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QUANTITATION OF NATURAL TRIACYLGLYCEROLS BY REVERSED-PHASE LIQUID CHROMATOGRAPHY WITH DIRECT LIQUID INLET MASS SPECTROMETRY

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SUMMARY

Using acetonitrile and propionitrile as eluting solvents and reagent gases 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. In the absence of pure structural isomers and mixed acid standards, preliminary calibration factors have been determined for total ion and specific ion current responses by comparing the peak area ratios obtained by liquid chromatography–mass spectrometry with the proportions of the molecular species known to be present in randomized oils and in natural oils of known chemical composition. Although the derived 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 $(MH - RCOOH)^+$ ions over a 1–3-fold range of intensities, while the nature of the fatty acid affected it over a range of 1.25-fold. After suitable calibration of the relative ion responses it was possible to determine the identities and amounts of the individual molecular species in natural fats and oils.

INTRODUCTION

Previous work has demonstrated[1,2] that reversed-phase high-performance liquid chromatography (HPLC) with direct liquid inlet mass spectrometry (MS) is well suited for the separation and indentification of molecular species of triacylglycerols in natural fats and oils. Although the total chemical ionization current profiles of the LC–MS separations of several of these oils approximated closely the elution profiles obtained for them by means of HPLC with refractometric detection[3,4], it was obvious that for accurate quantitation the LC–MS response to different molecular species of triacylglycerols required calibration with standards.

In this study we have obtained preliminary calibration factors for various mixed acid triacylglycerols by comparing the peak area proportions obtained by LC–MS with

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the known molar proportions of the mixed acid triacylglycerols in a sample of fully characterized corn oil and in randomized peanut oil. The calibration factors for both total ion current response and specific ion current response have been applied to the quantitative analysis of several natural oils and the results have been compared with those obtained by chemical analyses and computerized data fitting.

EXPERIMENTAL

Materials

The various natural and randomized oils and fats were available in the laboratory from previous studies[5,6]. They had been stored in closed containers in the solid state at 4°C for 3–4 years. Prior to LC–MS analysis they were purified by thin-layer chromatography as described previously[7].

HPLC instrumentation and chromatographic conditions

The HPLC analyses were performed with a Hewlett-Packard Model 1084B liquid chromatograph (Hewlett-Packard, Palo Alto, CA, U.S.A.) equipped with a Supelcosil LC-18 column (Supelco, Bellefonte, PA, U.S.A.) using a gradient of 30–90% propionitrile in acetonitrile (Fluka, Hauppauge, NY, U.S.A.). The columns were operated at a flow-rate of 1.5 ml/min and an oven temperature of 30°C. For injection the triacylglycerols were dissolved in propionitrile at 100–200 mg/ml and 10–25 μl were injected on to the column. The triacylglycerol peaks were detected by chemical ionization mass spectrometry using a direct liquid inlet interface as described below.

Mass spectrometric instrumentation and operating conditions

The mass spectrometry was performed with a Hewlett-Packard Model 5985B quadrupole mass spectrometer equipped with a Hewlett-Packard direct liquid inlet interface as described by Kenyon *et al.*[8]. About 1% of the HPLC column effluent was admitted to the mass spectrometer and full mass spectra (200–1000 mass units) were recorded every 7 sec over the entire elution profile. The data were analyzed by means of a Hewlett-Packard data system (Model HP 1000E) and a graphics terminal (Model HP 2648A) as described previously[9]. The total mass spectra were corrected for background by subtracting a scan made with propionitrile–acetonitrile alone. The LC–MS response to the various molecular species was determined by constructing mass chromatograms for the various ions or groups of ions of interest by means of the data system.

RESULTS AND DISCUSSION

Reproducibility of LC – MS profiles

HPLC with reversed-phase columns and appropriately selected solvent systems provides an extensive resolution of natural triacylglycerol mixtures including many "inseparable" pairs or multiplets of molecular species. Table I shows the reproducibility of the LC–MS elution profiles of peanut and soybean oils in the propionitrile–acetonitrile gradient. The peak areas represent the total chemical ionization current. The different runs were recorded on different days but under otherwise similar working conditions. It can be seen that the relative peak areas differed by less than 5% for peak

TABLE I

REPRODUCIBILITY OF LC–MS PROFILES OF SOME NATURAL MIXTURES OF TRIACYLGLYCEROLS

Results are area-%.

