Journal of Chromatography, 196 (1980) 279–293
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 12,876

FRACTIONATION OF OIL TRIACYLGLYCEROLS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

JEAN A. BEZARD* and MOUSSA A. OUEDRAOGO

Laboratoire de Physiologie Animale et de la Nutrition, Université de Dijon, B.P. 138, 21004 Dijon Cedex (France)

(First received January 21st, 1980; revised manuscript received April 3rd, 1980)

SUMMARY

A study of the conditions of triacylglycerol fractionation by reversed-phase high-performance liquid chromatography (HPLC) with [^{14}C]trioleoylglycerol showed that contamination by the adjacent peaks was very low (<0.6%); this was also proved by the high purity (>98%) of the re-chromatographed fractions.

Triacylglycerols from peanut and cottonseed oils eluted according to increasing partition numbers, the latter generally being very close to the even numbers expected. The percentages of the fractions determined from peak areas (detection by differential refractometer) were close to those determined by the internal standard procedure. A study of the fatty acid composition of the isolated fractions did not lead to a precise determination of the triacylglycerol composition, except for the first fraction, composed mainly of trilinoleoylglycerol, and the second fraction, composed of palmitoyldilinoleoyl- and oleoyldilinoleoylglycerol. These two triacylglycerol fractions represented respectively 4.3 and 11.5% of peanut oil and 27.6 and 11.2% of cottonseed oil. Cottonseed oil triacylglycerols were first fractionated by argentation thin-layer chromatography according to their degree of unsaturation. When the fractions were re-fractionated by reversed-phase HPLC, according to their carbon number, very simple mixtures were isolated, which will serve as useful substrates for further analysis of the isomers of fatty acid positions in triacylglycerols.

INTRODUCTION

Natural oils and fats are complex mixtures of triacylglycerols (TGs). The number and the proportion of the possible molecular species is large and a complete determination can be achieved only via several steps.

In a first step, the composition of TG types can be determined (three fatty acids are known but their positional distribution is not). This requires methods of fractionating the complex mixture of TGs into simpler mixtures, showing the same characteristics, either the same degree of unsaturation or the same chain length or both. For most oils, only one fractionation by silver ion (argentation) thin-layer

0021-9673/80/0000-0000/$02.25 © 1980 Elsevier Scientific Publishing Company

chromatography (TLC) according to the degree of unsaturation[1,2] into classes (TGs showing the same unsaturation) has been necessary. Gas chromatographic (GC) analysis of the component fatty acids and triacylglycerols of each class generally allows the precise composition to be calculated[2,3]. For more complex oils it has been necessary to utilize a second fractionation by GC[4,5], into groups (TGs showing the same chain length and the same unsaturation), before applying the calculation method.

In a second step, the composition of the TG types in *sn*-2-isomers can be established by means of pancreatic lipase[6,7]. For most oils, by hydrolysing the TGs of the same class, isolated by argentation TLC, the proportion of the different *sn*-2-isomers can be calculated, provided that the diglycerides formed during lipolysis were fractionated according to degree of unsaturation[8]. For more complex oils, the TGs of one class must first be fractionated into groups, which are then subjected to lipolysis[4,5].

In a third and last step, for the determination of the *sn*-1,3-isomers, snake venom phospholipase, which hydrolyses fatty acids esterified at the internal position of synthetic phosphatidylglycerols, can be used[9]. Whatever the oil, it will be necessary to work on the simplest mixtures of TGs, at least those obtained from combined fractionation according to unsaturation and chain length. In addition, some products of the reaction will have to be fractionated.

Therefore, in the study of triacylglycerol structures fractionation of the originally complex mixture of TGs is necessary in order to isolate much simpler mixtures. It also appears necessary in most instances to fractionate end-products of the enzymatic reactions. Because the calculation methods depend on data derived from analysis of the isolated fractions, very high purities of these fractions are required for accurate and precise determinations.

Fractions of TGs isolated by argentation TLC according to unsaturation are generally not very contaminated[2,3]. Fractionation according to chain length can be carried out by GC[10,11], but the collected fractions are sometimes highly contaminated[12].

Reversed-phase liquid chromatography has been widely used in the past for the separation of triacylglycerols[13,14]. Recently, great improvements, especially the introduction of chemically bonded phases and high-performance liquid chromatography (HPLC), have renewed interest in this technique.

The first paper dealing with the HPLC of triacylglycerols was published in 1975 by Pei *et al.*[15]. Simple TGs of medium chain length were separated on a reversed-phase column. Other workers extended its field of application to the analysis of long-chain TGs, on silicic acid columns, reversed-phase columns, or both[16–25]. Plattner *et al.*[16] presented the results of extensive work on the separation of TGs on reversed phases.

The fractionation of triacylglycerols by HPLC on reversed-phase columns was performed in 1977 independently by Plattner *et al.*[16] and Wada *et al.*[26]. Fractions of soybean oil were collected for GC analysis of fatty acids[16,26] and of TGs[26]. Exact compositions of the TGs were worked out for several[16] or all fractions[26] of this oil. Wada *et al.*[27] later reported similar work on beef TGs.

