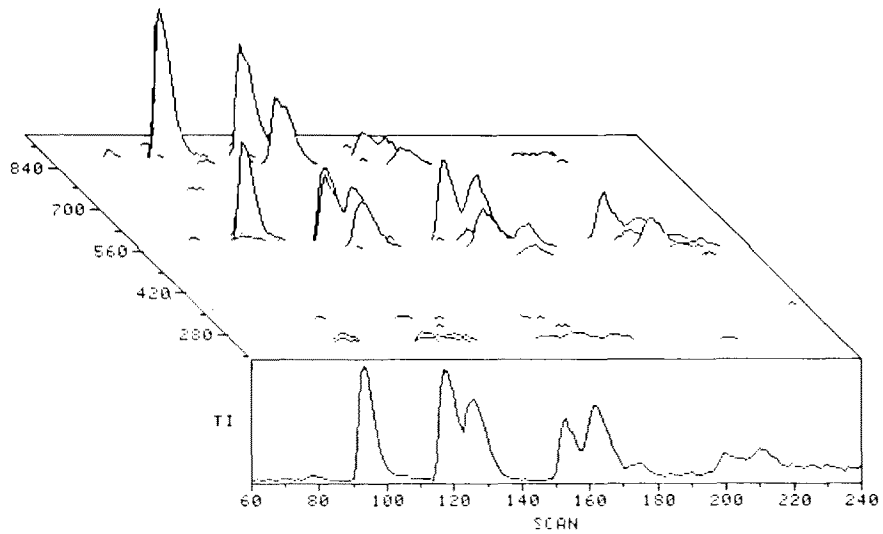

FIG. 4. Three-dimensional computer plot of the LC/MS data as obtained for natural corn oil triacylglycerols in Figure 4. LC/MS conditions as given in Figure 3.

calibration factors for total ion and specific ion current response by comparing the peak area ratios obtained by LC/MS with the proportions of the molecular species known to be present in randomized oils and in natural oils of known chemical composition (27,28). Although these factors include both chromatographic and mass spectrometric effects and are obtained with a gradient of reagent gases, they appear to be generally applicable. It was shown that positional isomers affected the yield of the $[MH\text{-}RCOOH]^+$ ions over a 1-3 fold range of intensities, while the nature of the fatty acid affected it over a range of 1-2 fold. After appropriate calibration of the relative ion response, it was possible to determine the identity and quantity of the individual molecular species in natural fats and oils with good precision. Table I compares the LC/MS estimates for the composition of the triacylglycerol species determined for randomized corn oil with that derived by calculation assuming a completely non-specific association and positional distribution of fatty acids in the alkali rearranged oil sample.

TABLE I

Total Ion Response for Triacylglycerols in Randomized Corn Oil

Molecular species	Ion sum[a] (area %)	Calculated (mol-%)[b]	Correction factor[c]
18:2 18:2 18:3	0.4	–	–
18:2 18:2 18:2	16.9	18.7	1.3
18:1 18:2 18:2	23.4	24.8	1.2
16:0 18:2 18:2	13.2	13.5	1.2
18:1 18:1 18:2 / 18:0 18:2 18:2	10.8	13.0	1.4
16:0 18:1 18:2	16.8	11.9	0.8
16:0 16:0 18:2	5.0	3.2	0.8
18:1 18:1 18:1	1.6	1.7	1.2
18:0 18:1 18:2 / 16:0 18:1 18:1	5.5	4.6	1.0
16:0 18:0 18:2 / 16:0 16:0 18:1	3.6	2.2	0.7
16:0 16:0 16:0	0.5	0.3	0.7
18:0 18:1 18:1	0.9	0.5	0.7
16:0 18:0 18:1	1.1	0.6	0.6

[a]Ion sum from m/z 500 to 1000.

[b]Calculated by the 1-random-2-random-3-random procedure.

