# Determination of Molecular Species of Oil Triacylglycerols by Reversed-Phase and Chiral-Phase High-Performance Liquid Chromatography

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**A method is described for the determination of molecular species of oil triacylglycerols. The method is based on the analytical separation of the enantiomeric *sn*-1,2- and *sn*-2,3-diacylglycerols, derived from triacylglycerols, by high-performance liquid-chromatography (HPLC) on a chiral column containing N-(R)-1-(α-naphthyl)ethylaminocarbonyl-(S)-valine as stationary phase. Model triacylglycerol molecules comprising three known fatty acids were isolated from peanut oil and cottonseed oil by a combination of argentation-TLC and reversed-phase HPLC and submitted to partial chemical deacylation. The derived *sn*-1,2(2,3)-diacylglycerols were analyzed and fractionated as 3,5-dinitrophenyl urethane derivatives by reversed-phase HPLC according to chainlength and unsaturation. From the *sn*-1,2(2,3)-diacylglycerol composition and the diacylglycerol *sn*-1,2- and *sn*-2,3-enantiomer composition, the individual molecular species of four peanut oil triacylglycerols and one cottonseed oil triacylglycerol were identified and quantitated. The method can be applied to triacylglycerols of any other oil or fat.**

KEY WORDS: Chiral-phase HPLC, diacylglycerols, enantiomers, molecular species, reversed-phase HPLC, triacylglycerols.

In stereospecific analysis of oil triacylglycerols, representative diacylglycerols should be produced by enzymatic or chemical partial deacylation and studied for fatty acid distribution (1). In the previous methods, the enantiomeric *sn*-1,2(2,3)-diacylglycerols, isolated by preparative thin-layer chromatography (TLC), were transformed into phospholipid-like molecules. The two enantiomeric synthetic phospholipids were then differentiated by the stereospecific action of phospholipase A (2) or phospholipase C (3). The diacylglycerol enantiomers are preferentially separated by high-performance liquid chromatography (HPLC) either as diastereomers on a classical silica column (4,5), or more currently as 3,5-dinitrophenylurethane (DNPU) derivatives on a chiral column (6–8).

In a previous study (9), we submitted pure peanut oil triacylglycerols isolated by argentation-TLC and reversed-phase HPLC to partial chemical deacylation, and we fractionated the pure enantiomeric diacylglycerols as DNPU derivatives by reversed-phase HPLC. The diacylglycerol enantiomers were consecutively separated by chiral-phase HPLC (8).

The present study reports the determination of molecular species of the major peanut oil diacid- and triacid-triacylglycerols from the data previously obtained. These results complete those of another study on triacylglycerol structure of an African peanut oil of the same origin (10).

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## EXPERIMENTAL PROCEDURES

*Samples.* Peanut oil was a crude oil produced in Burkina Faso (West Africa). Crude cottonseed oil was provided by IRCT (Institut de Recherche du Coton et des Textiles Exotiques, Montpellier, France). The triacylglycerol fraction was isolated from the crude oils by silicic acid column chromatography (11). Unique triacylglycerols (the three fatty acids are known) were prepared by a combination of argentation-TLC and reversed-phase HPLC (12). Triacylglycerols were first fractionated according to degree of unsaturation on silver nitrate-impregnated silica (10%) plates with hexane/diethyl ether/methanol (79:20:1) as developing solvent. Triacylglycerols from five bands of different unsaturation (011, 111 + 002, 012, 112, 022 + 122 + 222) were extracted from the scraped-off silica. Fifty plates (5–10 mg triacylglycerols per plate) were necessary to fractionate enough material for further analysis. Unique triacylglycerols were isolated from these five fractions by reversed-phase HPLC with an octadecyl/silyl column and acetonitrile/acetone (42:58) as developing solvent. In most cases, about 20 fractionations of 4–7 mg of triacylglycerols from each band were necessary to isolate enough material for the next steps of analysis. The isolated triacylglycerols were the following three diacid-triacylglycerols from peanut oil—palmitoyldioleoylglycerol (16:0 18:1 18:1), dioleoyllinoleoylglycerol (18:1 18:1 18:2) and oleoyldilinoleoylglycerol (18:1 18:2 18:2). The triacid-palmitoyloleoyllinoleoylglycerol (16:0 18:1 18:2) was isolated from both peanut oil and cottonseed oil for comparison.

*Enantiomeric diacylglycerols.* Triacylglycerols were submitted to partial Grignard degradation (13) as previously reported (8). Ethyl magnesium bromide in diethyl ether (0.25 mL, 1 M) from Aldrich Chemical Co. (Milwaukee, WI) was added to 5–10 mg triacylglycerols in 1 mL diethyl ether for 1 min at ambient temperature. The *sn*-1,2(2,3)-diacylglycerols were separated by boric acid-TLC with petroleum ether/diethyl ether (50:50) as developing solvent (13). The DNPU derivatives were prepared by reacting diacylglycerols (0.6–3 mg) in 4 mL dry toluene with 2–10 mg 3,5-dinitrophenyl isocyanate (Sumitomo, Osaka, Japan) in the presence of 40 μL dry pyridine, at ambient temperature for 1 hr (14). The crude urethane derivatives extracted from the medium were directly fractionated by reversed-phase HPLC without purification (8).

Triacylglycerols were also submitted to partial enzymatic deacylation for comparison. One to five mg were emulsified in 1 mL of 1 M TRIS buffer in the presence of calcium chloride and sodium taurocholate for 3 min at 0°C (B 12 Sonifier, Branson, CT). The emulsion was incubated at 37°C with 100 μL of buffer containing 4.25 mg $mL^{-1}$ of lyophilized porcine pancreatic lipase (Precibio, Paris, France). The *sn*-2-monoacylglycerols were separated by boric acid-TLC (13) and analyzed by capillary gas chromatgraphy for fatty acid composition.