The separation of triacylglycerols by reversed-phase HPLC is effected according to the integral partition number[26]. However, when applied to triacylglycerols with the same degree of unsaturation, a separation according to carbon number can be

expected, as with saturated TGs[15]. Hence reversed-phase HPLC could advantageously replace GC, which is not very satisfactory.

In our studies on triacylglycerol composition analysis, relatively large amounts of very simple mixtures of TGs of high purity were needed for analysis of the *sn*-1-, -2- and -3-isomers. This prompted us to investigate further the possibility of fractionating pure TG fractions by reversed-phase HPLC, especially after a preliminary fractionation by argentation TLC. This paper reports some of our results in this field.

EXPERIMENTAL

Triacylglycerols

[^{14}C]Trioleoylglycerol (50 mCi·mmol^{-1}) was purchased from Centre d'Etudes Nucléaires, Gif-sur-Yvette, France, diluted with cold trioleoylglycerol (Nu-Chek Prep., Elysian, MN, U.S.A.) to a specific activity of 23,500 dpm·mg^{-1} and purified by reversed-phase HPLC using an old column. The purified triacylglycerol was then dissolved in acetone (10 mg·ml^{-1}) for HPLC.

Peanut oil TGs were prepared from a crude oil from Upper Volta (Africa). Total lipids were extracted with 15 volumes of dimethoxymethane–methanol (4:1)[28], dried with absolute ethanol using a rotary vacuum evaporator and then subjected to silicic acid column chromatography[29] to isolate pure TGs. The purity of the TG fractions was checked by TLC[30].

Cottonseed oil was extracted from cotton seeds (Upper Volta) and TGs were prepared as above.

Argentation thin-layer chromatography

In one experiment, cottonseed oil triacylglycerols, extracted from a more recent batch of seeds, were fractionated by argentation TLC[1]. Glass plates (20 × 20 cm) were coated with a 0.25-mm layer of silica gel (Kieselgel G, Type 60; Merck, Darmstadt, G.F.R.) impregnated with 20% (w/w) of silver nitrate, dried at 100°C for 1 h, stored in the dark and activated at 120°C for 0.5 h before use. A sample of 5 mg of TGs in chloroform was applied and the plates were developed with chloroform–methanol (198.5:1.5), dried under nitrogen and sprayed with a 0.05% ethanolic solution of 2',7'-dichlorofluorescein. The bands, revealed under UV light (350 nm), were delineated, the silica gel was scraped off and the TGs were extracted from the powder with chloroform–methanol (90:10).

High-performance liquid chromatography

Samples of triacylglycerols (0.5–4 mg dissolved in acetone) were run on a Waters Assoc. ALC:GPC 201 liquid chromatograph equipped with a 30 cm × 3.9 mm I.D. stainless-steel μBondapak C_{18} column and a differential refractometer (R-401), both at room temperature. Analyses were carried out isocratically using acetonitrile–acetone (42:58) at a flow-rate of 1.3 ml·min^{-1}. The solvents, of analytical-reagent grade, obtained from Merck, were vacuum degassed for 20 min before use.

To collect a TG fraction, the solvent emerging from the detector was collected

through a 15 cm × 0.1 mm I.D. stainless-steel tube into one or two 5-ml vials, routinely 13 drops after the beginning of the registered peak on the chromatogram.

Gas–liquid chromatography

Fatty acids were analysed as butyl esters[31] on a Becker-Packard Model 417 gas chromatograph, with a 25 m × 0.4 mm I.D. glass capillary column coated[32] with Carbowax 20M (Applied Science Labs., State College, PA, U.S.A.) at a constant temperature of 200°C and a nitrogen flow-rate of 4.5 ml·min^{-1}, and equipped with a ROS injector[33] (S.P.I.R.A.L., Dijon, France) and a flame-ionization detector. The peak areas were measured with an ICAP 10 integrator (Lignes Télégraphiques Téléphoniques, Paris, France). Calibration factors for quantitative determinations were calculated using standard mixtures of fatty acids (Nu-Chek Prep.).

Radioactivity

Radioactivity was measured by liquid scintillation counting with Permafluor III (Packard Instruments, Paris, France) on a Packard PLD Tri-Carb spectrometer.

Calculations

Quantitation of the different fractions of peanut and cottonseed oil TGs collected by HPLC was performed in two ways. Firstly the HPLC peak areas were used, assuming that the areas were proportional to the weights of the TGs. Conversion of weights into moles was effected from the average molecular weights of the TGs, calculated from the fatty acid composition of each fraction. The second procedure used was internal standardization. To each fraction of TGs collected, a known amount of pentadecanoic acid (15:0) (Nu-Chek Prep.) was added before preparation of the butyl esters and their analysis by GC. The amount of the TG fatty acids was determined by comparison with the amount of internal standard (15:0), both proportional to the corrected peak areas. The amount of the TG fraction was calculated from that of the component fatty acids.

For the TGs of each fraction collected an integral partition number (*IPN*) was calculated and an equivalent partition number (*EPN*) was determined. The *IPN* was calculated from the carbon number (*C*) and the number of double bonds (*N*) using the equation[14]

$$IPN = C - 2N$$

The carbon number (number of carbon atoms in the acyl chains of a triacylglycerol)[14] was equal to three times the average chain length of the component fatty acids, calculated from the percentages in the fraction of these acids and from their chain lengths. Similarly, the number of double bonds was equal to the average number of double bonds in the component fatty acids.