[c]Correction factor = calculated value/ion sum and normalized to 1.3 for 18:2 18:2 18:2 (28).

Structural Analyses of Diacylglycerols

The early methods of complete determination of the molecular species of diacylglycerols were based on combined application of argentation TLC, high temperature GLC and positional enzymic analysis. The usefulness of mass spectrometry for a rapid analysis of minute amounts of molecular species of diacylglycerols was demonstrated by Barber et al. (29) and Casparini et al. (30), but practical applications of this method remained few because of the unavailability of the equipment. The first methods of GC/MS analysis of diacylglycerols used the TMS ethers (30), which are unstable and cannot be purified before analysis, or acetates (31), which do not yield sufficiently characteristic spectra for positive identification. We (32) along with others (33,34) have demonstrated that the t-BDMS ethers of diacylglycerols possess many of the mass spectrometric properties of the TMS ethers and yield a $[M\text{-}57]^+$ fragment, which can be utilized for accurate measurement of molecular weight. The t-BDMS ethers are stable to moisture and can be isolated from the reaction mixture, purified by TLC prior to analysis, and subjected to a meaningful further enzymic positional analysis, if necessary. We have shown (32) that the reverse isomers of the saturated diacylglycerols, when analyzed as the t-BDMS ethers by electron impact mass spectrometry, yield different proportions of the ions due to loss of the acyloxy radical $[M\text{-}RCOO]^+$ from positions 1 (or 3) and position 2, indicating the relative amounts of the reverse isomers, e.g. sn-1-palmitoyl 2-stearoyl and sn-1-stearoyl 2-palmitoyl-glycerols. For these isomers the ratio of m/z 427 (loss of stearoyl) to that of m/z 455 (loss of palmitoyl group) was 2.0 and 0.6, respectively. Comparable differences between the mass spectra of the reverse isomers are seen also for some mono- and di-unsaturated species. The above method of determination of reverse isomers by GC/MS cannot be applied to the polyunsaturated species, because the relative intensity of the [M-acyloxy] fragment falls off sharply with increasing unsaturation. This problem can be overcome, however, by examining the appropriate molecular species of the diacylglycerols following hydrogenation, provided the fatty acids in the sn-1- and sn-2-positions differ in their chain length. We have pointed out (35), that a reduction of the double bonds with deuterium gas in the presence of a selective catalyst would allow the subsequent identification of the fatty acids in the primary and secondary positions on the basis of chain length and the content of the deuterium

atoms. This method now has been successfully applied to a practical quantitation of the diacylglycerol moieties of natural glycerophospholipids by Dickens et al. (36).

Since the relative intensities of the characteristic ions are much higher in the chemical ionization spectra than in the electron impact ionization spectra, we have sought the possibility of distinguishing between the reverse isomers of all types of diacylglycerols by combining GLC or HPLC with chemical ionization mass spectrometry. Figure 5 shows the GC/MS spectra of the t-BDMS ethers of 16:0 18:2 and 16:0 22:6 species in the hydrogen ionization mode. The fatty acids appear to be released at markedly different rates from the sn-1- and the sn-2-positions, but analyses of the reverse isomers would be necessary to rule out the effect of unsaturation. It would appear that the milder method of ionization allows a more effective exploitation of the limited differences in the relative ionization of the two ester bonds.

The X-1,3-isomers of diacylglycerols are readily separated from the X-1,2-diacylglycerols. The X-1,3-isomers also yield the M-acyloxymethylene fragments which are absent in the X-1,2-isomers (32).