Peak No.	*Peanut oil*				*Peak No.*	*Soybean oil*		
	1	*2*	*3*	*Av.**		*1*	*2*	*Av.***
1	3.6	3.2	4.8	3.9±0.8	1	3.5	4.0	3.8±0.3
2	1.2	1.2	1.4	1.3±0.1	2	15.0	14.8	14.9±0.1
3	14.0	13.5	16.6	14.7±1.7	3	2.3	3.0	2.7±0.3
4	8.3	7.4	8.9	8.2±0.8	4	13.0	12.5	12.8±0.2
5	1.4	1.1	1.0	1.2±0.2	5	15.8	16.4	16.1±0.3
6	30.4	32.0	31.8	31.4±0.9	6	9.1	9.2	9.2±0.05
7	19.3	19.0	16.6	18.3±1.5	7	16.3	15.3	15.8±0.3
8	2.9	2.0	1.9	2.3±0.6	8	4.5	3.9	4.2±0.3
9	0.9	0.9	0.7	0.8±0.1	9	4.1	4.2	4.2±0.05
10	7.7	8.7	7.6	8.0±0.6	10	13.2	12.6	12.9±0.3
11	2.1	1.8	1.4	1.8±0.4	11	1.8	2.0	1.9±0.1
12	3.1	3.4	3.0	3.2±0.2	12	1.4	1.6	1.5±0.1
13	1.2	1.2	1.0	1.1±0.1				
14	2.2	2.3	2.1	2.2±0.1				
15	0.9	1.0	0.6	0.8±0.2				
16	0.5	0.7	0.4	0.5±0.1				
17	0.3	0.4	0.1	0.3±0.1				

* Mean ± S.D.

** Average ± range/2.

areas making up more than 10% of the total peak area and by less than 10% for peak areas making up 1–10% of total peak area. For peaks constituting less than 1% of the total peak area the relative error could be much higher than 10%. Comparable relative errors were recorded for consecutive HPLC runs using the direct liquid inlet LC–MS as a detector. The nature of the molecular and fragment ions produced has been described elsewhere[1,2].

Quantitation of resolved peaks

Fig. 1 shows the total ion current profile of an LC–MS separation of natural corn oil triacylglycerols. Examination of the mass spectra of each peak indicates that most of the peaks contain single components and that only a few are made up of two or more molecular species in significant amounts. As the oil sample has been chemically analyzed[5], the positional distribution of the fatty acids is known and the exact molecular species composition can be obtained by the 1-random–2-random–3-random calculation. Table II compares the calculated molar-% composition of the corn oil triacylglycerols with the composition derived by the total chemical ionization current recorded as an area-% for each peak. Any overlapping peaks are summed for the present purposes. There appears to be excellent agreement. The minor discrepancies remaining can be corrected by means of appropriate factors, which can be calculated from the data in Table II. It can be seen that the relative correction factors range from about 1.3 for 18:2 18:1 18:2 to approximately 0.5 for 16:0 16:0 18:1 and 16:0 16:0 18:2. Clearly the response decreases with increasing overall unsaturation of the triacylglycerol molecule. Compa-