The equivalent partition number, defined as the carbon number of the equivalent *n*-saturated triacylglycerol which elutes at the same point as the TG fraction[14], was determined graphically in the following manner. A mixture of six simple saturated triacylglycerols (carbon number 18–48) were analysed by HPLC under the conditions routinely used. The plot of carbon number *versus* retention volume showed a linear relationship similar to that found in reversed-phase partition chromatography[34]

and in reversed-phase HPLC[16]. From this linear relationship a carbon number (the *EPN*) can be determined for each TG fraction by comparison with its retention volume.

RESULTS AND DISCUSSION

Study and control of the conditions of fractionation

Conditions of peak collection. The best conditions of fractionation were determined in order to collect the maximum amount of the fraction while avoiding significant contamination.

Purified [^{14}C]trioleoylglycerol (1 mg in 100 μl of acetone) was analysed. The solvent emerging from the detector was collected in fractions of 10 drops for 3 min, from *ca.* 0.25 min before the registered peak started until *ca.* 1 min after it had finished.

Fig. 1 shows the peak (chromatogram) registered at a high chart speed (5 cm·min^{-1}) and the peak of radioactivity (radiogram), drawn from the values measured in the different fractions collected. The shift between the two peaks (0.25 min) corresponded to 20–25 drops of solvent. Contamination was minimal when collection started only 10–15 drops after the registration started. The collected peak showed no appreciable tailing, the latter being responsible for overlapping of peaks and contamination.

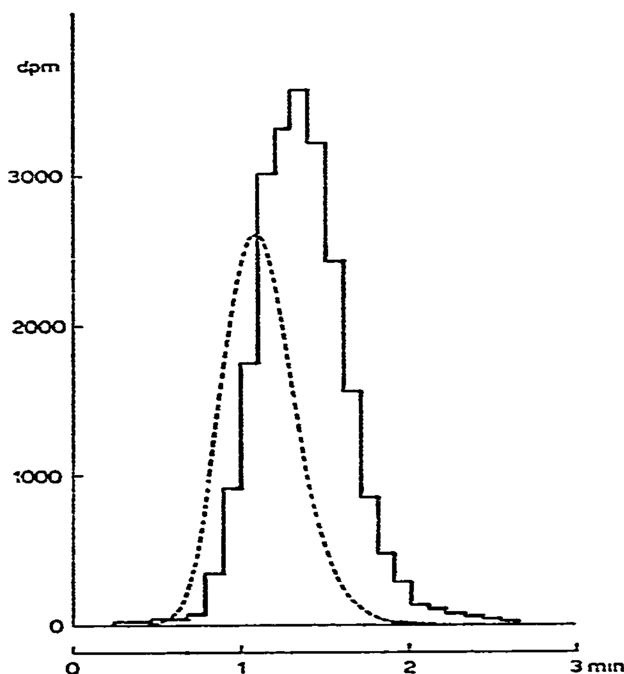


Fig. 1. Delay and shape of the collected peak of [^{14}C]trioleoylglycerol (radiogram, solid line). The registered peak (chromatogram, broken line) is expressed in arbitrary units.

Purity of the fractions collected. The collection of 0.6, 0.75 and 1 mg of [^{14}C]trioleoylglycerol showed that 98.5, 96.2 and 97.6%, respectively, of the injected radioactivity was recovered, proving that virtually no radioactive material which might cause further contamination was retained on the column.

In fact, this contamination was very low, as shown by the following experiments. When 3 mg of peanut oil TGs together with [^{14}C]trioleoylglycerol (20%, w/w) were separated into eight fractions (Fig. 2), only 0.5–0.6% of the radioactivity collected with the [^{14}C]TG peak was found in the fractions collected before and after the

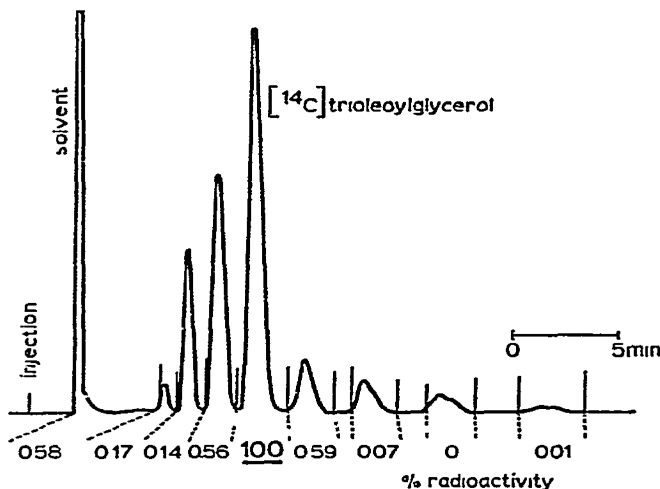


Fig. 2. Chromatogram recorded during fractionation of peanut oil triacylglycerols with [^{14}C]trioleoylglycerol added and percentage radioactivity of the nine fractions collected.

peak, respectively. In another experiment, three samples of 4 mg of peanut oil were fractionated and the eight fractions collected were re-chromatographed. Only very slight contamination by the preceeding peak (Fig. 3) could be observed in fractions 3–5, and even in fraction 4, which eluted just after the major peak.