The free sn-1,2-, and sn-2,3-diacylglycerols are readily resolved by HPLC on reversed phase columns to give molecular species of uniform carbon number and degree of unsaturation (27). It is convenient, however, to convert the free diacylglycerols into the t-BDMS ethers, which are more stable under the HPLC conditions and which also yield excellent separations on the reversed phase systems. Recently, Nakagawa and Horrocks (37) have reported excellent HPLC resolution of diacyl-, alkenylacyl- and alkylacylglycerols as the acetates, while Myher and Kuksis (38) have reported effective GLC resolution of the TMS and t-BDMS ethers of alkenylacyl- and diacylglycerols on polar capillary columns. Figure 6 shows an LC/MS elution pattern obtained for the sn-1,2-diacylglycerol moieties derived from egg yolk phosphatidylcholines using a reversed phase column and a 30-90% gradient of propionitrile in acetonitrile (39), along with the complete mass spectrum of one of the major peaks (Peak 11). The composition of the individual peaks is derived from the full chemical ionization spectra recorded at 7 sec intervals throughout the elution curve. Thus, the mass spectrum of Peak 11 indicates that it contains mainly the t-BDMS ether of sn-1-palmitoyl-2-linoleoylglycerol. The spectrum shows a significant protonated molecular ion (m/z 707) as well as a fragment representing the intact diacylglycerol (m/z 575). The major intensities are due to a loss of the palmitoyl (m/z 451) and of linoleoyl (m/z 427) radicals from the parent molecule. However, both acids are not lost at the same rate. We have shown (39) that the relative yields of the various ion fragments vary from species to species, depending on the degree of unsaturation and the positional distribution of the fatty acids. Thus, the relative increase in the abundance of the $[MH]^+$ ion with unsaturation indicates that the presence of a polyunsaturated acyl chain tends to stabilize this ion. Furthermore, in all species the fatty acid in the sn-2-position is lost to a greater extent (3-4 fold) than the acid in the sn-1-position, which suggests that the reverse isomers may yield different spectra in the LC/MS system. Thus, while the 18:1 and 18:2 acids are released at comparable rates from the sn-2-position, the 18:2 acid is lost 3-4 times more readily than the 18:1 acid, when the latter is in the sn-1-position, as in sn-glycerol-1-oleate-2-linoleate. It may therefore be possible to determine the ratios of the reverse isomers in both sn-1,2- and sn-2,3-diacylglycerols, when the enantiomers are analyzed separately, provided appropriate calibrations with standard diacylglycerols have been made.

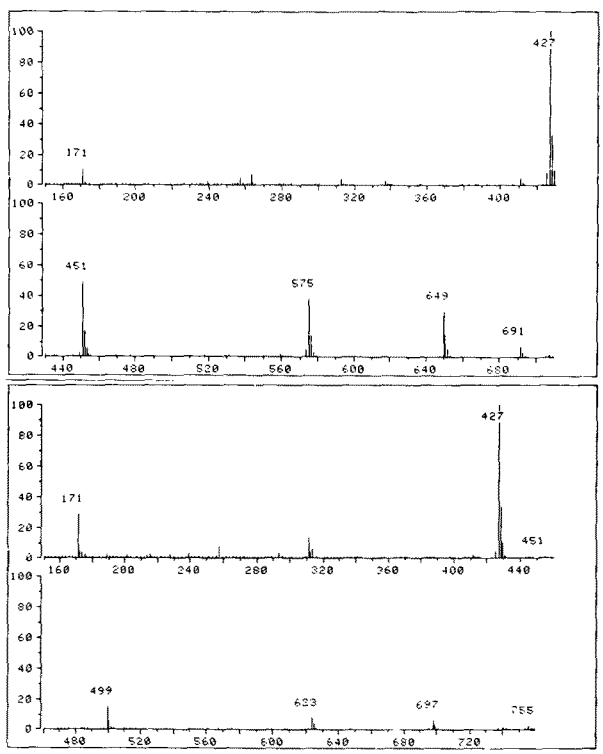

FIG. 5. GC/MS chemical ionization spectra of the t-BDMS ether of sn-1-palmitoyl-2-linoleoylglycerol (upper spectrum) and of sn-1-palmitoyl-2-docosahexaenoylglycerol (lower spectrum). GC/MS conditions as described elsewhere (32), except that the mass spectra were recorded with hydrogen as the ionization gas and with the instrument in the chemical ionization mode.