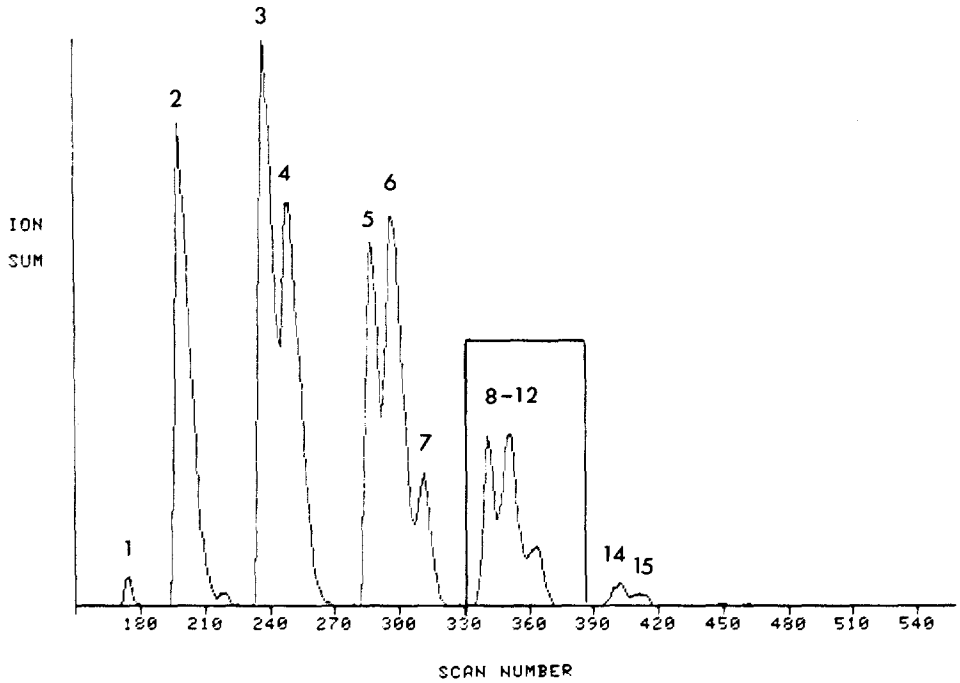

Fig. 1. Total ion tracings of an LC–MS separation of corn oil triacylglycerols. Peaks are identified as in Table II: 1, 18:2 18:2 18:3; 2, 18:2 18:2 18:2; 3, 18:1 18:2 18:2; 4, 16:0 18:2 18:2; 5, 18:1 18:1 18:2; 6, 18:0 18:2 18:2 + 16:0 16:1 18:2; 7, 16:0 16:0 18:2; 8, 18:1 18:1 18:1; 9, 18:0 18:1 18:2; 10, 16:0 18:1 18:1; 11, 16:0 18:0 18:2; 12, 16:0 16:0 18:1; 13, 16:0 16:0 16:0; 14, 18:0 18:1 18:1; 15, 16:0 18:0 18:1. LC–MS conditions as given in the text.

TABLE II

TOTAL ION RESPONSE FOR THE TRIACYLGLYCEROLS IN NATURAL CORN OIL

Peak No.	*Molecular species*	*Ion sum* (area-%)*	*Calculated** mol-%*	*Correction*** factor*
1	18:2 18:2 18:3	0.5		
2	18:2 18:2 18:2	16.9	17.8	1.3
3	18:1 18:2 18:2	19.0	24.6	1.7
4	16:0 18:2 18:2	17.3	15.5	1.2
5	18:1 18:1 18:2	10.4	11.2	1.4
6	18:0 18:2 18:2	16.4	15.3	1.2
	16:0 18:1 18:2			
7	16:0 16:0 18:2	4.3	3.0	0.9
8	18:1 18:1 18:1	4.2	1.7	0.5
9	18:0 18:1 18:2	6.7	5.0	1.0
10	16:0 18:1 18:1			
11	16:0 18:0 18:2	2.4	2.0	1.1
12	16:0 16:0 18:1			
13	16:0 16:0 16:0	0.1	0.1	1.3
14	18:0 18:1 18:1	0.8	0.6	1.0
15	16:0 18:0 18:1	0.5	0.4	1.1

* Ion sum from *m/z* 500 to 1000.

** Calculated by the 1-random–2-random–3-random procedure.

*** Correction factor = calculated value/ion sum and normalized to 1.0 for peak 9.

TABLE III

RELATIVE YIELDS OF PROTONATED MOLECULAR ION FOR SELECTED TRIACYLGLYCEROL SPECIES

Molecular species	*$MH^+/[MH^+ - RCOOH]$ abundance ratio*
16:0 16:0 16:0	Not detected
16:0 16:0 18:1	0.014
16:0 16:0 18:2	0.010
16:0 18:1 18:1	0.029
18:1 18:1 18:1	0.050
16:0 18:1 18:2	0.011
18:1 18:1 18:2	0.15
16:0 18:2 18:2	0.43
18:1 18:2 18:2	0.47
18:2 18:2 18:2	0.87

rable sets of relative response factors were obtained from the analyses of other mixtures of randomized triacylglycerols (see below).

Alternatively, the proportions of the individual triacylglycerol species in the LC–MS peaks could be quantitated on the basis of their molecular ions, although the latter make up only a small proportion of the total ion current. This method of triacylglycerol quantitation has been employed in the past for both direct probe sublimations and gas chromatography–mass spectrometry (GC–MS)[10–14]. As the relative proportions of the masses of the molecular species for the corn oil are known from calculation, it is possible to obtain appropriate calibration factors as shown above for the total ion current. Table III shows the abundance ratio of protonated molecular ion to $(MH - RCOOH)^+$ ion for a number of molecular species of triacylglycerols. It is apparent that the relative yield of MH^+ is very sensitive to the degree of unsaturation, being high for trilinoleoylglycerol and negligible for the saturated triacylglycerols. It would therefore appear that the molecular ion responses are not as well suited for triacylglycerol quantitation as the total ion current response, which varies much less. The total ion current response, however, cannot be used for quantitation of overlapping species of triacylglycerols and both molecular ions and characteristic fragment ions must be measured and calibrated for accurate determination (see below).