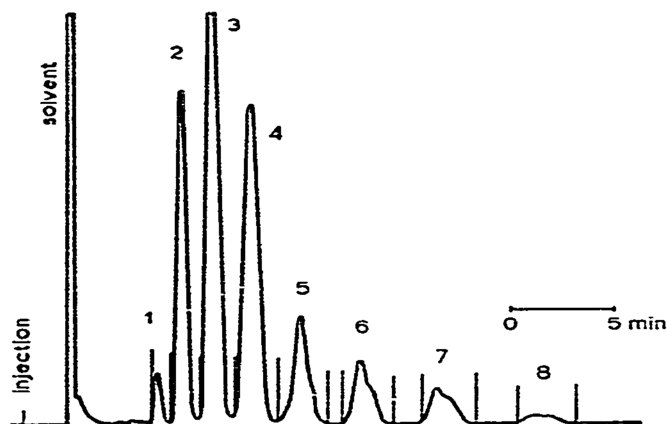
It can therefore be concluded that the fractions of TGs collected under the conditions chosen were of high purity and are suitable for further analysis.

Composition of the fractions collected

Peanut oil triacylglycerols. The eight fractionated peaks of peanut oil TGs were analysed for fatty acid composition by GC. The results are reported in Table I.

The percentages of these fractions were determined from the HPLC peak areas (first line) and from the amount of added internal standard (second line). The two series of values are similar. The differences (5% for the three main fractions) may be due to differences in the refractive index responses. Similarly, the percentages of the six fractions of soybean oil TGs calculated by Wada *et al.*[26] from the peak areas were close to those calculated by Plattner *et al.*[16] by the internal standard procedure.

Also in Table I, the fatty acid compositions of the fractions, their carbon



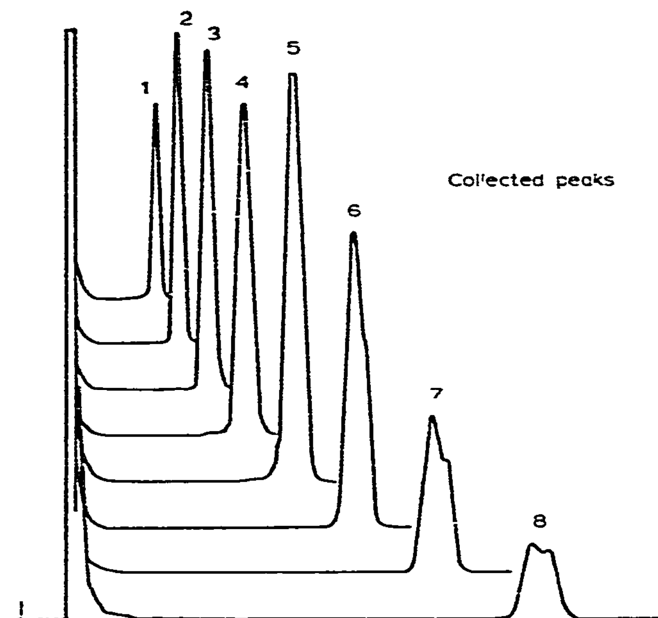


Fig. 3. Fractionation of peanut oil triacylglycerols and re-chromatography of the eight fractions collected.

number and the number of double bonds show that the unsaturation was very high in the first fraction and then decreased regularly, while the chain length increased sharply from fraction 5, following a very slow decrease.

The integral partition numbers, calculated by assuming that a double bond was exactly equivalent to a reduction of two carbon atoms, were very close to even numbers for fractions 2–7 (44–54), but different for the last fraction (55.02 instead of 56). The graphically determined equivalent partition numbers exhibited some differences from the calculated values. For the first four regular-shaped peaks the

TABLE I

PERCENTAGE AND FATTY ACID COMPOSITION OF THE EIGHT FRACTIONS OF PEANUT OIL TRIACYLGLYCEROLS FRACTIONATED BY REVERSED-PHASE HPLC

Parameter	*Fraction**								*Total fatty acids (mol%)*		
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*Reconstituted****		*Experimental*
									Areas	*15:0*	
Mol% (areas)	2.2	16.8	33.6	28.0	8.0	5.5	4.4	1.5			
Mol% (15:0)	1.9	15.8	32.0	26.8	9.4	7.3	5.4	1.4			
Fatty acids (mol%)**:											
16:0	1.7	9.1	16.8	16.7	8.8	9.0	10.4	10.7	13.7	13.5	13.9
18:0	1.1	0.2	1.4	5.9	17.3	4.0	2.9	7.6	4.0	4.2	4.2
18:1	2.8	24.2	45.8	65.8	37.3	28.9	39.7	46.1	44.9	44.6	44.7
18:2	94.4	66.3	35.6	9.4	20.5	25.1	12.4	5.0	31.3	30.7	30.9
20:0				0.9	7.3	9.3	1.3	–	1.4	1.6	1.6
20:1				1.3	2.9	0.4	0.3	0.6	1.0	1.1	0.8
22:0					5.9	20.0	22.9	6.2	2.7	3.2	2.9
24:0						3.3	10.1	23.8	1.0	1.1	1.0
Carbon number§	53.90	53.44	53.07	53.13	54.79	57.04	58.04	58.42			
Number of double bonds§	5.75	4.70	3.52	2.58	2.44	2.39	1.94	1.70			
Integral partition number§	42.40	44.04	46.03	47.97	49.91	52.26	54.16	55.02			
Equivalent partition number§	40.6	42.6	44.8	47.0	49.2	51.5	53.7	55.7			

* The fractions correspond to the peaks on the chromatogram (Fig. 3), numbered in order of elution. Their molar percentages (see below) were calculated either from the peak areas ("Areas") on the chromatogram or from pentadecanoic acid (15:0) added as an internal standard.