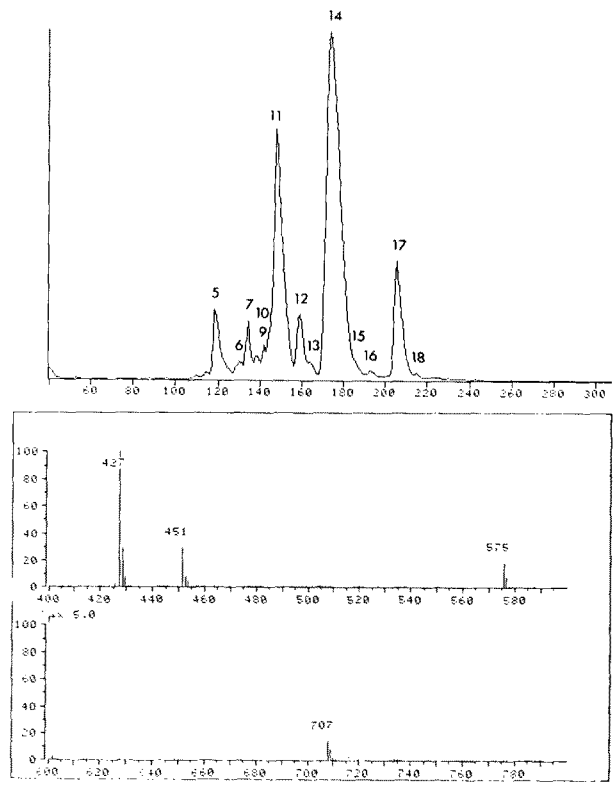

FIG. 6. LC/MS elution profile of the t-BDMS ether of the sn-1-2-diacylglycerol moieties of egg yolk phosphatidylcholines, along with the mass spectrum of one of the major peaks (Peak 11). Major peak identity: 5, 16:1 18:2; 7, 16:0 20:4; 11, 16:0 18:2; 12, 18:0 20:4; 14, 16:0 18:1; 18:0 18:2; 18:1 18:1; 17, 18:0 18:1. LC/MS conditions as given elsewhere (39).

This approach would avoid the hydrogenation or deuteration of the polyunsaturated species needed to insure adequate yields of the M-acyloxy ions in the electron impact mass spectrometry.

Examination of the mass spectra of each HPLC peak in Figure 6 indicates that most peaks contain single components and that only a few are made up of two or more molecular species in significant amounts. In order to obtain an LC/MS estimate of the concentration of the unresolved components within each HPLC peak, it is necessary to quantitate the intensities of the characteristic ions. Generally, the intensity of one or both $[MH\text{-}RCOOH]^+$ ions can be unambiguously assigned to a particular molecular species. When only one of a pair can be measured the other can be calculated from the known fragmentation pattern of reference compounds. The $[MH\text{-}132]^+$ ions at m/z 577 and 603 in the mass chromatograms in Figure 7 show that Peak 14 (Figure 6) is composed of 16:0 18:1 and 18:0 18:2 (and/or 18:1 18:1) species, which overlap. The relative proportions of the 16:0 18:1 and of the 18:0 18:2 plus any 18:1 18:1 species can be obtained from the relative intensities of the $[MH\text{-}132]^+$ ions, since these species yield comparable proportions of the total ion current in the form of the $[MH\text{-}132]^+$ ions. The relative content of the 18:1 18:1 species can be calculated from the relative proportions of the various $[MH\text{-}RCOOH]^+$ ions at m/z 453 (oleate retained), 451 (linoleate retained), 427 (palmitate retained) and 455 (stearate retained) for the diacylglycerol species overlapping in Peak 14. As a result, we have been able to obtain excellent resolution and quantitation of the diacylglycerol moieties of natural glycerophospholipids from a variety of sources. This technique is similarly applicable to the resolution and quantitation of the sn-2,3-diacylglycerol moieties recovered from natural mixtures of free diacylglycerols or generated by Grignard degradation during structural analyses of triacylglycerols.