Quantitation of unresolved peaks

Fig. 2 illustrates the problems involved in the quantitation of unresolved or partially resolved triacylglycerol peaks. In this example, taken from the corn oil profile, five triacylglycerol species form three peaks in the LC–MS elution curve. These species can be readily identified by their $(MH - RCOOH)^+$ ions and the exact elution profile of each species traced by means of appropriate mass chromatograms. When these species differ in molecular weight, they can be identified and quantitated by calibrating the response for the parent ions. Where the overlapping peaks possess identical molecular weights, it is necessary to quantitate them by calibrating the characteristic $(MH - RCOOH)^+$ fragments.

Table IV shows that the relative yields of the $(MH - RCOOH)^+$ ions vary with

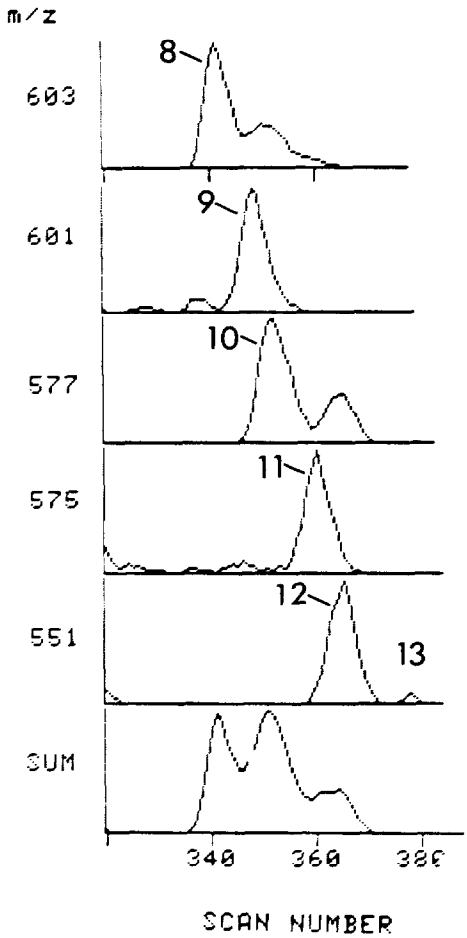

Fig. 2. $(MH - RCOO)^+$ ion profiles for peaks 8–12 in LC–MS separation of corn oil. Peaks are identified as in Table II: 8, 18:1 18:1 18:1; 9, 18:0 18:1 18:2; 10, 16:0 18:1 18:1; 11, 16:0 18:0 18:2; 12, 16:0 16:0 18:1; 13, 16:0 16:0 16:0. LC–MS conditions as given in the text.

the fatty acid composition of the triacylglycerol molecule. For the triacylglycerol types shown all the isomers are present in equal proportions. For the triacylglycerol species having two different fatty acids, the abundance of the fragment containing both acids is divided by two. Although an exact comparison is difficult, the results are consistent with the following relative order of release of the fatty acids from all positions of the triacylglycerol molecule with the above range of mixed acid triacylglycerols investigated: 16:0 > 18:2 > 18:1. From a triacylglycerol made up of all three fatty acids, the oleate from linoleate were released at about the same rate, but about 20% slower than the palmitate: 16:0 > 18:2 ≥ 18:1.

The relative yields of the $(MH - RCOOH)^+$ fragments from a natural oil, however, would also vary with the positional distribution of the fatty acids. Table V shows the effect of positional isomerism on the relative yields of the $(MH - RCOOH)^+$ from

TABLE IV

RELATIVE YIELDS OF [MH$^+$ – RCOOH] IONS FROM SELECTED TRIACYLGLYCEROL SPECIES

*Molecular species**	*Acyl pairing of [MH$^+$—RCOOH] ion*	*Relative abundance***
18:1 18:2 18:2	18:2 18:2	1.00
	18:1 18:2	0.76
16:0 18:2 18:2	18:2 18:2	1.0
	16:0 18:2	0.75
18:1 18:1 18:2	18:1 18:2	1.0
	18:1 18:1	0.86
16:0 18:1 18:2	16:0 18:2	0.84
	16:0 18:1	0.88
	18:1 18:2	1.00
16:0 16:0 18:1	16:0 16:0	0.80
	16:0 18:1	1.00

* All isomers present in equal proportions.