** Traces of 16:1, 18:3 and 22:1 were also found, in particular 0.2% of 16:1 in fraction 2.

*** Reconstituted from the fatty acid composition of the eight fractions and from their percentages calculated in the two different ways (Areas and 15:0).

§ For definitions, calculations and graphical determinations, see Experimental. Palmitic acid (16:0), palmitoleic acid (16:1), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), linolenic acid (18:3), arachidic acid (20:0), eicosenoic acid (20:1), behenic acid (22:0), docosenoic acid (22:1), lignoceric acid (24:0).

difference decreased from 1.8 to 1.0, as the number of double bonds decreased. This indicates that the assumption used for the calculation is not completely valid, especially with the solvent system selected for this work[14,22].

Finally, the reconstituted fatty acid composition of the peanut oil TGs was found to be very close to the experimentally determined one, demonstrating the validity of the analyses.

For each fraction we can tentatively determine the nature and the proportions of the component TG types.

The first fraction was found to be composed almost solely of linoleoyl groups and probably represented pure trilinoleoylglycerol. As already shown by several workers, very pure trilinoleoylglycerol can be prepared by thin-layer, paper and column liquid partition chromatography[14] from oils in which the most unsaturated fatty acid is linoleic acid (and the shortest is palmitic acid). However, reversed-phase HPLC is more rapid, more accurate and much larger amounts of starting material can be used.

The second fraction contained about 67% of linoleoyl groups and 33% of palmitoyl plus oleoyl groups. Assuming that on a reversed phase palmitic acid is equivalent to oleic acid, this fraction consisted of $9.1 \times 3 = 27.3\%$ of 16:0 18:2 18:2 and $24.2 \times 3 = 72.6\%$ of 18:1 18:2 18:2. These are the only two TG types for which the partition number is 44. As this fraction represented 15.8% of the total TGs (internal standard procedure), these two TGs represented 4.3 and 11.5%, respectively, of peanut oil.

As for the third fraction, if we tentatively apply the method of calculation worked out for other oils[4,35], the necessary independent equations are not available for solving all the possible types. Only the percentage of 18:0 18:2 18:2 could be calculated from that of 18:0, only present in this TG, namely $1.4 \times 3 = 4.2\%$. The same is true for the next three fractions: the exact percentage of only one TG type in each fraction could be determined, as follows:

fraction 4: 20:0 18:2 18:2 (2.7% of the fraction, 0.7% of the oil);
fraction 5: 22:0 18:2 18:2 (17.7 and 1.7%, respectively);
fraction 6: 24:0 18:2 18:2 (9.0 and 0.7%, respectively).

Additional GC analysis for TGs in these last fractions according to carbon number would allow a more precise composition to be determined[26]. However, these mixtures are not simple enough for a complete analysis to be successful.

Cottonseed oil triacylglycerols. Cottonseed oil TGs of simpler fatty acid composition were fractionated under the same conditions as above. The results are reported in Table II.

The same remarks as for peanut oil can be made here. Proportions of the fractions calculated from the peak areas were close to those calculated by the internal standard procedure. The carbon numbers decreased from fraction 1 to fraction 4 and increased for fraction 5, as did the numbers of double bonds. The integral partition numbers were close to even numbers (42–48), except for the last fraction (close to 49), which was rich in stearoyl groups. The graphically determined equivalent partition numbers were found to be lower than the preceding ones for the first four fractions, and the difference decreased from 1.6 to 0.2 as the number of double bonds decreased. The reconstituted fatty acid compositions of the oil TGs were close to those determined experimentally.

TABLE II

PERCENTAGE AND FATTY ACID COMPOSITION OF THE FIVE FRACTIONS OF COTTONSEED OIL TRIACYLGLYCEROLS FRACTIONATED BY REVERSED-PHASE HPLC

Parameter	*Fraction**					*Total fatty acids (mol%)*		
	1	*2*	*3*	*4*	*5*	*Reconstituted***		*Experimental*
						Areas	*15:0*	
Mol% (areas)	10.9	36.2	35.4	13.1	4.4			
Mol% (15:0)	10.5	38.6	35.3	13.3	2.3			
Fatty acids (mol%):								
14:0	3.6	1.4	0.5			1.1	1.1	0.9
16:0	1.1	23.8	41.2	44.2	26.2	30.2	30.3	30.7
16:1	1.2	1.0	0.3			0.6	0.6	0.8
18:0			1.8	8.4	25.7	2.9	2.4	2.2
18:1	0.6	9.7	22.0	36.8	35.2	17.7	17.3	18.0
18:2	93.5	64.1	34.2	10.6	12.9	47.5	48.3	47.4
Carbon number**	53.42	52.36	51.44	51.35	52.42			
Number of double bonds*'	5.66	4.17	2.72	1.74	1.83			
Integral partition number**	42.10	44.02	46.00	47.87	48.76			
Equivalent partition number**	40.5	42.9	45.4	47.7	49.6			

* These five fractions correspond to the HPLC peaks, numbered in order of elution.