Using propionitrile and acetonitrile as eluting solvents and reagent gases, the yields of both quasi-molecular and fragment ions are found to vary with the degree of unsaturation and positional distribution of the fatty acids in the diacylglycerol molecules and appropriate calibration factors are necessary for accurate quantitation. We have reported (39) preliminary calibration factors for total and specific ion current responses by comparing the peak area ratios obtained by LC/MS with the weight and mole proportions of molecular species known to be present in the samples from detailed analyses by capillary GLC on polar liquid phases. It was found that the total chemical ionization current response agreed closely with the weight composition of the molecular species. The relative yields of the $[MH\text{-}132]^+$ ions varied over a 1-3 fold, while those of the $[MH\text{-}RCOOH]^+$ ions varied over a 3-4 fold range of intensities. After appropriate calibration of the relative response, we determined the identity and quantity of the molecular species of the diacylglycerols, except the reverse isomers and enantiomers.

Structural Analyses of Monoacylglycerols

The molecular species of monoacyl (1,40) and monalkyl (40) glycerols are readily resolved by GLC on polar liquid phases to yield components of uniform molecular weight, degree of unsaturation and positional distribution. The X-1- and 2-monoacyl and monoalkylglycerols yield significantly different retention times, which can be readily established by means of appropriate reference standards. Likewise, various chromatographic systems have been developed for the resolution of the monoalkyl-2-monoalkenyl- and monoacylglycerols (40). Furthermore, all of these compounds yield characteristic mass spectra (40),

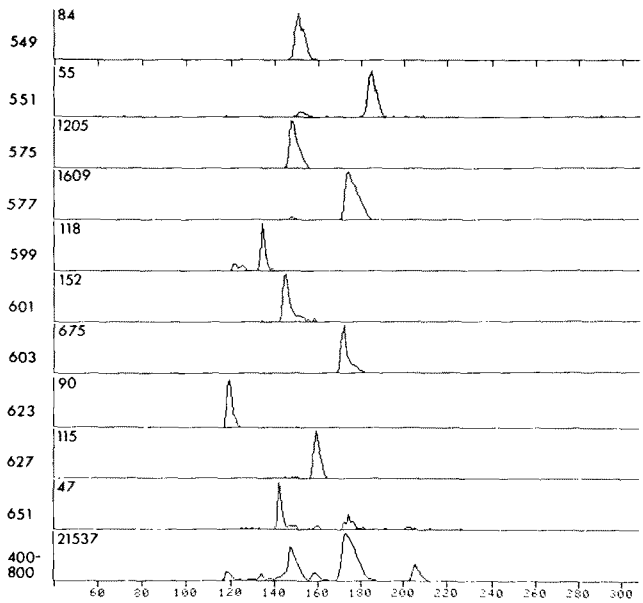

FIG. 7. Mass chromatograms of the $[MH\text{-}RCOOH]^+$ ions of the sn-1,2-diacylglycerol-3-t-BDMS ethers derived from egg yolk phosphatidylcholines as obtained by HPLC with direct liquid inlet mass spectrometry. Ordinates, fragment ion intensities for each ion indicated. Abscissa, consecutive mass spectrum numbers (scan numbers). The relative intensity values given in the upper left hand corner of the ion chromatograms refer to the major peak in each elution pattern. LC/MS conditions as given in Figure 1.

thus eliminating any uncertainty in the identification of any of these species by presently available methods.