** For triacylglycerol species having two different fatty acids, the abundance of the fragment containing both acids is divided by 2.

a representative isomeric pair of triacylglycerols. The (MH – RCOOH)$^+$ ion that results from the loss of a fatty acid from either of the *sn*-1- or the *sn*-3-position is about four times more abundant than the equivalent ion resulting from a loss of a fatty acid from the *sn*-2-position. The yields observed for the two positional isomers are closely similar for all molecular species tested. Therefore, positional isomerism affects the ion yield much more than does the nature of the fatty acid, and an accurate quantitation of molecular species of triacylglycerols by the intensities of the (MH – RCOOH)$^+$ ions requires a knowledge of the positional isomer composition.

Composition of randomized peanut oil

The methods of quantitation of both resolved and unresolved triacylglycerols were tested by comparing the relative chemical ionization response of the triacylglycerols from randomized peanut oil with the relative molar percentages obtained by calculation from the known fatty acid composition of the oil. Fig. 3 shows the LC–MS profile of randomized peanut oil. The molecular species of the triacylglycerols making up the various peaks were identified and quantitated as described for corn oil. Table

TABLE V

EFFECT OF POSITIONAL ISOMERISM ON THE RELATIVE YIELDS OF THE [MH$^+$ – RCOOH] IONS

The yields observed for these two isomers are typical of all such isomers. The [MH$^+$ – RCOOH] ions that results from the loss of a fatty acid from either of the *sn*-1- or *sn*-3-positions is approximately four times more abundant than the equivalent ion that results from loss of a fatty acid from the *sn*-2-position (7.6:2 = 3.8).

[MH$^+$ – RCOOH] ion		*Triacylglycerol isomer*	
m/z	*Acyl pairing*	*16:0 18:1 18:1*	*18:1 16:0 18:1*
577	16:0 18:1	1.1	7.6
603	18:1 18:1	1.0	1.0

TABLE VI

TOTAL ION RESPONSE FOR THE TRIACYLGLYCEROLS IN RANDOMIZED PEANUT OIL

Peak No.	*Molecular species**	*Ion sum*** *(area-%)*	*Calculated**** *mol-%*	*Correction factor*§
1	18:2 18:2 18:2	1.3	2.2	1.9
2	18:1 18:2 18:2	8.5	11.9	1.6
3	16:0 18:2 18:2	2.5	2.8	1.2
4	18:2 18:2 20:1	0.3	0.3	
	18:1 18:1 18:2	18.6	21.7	1.3
5	18:0 18:2 18:2	0.7	0.6	
	16:0 18:1 18:2	12.4	9.9	0.9
6	16:0 16:0 18:2	2.2	1.1	0.6
7	18:1 18:2 20:1	1.2	1.1	
	18:1 18:1 18:1	14.1	13.2	1.0
8	18:0 18:1 18:2	1.3	2.3	
	16:0 20:1 18:2	0.2	0.3	
	16:0 18:1 18:1	14.4	8.9	0.7
9	16:0 18:0 18:2	0.6	0.5	
	16:0 16:0 18:1	3.8	2.0	0.6
9.5	16:0 16:0 16:0	0.4	0.2	
10	18:1 18:1 20:1	1.0	1.0	1.1
11	18:2 18:2 22:0	0.4	0.6	1.6
	18:1 18:2 20:0	0.9	1.2	1.4
	16:0 18:1 20:1	0.8	0.5	0.7
	18:0 18:1 18:1	2.3	2.1	1.0
12	16:0 16:0 20:1	0.1	0.05	
	16:0 18:0 18:1	1.7	1.0	0.7
13	18:2 18:2 24:0	0.2	0.3	
	18:1 18:2 22:0	1.5	2.2	1.7
	18:1 18:1 20:0	1.3	1.1	1.0
14	16:0 18:2 22:0	0.6	0.5	1.0
	16:0 18:1 20:0	0.8	0.5	0.7
15	18:1 18:2 24:0	0.6	0.9	1.7
	18:1 18:1 22:0	1.9	2.0	1.2
16	18:0 18:2 22:0	0.1	0.1	
	16:0 18:1 22:0	1.4	0.9	0.8
17	18:1 18:1 24:0	1.0	0.7	0.8
18	18:0 18:1 22:0	0.7	0.6	
	16:0 18:1 24:0			

* No distinction made among the *sn*-1-, *sn*-2- and *sn*-3-positions of the triacylglycerol molecule.