** See footnotes to Table I and Experimental; myristic acid (14:0).

We can also try to determine the TG composition of each fraction from its fatty acid composition.

Fraction 1 was composed mainly of linoleoyl groups and thus contained at least 90% of trilinoleoylglycerol, which accounted for nearly 10% of the cottonseed oil TGs. Fraction 2 contained approximately 67% of linoleoyl groups and 33% of palmitoyl plus oleoyl groups and the TG composition was 16:0 18:2 18:2 = 23.8 × × 3 = 71.4% of the fraction and 27.6% of the oil, and 18:1 18:2 18:2 = 9.7% × 3= = 29.1% of the fraction and 11.2% of the oil.

For the last three fractions the number of all possible TG types was too high for an accurate composition to be calculated, even with additional GC analysis of TGs. Additionally, they are not simple enough to be further analysed for fatty acid position[6,9].

Classes of cottonseed oil triacylglycerols. Table III reports results obtained in the HPLC fractionation of class 012 triacylglycerols, isolated by argentation TLC.

As expected, the three fractions collected showed the same degree of unsaturation, that of the class (012), and they were separated according to the carbon number, *i.e.*, 50, 52 and 54.

TABLE III

PERCENTAGE AND FATTY ACID COMPOSITION OF CLASS 012 TRIACYLGLYCEROLS FROM COTTONSEED OIL ISOLATED BY ARGENTATION THIN-LAYER CHROMATOGRAPHY

Class 012 triacylglycerols are triacylglycerols with the same degree of un saturation (0 = saturated, 1 = monounsaturated, 2 = diunsaturated fatty acid).

Parameter	*Fraction**			*Total fatty acids (mol%)*	
	1	*2*	*3*	*Reconstituted*	*Experimental*
Mol% (15:0)	5.5	82.9	11.6		
Fatty acids (mol%)					
14:0	16.0			0.9	0.9
16:0	18.8	34.7	3.4	30.2	27.7
16:1	14.4			0.8	0.9
18:0			30.2	3.5	2.4
18:1	16.9	32.9	32.8	32.0	33.8
18:2	33.9	32.4	33.6	32.6	34.3
Carbon number**	50.09	51.92	53.80	52.03	52.17
Number of double bonds**	2.97	2.93	3.00	2.94	3.09
Integral partition number**	44.15	46.06	47.80	46.15	46.00

* The fractions correspond to the three HPLC peaks, numbered in order of elution.
** See footnotes to Table I.

The TG composition of each fraction was easily determined starting from its fatty acid composition. In fraction 1 (carbon number 50) 14:0 is necessarily associated with 18:1 and 16:0 with 16:1, as 18:2 was the only diunsaturated fatty acid present. For the first pair, the respective percentages were nearly the same (16.0 and 16.9%). However, the percentages of 16:0 and 16:1 were different. Using average values, the proportions of the only two possible TG types of fraction 1 were calculated. These are reported in Table IV (class 012).

TABLE IV

ANALYSIS OF THE TRIACYLGLYCEROLS OF COTTONSEED OIL BY REVERSED-PHASE HPLC IN COMBINATION WITH ARGENTATION THIN-LAYER CHROMATOGRAPHY

*Class** *(mol%)*	*Fraction*** *(mol%)*	*Triacylglycerol type****	*Mol%*		*Integral partition number*§	*Equivalent partition number*§
			In the fraction	*In the oil*		
001 (4.5)	2 (75.7)	16:0 16:0 18:1	100	3.4	48.0	47.3
011 (3.2)	2 (86.4)	16:0 18:1 18:1	100	2.8	48.1	47.0
002 (10.8)	1 (83.4)	16:0 16:0 18:2	100	9.0	46.0	45.3
012 (19.8)	1 (5.5)	{14:0 18:1 18:2	49.8	0.5	44.2	42.5
		{16:0 16:1 18:2	50.2	0.6		
	2 (82.9)	16:0 18:1 18:2	100	16.4	46.1	44.9
	3 (11.6)	18:0 18:1 18:2	100	2.3	47.8	46.7
112 (5.9)	2 (95.6)	18:1 18:1 18:2	100	5.6	45.9	44.4
022 (27.6)	2 (90.1)	16:0 18:2 18:2	100	24.9	44.0	42.8
122 (13.7)	2 (95.2)	18:1 18:2 18:2	100	13.0	44.0	42.5
222 (13.8)	1 (99.9)	18:2 18:2 18:2	100	13.8	42.4	40.5

* Triacylglycerols with the same degree of unsaturation separated on argentation TLC plates. In parentheses the percentages (mol%) in the total oil of the classes of triacylglycerols that were determined by the internal standard procedure (see Experimental).

** Correspond to the HPLC peaks, numbered in order of elution. Only the major fraction (except for class 012), generally corresponding to the second fraction, was collected. Its percentage in the fraction (indicated in parentheses as mol%) was determined from the internal standard (see Experimental).