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⁂Sterols, Methyl Sterols, Triterpene Alcohols and Fatty Acids of the Kernel Fat of Different Malagasy Mango *(Mangifera indica)* Varieties

EMILE M. GAYDOU, Laboratoire de Phytochimie and Ecole Supérieure de Chimie de Marseille, Université de Droit, d'Economie et des Sciences, Centre de Saint Jérôme, rue Henri Poincaré, 13397 Marseille Cédex 13, France and **PHILIPPE BOUCHET,** Etablissement d'Enseignement Supérieur Polytechnique, Département de Chimie, BP 1500, Antananarivo, République Démocratique de Madagascar

ABSTRACT

The kernel fat content of 16 different mango varieties collected from the Northwestern part of Madagascar island were examined. The fat content (22-54%) was determined by chloroform/methanol extraction. Investigation by gas liquid chromatography (GLC) revealed 15 fatty acids, mainly palmitic (7-12%), stearic (22-40%), oleic (41-48%) and linoleic (7-17%). Significant correlations were observed among the main fatty acids. Testing for the sterol fraction in 15 mango varieties allowed us to separate and quantitatively analyze 7 sterols by GLC. The main sterols were β-sitosterol (47-76%), stigmasterol (12-23%) and campesterol (7-12%). The stigmasterol/campesterol ratio (1.2:2.3) was lower in mango kernel fat than in cocoa butter. Among the 4-methyl sterol fractions, gramisterol, lophenol, obtusifoliol and citrostadienol were tentatively identified by GLC. Lupeol, cycloartenol, α- and β-amyrins and friedelinol were tentatively identified by GLC in the triterpene alcohols fractions.

INTRODUCTION

Mango *(Mangifera indica)* fruits are widely grown in tropical countries. The world mango production amounted to some 13 million tons in 1978 (1). On Madagascar island, more than 7 native varieties are found and 33 ameliorated varieties have been introduced (2,3). The Malagasy mango production is higher than 200,000 tons a year and in this country, after consumption of the mango fruit, the mango stones remain as waste. Studies of the fat content extracted from these mango kernels have been recently investigated (1,4-7). The composition of mango kernel fat is similar to that of cocoa butter, Borneo tallow, illipe *(Shorea stenoptera)* butter and karite or shea *(Butyrospermum parkii)* butter and could be used in the food industry as a substitute for these fats (8-10). Taking into account its low commercial value, partial substitution for cocoa butter represents an interesting possibility. In a recent study, Baliga and Shitole (8) have fractioned mango fat from acetone at low temperature in 1 or 2 stages to segregate suitable solid fractions having physical properties close to cocoa butter. Although the positional distribution of the fatty acids in the triglycerides of mango kernel fat varieties of African origin has been studied by Van Pee et al. (1,11, 12), we know nothing about the unsaponifiable matter composition of the kernel fat. Some triterpenoids of the leaves (13) and the root bark (14) of *M. indica* have been investigated. This paper presents results on several characteristics of mango kernel fats. Sterols, methyl sterols, triterpene alcohols and fatty acids of kernel fats of 16 mango varieties found in Madagascar were examined.

EXPERIMENTAL

Materials

Sixteen ripe mango fruit varieties were collected from the Mangatsika Station (Mahajanga area) in December 1981. The physical and chemical characteristics of the ripe mango fruits and kernels are given in Table I. The determinations were made from at least 6 randomly selected fruits. Moisture of mango kernels and unsaponifiable matter of kernel fats were determined according to NFT 60-201 and 60-205 Norms (15). The fat content was determined after extracting the powdered kernels in a Soxhlet apparatus with a chloroform/methanol mixture (2:1, v/v) for 8 hr.

For the tentative identification of fatty acid methyl esters, commercial saturated even-numbered methyl esters (Fluka, Buchs, Switzerland) and unsaturated and polyunsaturated methyl esters (Sigma Chemical Co., St. Louis, Missouri) were used as standards. For the tentative identification of sterols, commercial cholesterol and lanosterol (Fluka) were used as standards.

Methyl Ester Preparation

The methyl esters of the 16 mango fats were prepared by transesterification of the oils in methanol containing 1%