** Ion sum from *m/z* 500 to 1000. Overlapping species were calculated using the abundances of the appropriate $[MH^+ - RCOOH]$ ions.

*** Calculated via the 1,2,3-random procedure.

§ Correction factor = calculated value/ion sum and normalized to 1.0 for triolein.

VI gives the area percentages obtained by LC–MS together with the calculated molar proportions of the molecular species of randomized peanut oil. There is reasonably close agreement between the calculated proportions for the molecular species and those determined from the total chemical ionization current.

Further, the proportions of the unresolved peaks appear to have been correctly calculated from the $(MH - RCOOH)^+$ fragment ions. This is due to the fact that in the randomized oil the "diacylglycerol" fragments of interest are present in equal amounts, and can be calculated by appropriate apportioning of the ion response. Nev-

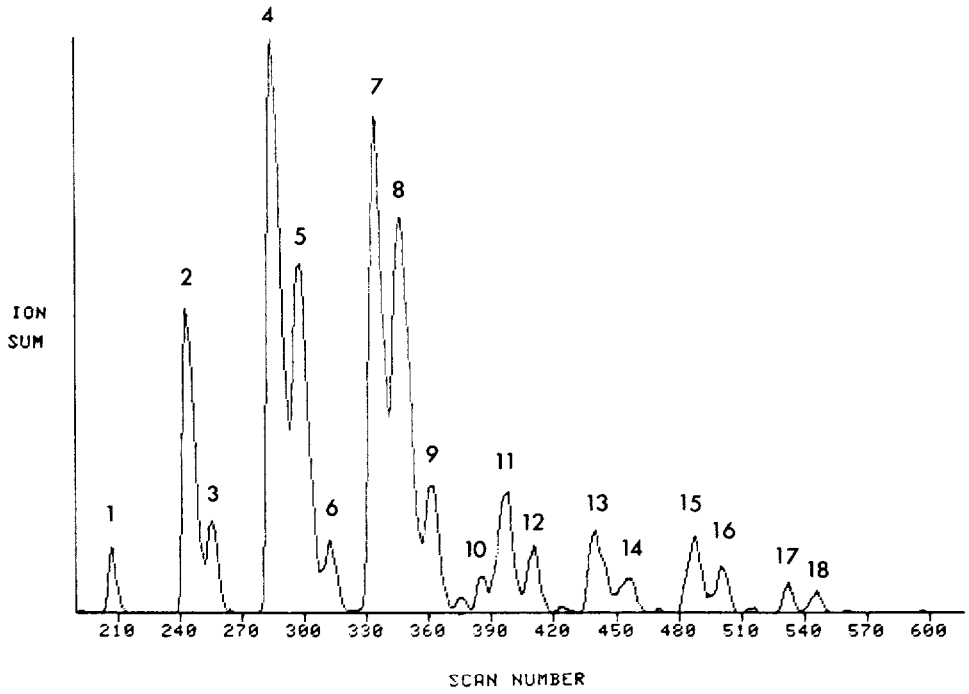

Fig. 3. Total ion tracings of an LC–MS separation of randomized peanut oil. Peaks are identified as in Table V. Major peaks: 1, 18:2 18:2 18:2; 2, 18:1 18:2 18:2; 3, 16:0 18:2 18:2; 4, 18:1 18:1 18:2; 5, 16:0 18:1 18:2; 6, 16:0 16:0 18:2; 7, 18:1 18:1 18:1; 8, 16:0 18:1 18:1; 9, 16:0 16:0 18:1; 10, 18:1 18:1 20:1; 11, 18:0 18:1 18:1; 12, 16:0 18:0 18:1; 13, 18:1 18:2 22:0 + 18:1 18:1 20:0; 14, 16:0 18:2 22:0 + 16:0 18:1 20:0; 15, 18:1 18:1 22:0; 16, 16:0 18:1 22:0; 17, 18:1 18:1 24:0; 18, 18:0 18:1 22:0. LC–MS conditions as given in the text.

ertheless, there remains a need for calibration of the ion current response for accurate quantitation. This can be done by dividing the calculated molar percentage by the corresponding peak area percentage derived from the ion current response and normalizing to trioleoylglycerol response taken as 1.0. These correction factors are closely similar to those derived for the randomized corn oil and indicate that they are generally applicable.