*** Triacylglycerols of which the three component fatty acids were identified but their positioning was not. Their percentages in the fraction and in the total oil are given in the next two columns.

§ For definitions and determinations, see Experimental.

In fraction 2, the three fatty acids (16:0, 18:1 and 18:2) showed the same proportion of about 33%. Only one triglyceride type could therefore exist in this fraction (16:0 18:1 18:2) and the percentage in the oil was 16.4% (Table IV, class 012, fraction 2). In the last fraction (carbon number 54), still only one fraction was possible (18:0 18:1 18:2), with a percentage of 2.3% in the oil.

The other seven classes of cottonseed oil TG were also fractionated by HPLC and the main fraction (75–100% of the class) was collected and analysed for fatty acids by GC. For each fraction the fatty acid composition was very simple (Table V), so that only one TG type was possible. These seven TG types are listed in Table IV, together with those present in class 012. The eight main TG types, those from the eight classes, represented nearly 90% of the cottonseed oil TGs. The three last TGs together formed half. The main TG type, *i.e.*, palmitoyldilinoleoylglycerol (class 022), alone represented 25% of cottonseed oil. The TG composition reported here was close to that determined by other workers[36]. The origin of the oil may account

TABLE V

FATTY ACID COMPOSITION (mol%) OF THE MAJOR FRACTIONS ISOLATED BY REVERSED-PHASE HPLC FROM THE SEVEN CLASSES OF COTTONSEED OIL TRIACYLGLYCEROLS FRACTIONATED BY ARGENTATION THIN-LAYER CHROMATOGRAPHY

*Class**	*16:0*	*18:0*	*18:1*	*18:2*	*Carbon number**	*Number of double bonds**	*Integral partition number**
001	68.6		31.4		49.88	0.94	48.00
011	36.3	1.4	62.3		51.82	1.87	48.08
002	66.1		0.8	33.1	50.03	2.01	46.01
012	34.7		32.9	32.4	51.92	2.93	46.06
112			65.3	34.7	54	4.04	45.92
022	34.0			66.0	51.96	3.96	44.04
122			32.5	67.5	54.	5.02	43.96
222			5.6	94.4	54	5.83	42.35

* See footnotes to Tables I and III.

for the few differences observed, as is the case with the oils of two different batches of seeds analysed in this work: the oil prepared from more recent seeds and fractionated into classes showed a larger amount of trilinoleoylglycerol.

In the last two columns of Table IV, for the eight main TG types, the calculated integral partition number and the graphically determined equivalent partition number can be compared. It is apparent that the corresponding values differed, and the higher the degree of unsaturation, the larger was the difference. For example, for the calculated partition number 46 (classes 002, 012 and 112) three values of equivalent partition numbers were found (45.3, 44.9 and 44.4) for TGs containing 2, 3 and 4 double bonds per molecule, respectively. The size of the contribution of a double bond therefore seems to depend on the presence of other double bonds. This may be due to the solvent system used, as Herslöf *et al.*[22] showed that the assumption that one double bond is equal to two carbon atoms was better fulfilled in a methanol–acetone system, greater variations being apparent in the acetonitrile–acetone system.

CONCLUSION

To solve the problem of determinating the TG composition of oils, a single fractionation using reversed-phase HPLC is not sufficient, especially when only the component fatty acids of the fractions collected are analysed. If additional GC analysis of the TG fraction is carried out, more information is obtained, as shown by Wada and co-workers[26,27], but a precise and complete composition cannot be calculated because the mixtures of TGs collected are still too complex. To develop a complete determination, a second fractionation has to be made, either by GC according to carbon number[10] or by argentation TLC according to degree of unsaturation[1], followed by GC analysis of the fatty acids in the new fractions collected.

However, the preparative problem of isolating the simplest mixtures of TGs for further analysis of isomers is not completely solved with this double fractionation, because reversed-phase HPLC fractionation does not lead to TGs with perfectly determined characteristics, either chain length or degree of unsaturation.

If a fractionation is performed by argentation chromatography according to the degree of unsaturation and if the fractions collected are then analysed for fatty acids and triglycerides, a precise triglyceride composition can be determined, as was demonstrated for cottonseed oil[37] and even for the more complex peanut oil[3]. It is not necessary to proceed to a second fractionation as is generally the case with reversed-phase HPLC.

However, if a second fractionation by HPLC is carried out on the fractions isolated by argentation TLC, new fractions are now separated according to carbon number, as with GC, and these are extremely simple, as seen with cottonseed oil, for which each new fraction generally contained only one type of TG. These very simple mixtures can be submitted to other analyses, *e.g.*, for determination of the *sn*-2-isomers by means of pancreatic lipase[6,7], or for determination of the *sn*-1,3-isomers by means of snake venom phospholipase[9].

The second fractionation can also be effected by GC, but this technique is not satisfactory for high-molecular-weight triacylglycerols[12], whereas with HPLC several milligrams of TGs can be fractionated in each run, without any appreciable contamination. Moreover, fractionation is performed at room temperature and the TGs collected are at all times in solution; these conditions prevent their thermal or oxidative degradation.

To preserve these favourable conditions, the first fractionation by argentation TLC can be advantageously replaced with fractionation by HPLC using a column packed with silicic acid coated with silver nitrate[38,39], as for fatty acid methyl esters[40]. With this double fractionation by HPLC, large amounts of very simple mixtures of triacylglycerols can be obtained rapidly in high purity for further analysis of the isomers. We shall continue our work along these lines.

ACKNOWLEDGEMENTS

We are indebted to Mr. R. Barbès and Mr. G. Bondoux of Waters S. A., Paris, France, who provided a liquid chromatograph. We acknowledge the skillful technical assistance of J. Gresti with the GC analysis of fatty acids.

REFERENCES

1 C. B. Barrett, M. S. J. Dallas and F. B. Padley, *J. Amer. Oil Chem. Soc.*, 40 (1963) 580.
2 J. Bézard and M. Bugaut, *J. Chromatogr. Sci.*, 10 (1972) 451.
3 G. Semporé and J. Bézard, *Rev. Fr. Corps Gras*, 12 (1977) 611.
4 J. Bézard, J. P. Moretain and M. Bugaut, *Fette, Seifen, Anstrichm.*, 79 (1977) 399.
5 J. P. Moretain and J. Bézard, *Rev. Fr. Corps Gras*, 24 (1977) 303.
6 F. H. Mattson and L. W. Beck, *J. Biol. Chem.*, 214 (1955) 115.
7 P. Savary and P. Desnuelle, *C.R. Acad. Sci.*, 240 (1955) 2571.
8 J. Bézard, G. Semporé, G. Descargues and A. Sawadogo, *Fette, Seifen, Anstrichm.*, in press.
9 M. Yurkowski and H. Brockerhoff, *Biochim. Biophys. Acta*, 125 (1966) 55.
10 A. Kuksis and J. Ludwig, *Lipids*, 1 (1966) 202.
11 M. Bugaut and J. Bézard, *J Chromatogr. Sci.*, 8 (1970) 380.
12 M. Bugaut and J. Bézard, *J. Chromatogr. Sci.*, 11 (1973) 36.
13 F. B. Padley, *Chromatogr. Rev.*, 8 (1966) 208.
14 C. Litchfield, *Analysis of Triglycerides*, Academic Press, New York and London, 1972, p. 68.
15 T. Pei, R. Henly and S. Ramachandran, *Lipids*, 10 (1975) 152.
16 R. D. Plattner, G. F. Spencer and R. Kleinman, *J. Amer. Oil Chem. Soc.*, 54 (1977) 511.

17 A. Karleskind, *Rev. Fr. Corps Gras*, 24 (1977) 419.
18 B. Vonach and G. Schomburg, *J. Chromatogr.*, 149 (1978) 417.
19 N. A. Parris, *J. Chromatogr.*, 149 (1978) 615.
20 A. Karleskind, G. Valmalle, O. Midler and M. Blanc, *Rev. Fr. Corps Gras*, 25 (1978) 551.
21 R. D. Plattner, K. Wade and R. Kleinman, *J. Amer. Oil Chem. Soc.*, 55 (1978) 381.
22 B. Herslöf, O. Podlaha and B. Töregård, *J. Amer. Oil Chem. Soc.*, 56 (1979) 864.
23 R. D. Plattner and K. Payne-Wahl, *Lipids*, 14 (1979) 152.
24 K. Payne-Wahl, R. D. Plattner, G. F. Spencer and R. Kleinman, *Lipids*, 14 (1979) 601.
25 N. A. Parris, *J. Chromatogr. Sci.*, 17 (1979) 541.
26 S. Wada, C. Koizumi and J. Nonaka, *Yukakagu*, 26 (1977) 95.
27 S. Wada, C. Koizumi, A. Takiguchi and J. Nonaka, *Yukakagu*, 27 (1978) 579.
28 J. Delsal, *Bull. Soc. Chim. Biol.*, 26 (1944) 99.
29 D. L. Fillerup and J. F. Mead, *Proc. Soc. Exp. Biol. Med.*, 83 (1953) 574.
30 E. Stahl, G. Shroter, G. Kraft and R. Renz, *Pharmazie*, 11 (1956) 633.
31 G. Clément and J. Bézard, *C.R. Acad. Sci.*, 253 (1961) 564.
32 A. F. Prévot and F. X. Mordret, *Rev. Fr. Corps Gras*, 23 (1976) 409.
33 A. Ros, *J. Gas Chromatogr.*, 3 (1965) 252.
34 E. C. Nickell and O. S. Privett, *Separ. Sci.*, 2 (1967) 307.
35 J. Bézard, M. Bugaut and G. Clément, *J. Amer. Oil Chem. Soc.*, 48 (1971) 134.
36 G. Jurriens and A. C. J. Kroesen, *J. Amer. Oil Chem. Soc.*, 42 (1965) 9.
37 M. Ouedraogo and J. Bézard, *Rev. Fr. Corps Gras*, submitted for publication.
38 A. Monseigny, P. Y. Vigneron, M. Levacq and F. Zwobada, *Rev. Fr. Corps Gras*, 26 (1979) 107.
39 E. C. Smith, A. D. Jones and E. W. Hammond, *J. Chromatogr.*, 188 (1980) 205.
40 C. R. Scholfield, *J. Amer. Oil Chem. Soc.*, 56 (1979) 510.