Linearity of response

In the absence of pure standards the linearity of the LC–MS response must be assessed indirectly. It is obvious that both major and minor components are recovered in approximately the correct proportions from the randomized oils and from triacylglycerol mixtures of known structure. The relative concentrations of these components vary from 0.1 to 25% of the total triacylglycerol mixture, which represents a range of 1–250. As both minor and major components differ in composition from one oil to the other, it must be concluded that all the components give a linear response over the investigated concentration range. In most instances about 1 mg of total triacylglycerol mixture was injected and about 1% of the column effluent was admitted to the mass spectrometer. Therefore, the absolute concentration range over which a linear response was observed in the present experiments may be calculated to be 10 ng to 2.5 μg.

TABLE VII

MOLECULAR SPECIES OF TRIACYLGLYCEROLS IN GENETIC VARIETIES OF PEANUT OILS

Peak No.	*Molecular species*	*North American*		*South American*		*African*	
		*HPLC–MS**	*Fitted***	*HPLC–MS*	*Fitted*	*HPLC–MS*	*Fitted*
1	18:2 18:2 18:2	2.3	1.2	4.9	3.3	1.8	0.7
2	18:1 18:2 18:2	10.6	10.7	19.0	16.6	6.1	6.2
3	16:0 18:2 18:2	3.8	2.3	6.6	4.8	2.3	1.1
4	18:2 18:2 20:1 18:1 18:1 18:2	18.3	20.6	16.4	18.9	14.8	18.4
5	18:0 18:2 18:2 16:0 18:1 18:2	10.5	8.3	13.0	12.7	8.1	6.7
6	16:0 16:0 18:2	1.5	0.9	1.9	1.5	1.0	0.3
7	18:1 18:2 20:1 18:1 18:1 18:1	15.5	16.1	7.8	7.3	24.2	25.9
8	18:2 18:2 20:0 18:0 18:1 18:2 16:0 20:1 18:2 16:0 18:1 18:1	10.5	12.5	7.3	10.9	14.8	13.6
9	16:0 18:0 18:2 16:0 16:0 18:1	2.1	2.5	1.5	2.6	1.9	2.1

* Ion sum × correction factor

** Best computer fit of previous data[15,16].

TABLE VIII

MOLECULAR SPECIES OF TRIACYLGLYCEROLS OF NATURAL SOYBEAN OIL

Peak No.	*Molecular species*	*Ion sum* (area-%)*	*Corrected** mol-%*	*Calculated*** mol-%*
1	18:2 18:2 18:3	4.0	5.2	7.3
2	18:2 18:2 18:2	10.0	13.0	15.3
3	18:1 18:2 18:3	6.4	8.3	6.6
4	16:0 18:2 18:3	2.4	2.4	2.6
5	18:1 18:2 18:2	12.5	16.7	21.4
6	18:1 18:1 18:3 16:0 18:2 18:2 18:0 18:3 18:2 16:0 18:3 18:1	16.3	15.9	12.5
7	18:1 18:1 18:2	8.2	9.0	9.6
8	18:0 18:2 18:2 16:0 18:1 18:2	15.4	13.1	12.1
9	16:0 16:0 18:2	4.6	2.8	1.4
10	18:1 18:1 18:1	4.1	2.5	1.4
11	18:0 18:1 18:2 16:0 18:1 18:1	8.9	6.1	6.0
12	16:0 18:1 16:0	3.5	2.4	0.5
13	18:0 18:1 18:1	2.0	1.6	0.9
14	16:0 18:0 18:1	1.5	1.0	0.4

* Ion sum from *m/z* 500 to 1000.

** Ion sum × correction factor.

*** Calculated by the 1-random–2-random–3-random procedure.

Quantitation of natural triacylglycerol mixtures

By means of the calibration factors derived from corn oil and randomized peanut oil we have determined the molecular species composition of selected natural oils and have compared it with that derived on the basis of a computer fitting of the best chemical data available. Table VII gives the composition of four genetic varieties of peanut oil assessed in this way. Overall, the agreement between the results is good. Certain discrepancies, however, remain and may be attributed to the use of secondary correction factors derived from complex natural oils rather than primary calibrations that could eventually be derived from synthetic triacylglycerols of known structure. Alternatively, the differences may be real and may reflect the incompleteness of the chemical analyses and hence errors in the computerized fitting of the distribution. For some of the oils the agreement is closer than for others. The significance of these differences remains to be established.

Table VIII gives the estimates of the molecular species composition of soybean oil triacylglycerols as derived by LC–MS and by calculation from the 1-random–2-random–3-random distribution[17]. In the calculation of the unresolved species by means of the $(MH - RCOOH)^+$ ions, the positional isomer proportions were calculated on the basis of stereospecific analyses, which gave estimates similar to those obtained for a random distribution.

The generally good agreement obtained between the computer-fitted chemical data and the LC–MS estimates following calibration indicates that the latter method of quantitation yields valid data despite the use of a gradient system for peak elution and chemical ionization in the mass spectrometer. Apparently the exact composition of the acetonitrile–propionitrile mixture is not critical for this work as both of these reagents possess comparable potential for chemical ionization of the glycerolipid molecules.

Previous work

Prior to this work, molecular species of natural triacylglycerols have been quantitated on the basis of the intensities of the molecular ions following direct probe or gas–liquid chromatographic (GLC) methods of sample introduction. Hites[10] performed the electron impact mass spectrometric analysis of triacylglycerols with a direct inlet system, and compared the observed values of theoretical values obtained by 1,3-random–2-random calculation for natural oils and 1,2,3-random calculation for randomized oils. A high overall correlation was obtained with an absolute error of less than 10%. As various structurally different triacylglycerols were represented by the same molecular weight, no effort was made to derive unique calibration factors or to relate the peak intensity at a given mass to the concentration of the compound represented by that mass. Murata and Takahashi[11] employed a combined GC–MS system in the electron impact mode for the analysis of molecular species of several natural oils. This method yielded quantitative results for various combinations of fatty acids (except positional isomers) in the triacylglycerol peaks resolved on the basis of carbon number by GLC. The GC–MS results obtained for coconut oil triacylglycerols were compared with the results of the chemical analyses reported by Bezard *et al.*[18]. The overall agreement between the GC–MS and chemical data was reasonable, but major differences (up to 20-fold) were noted for some species. No calibration of the GC–MS response was made for either the molecular ions or the $(M - RCOO)^+$ ions. It was assumed that the fatty acids from the three positions of the triacylglycerol molecule were released at

equal rates during the electron impact ionization. Barber *et al.*[19] had reported that the fatty acids from 1-myristoyl-2-stearoyl-3-palmitoylglycerol are released at equal rates. However, the abundances of ions characteristic of the component acids $(M - RCOO)^+$ increased with increasing chain length. According to Lauer *et al.*[20], the size of the group rather than its location appeared to determine the relative amounts of the ions in the fully saturated triacylglycerol series. These observations are in contrast to the present finding of a 3–4-fold greater loss of the fatty acids from the *sn*-1- and *sn*-3- in comparison with the *sn*-2-positions during chemical ionization LC–MS. Apparently the softer chemical ionization is more sensitive to differences in the chemical structure of the triacylglycerol molecules. Murata[13] employed chemical ionization for the GC–MS analysis of natural triacylglycerols but did not investigate differences in the yield of the $(MH - RCOOH)^+$ ions from positional isomers of triacylglycerols. Murata[13] also did not comment on the decreasing yields of the molecular ions with increasing molecular weight of the triacylglycerol, all GC–MS quantitations being reported within a carbon number, with the carbon number distribution being obtained from GLC with flame-ionization detection. The progressive decrease in the intensity of the molecular ion with increasing chain length during ammonia chemical ionization has been documented by Schulte *et al.*[14], who also observed that the response was nearly independent of the number of double bonds. We have confirmed the progressive decrease in the total ion current of the chemical ionization with increasing chain length of the triacylglycerols during LC–MS, but in our case the yield of the molecular ions varied greatly with the degree of unsaturation of the molecule. Clearly, each method of ionization appears to give its own characteristic yield of ion intensities, which must be calibrated with appropriate structural and chemical isomers for accurate quantitative determination of natural triacylglycerols by either GC–MS, LC–MS or direct probe MS. As LC–MS provides the most extensive resolution of the individual molecular species of triacylglycerols, it requires a more accurate calibration than the other techniques, which deal with relatively large numbers of isomeric species within each molecular weight or carbon number class. The synthesis of mixed acid triacylglycerols without rearrangement is difficult[21], as samples regarded as pure may be contaminated with small amounts of rearranged products. However, the characteristic structural differences recognized by LC–MS should eventually help to establish the true purity of the standards and the appropriate calibration factors.

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