*Reversed-phase HPLC.* The analytical and preparative HPLC separations of *sn*-1,2(2,3)-diacylglycerols as DNPU derivatives according to chainlength and unsaturation were performed on a Waters model 6000 A Liquid Chromatograph (Waters Associates, Milford, MA) equipped with an octadecyl silyl column (25 cm × 4 mm ID) containing Lichrospher 100 CH-18/11 Super (4-μm particles) from Merck (Darmstadt, Germany). Analyses were carried out at temperatures of 12°C, 16°C or 20°C, depending on the diacylglycerols, with an isocratic solvent system of acetonitrile/acetone (55:4 or 60:40) at constant flow rate of 0.9 to 1.2 mL $min^{-1}$, depending on the diacylglycerols (8). Peak areas were measured by means of an Enica 21 integrator-calculator (Delsi, Suresnes, France).

*Chiral-phase HPLC.* The analytical HPLC separation of enantiomeric *sn*-1,2- and *sn*-2,3-diacylglycerols as DNPU derivatives were performed on the Waters Liquid Chromatograph equipped with a Sumipax OA-4100 chiral column (25 cm × 4 mm ID) containing N-(R)-1-(α-naphthyl)ethylaminocarbonyl-(S)-valine bonded to γ-aminopropyl silanized silica (5-μm particles) from Sumitomo Chemical (Osaka, Japan). The analyses were carried out at ambient temperature of *ca.* 20°C with an isocratic solvent system of hexane/ethylene dichloride/ethanol (80:20:1) at a flow rate of 0.9 to 1.2 mL $min^{-1}$ depending on the samples. Peak areas were measured by means of an Enica integrator-calculator. Other conditions were as reported previously (9).

*Gas chromatography.* Fatty acid compositions of *sn*-1,2(2,3)-diacylglycerols and *sn*-2-monoacylglycerols were determined by capillary gas chromatograph (GC) of methyl esters on a Becker-Packard model 417 gas chromatograph (Packard, Rungis, France) equipped with a laboratory-made 30 m × 0.4 mm ID glass capillary column, coated with Carbowax 20 M, at a constant temperature of 195°C and a nitrogen flow rate of 3 mL $min^{-1}$. Other conditions were as reported previously (8).

## RESULTS

Table 1 reports the composition of the *sn*-1,2(2,3)-diacylglycerols derived from the five studied triacylglycerols by Grignard degradation and the enantiomer composition of these enantiomeric diacylglycerols. The figures are means of three to seven values obtained in different ways as previously reported (8,9).

The proportion of the two *sn*-1,2(2,3)-diacylglycerols derived from 16:0 18:1 18:1 and 18:1 18:2 18:2 were very close to 45:55 in percentages. The monoacid-diacylglycerol was in slightly lower proportion than the diacid-diacylglycerol. The proportion of the three *sn*-1,2(2,3)-diacylglycerols of 16:0 18:1 18:2 was different depending on the triacylglycerol origin, except for 18:1 18:2 (*ca.* 49% in both cases).

The enantiomer composition of the *sn*-1,2(2,3)-diacylglycerols was rarely that of a racemic mixture (50:50) except, curiously, for *sn*-1,2(2,3)-16:0 18:2 derived from cottonseed oil (16:0 18:1 18:2) partial deacylation. Thus if the more unsaturated fatty acid was preferentially esterified at the *sn*-2-position, the other one did not indifferently occupy the *sn*-1- or the *sn*-3-position.

From the data reported in Table 1, the different molecular species (1,2,3-triacyl-*sn*-glycerols) present in the five analyzed triacylglycerols could be mathematically determined by solving a set of equations. The determination was easy for the diacid-triacylglycerols because only three molecular species could exist. An example is given in Table 2 concerning the triacylglycerol 18:1 18:2 18:2 from peanut oil. The percentage (mole %) of the three possible 1,2,3-triacyl-*sn*-glycerols (listed in the third column) are assumed to be a, b, c (fourth column). The chemical

**TABLE 1**

**Enantiomer Composition of the *sn*-1,2(2,3)-Diacylglycerols Issued from Chemical Deacylation of Diacid- and Triacid-Triacylglycerols Isolated from Peanut Oil and Cottonseed Oil by Combined Argentation-TLC and Reversed-Phase HPLC**

| | Peanut oil | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Triacylglycerols | 16:0 18:1 18:1 | | | | 18:1 18:1 18:2 | | | | 18:1 18:2 18:2 | | | |
| *sn*-1,2(2,3)-diacylglycerols[a] | 18:1-18:1 | | 16:0-18:1 | | 18:1-18:2 | | 18:1-18:1 | | 18:2-18:2 | | 18:1 18:2 | |
| Mole %[b] | 46.52 | | 53.48 | | 77.77 | | 22.23 | | 44.03 | | 55.97 | |
| Enantiomers | *sn*-1,2- | *sn*-2,3- | *sn*-1,2- | *sn*-2,3- | *sn*-1,2- | *sn*-2,3- | *sn*-1,2- | *sn*-2,3- | *sn*-1,2- | *sn*-2,3- | *sn*-1,2- | *sn*-2,3- |
| Mole %[c] | 38.04 | 61.96 | 58.95 | 41.05 | 56.17 | 43.83 | 23.40 | 76.60 | 81.91 | 18.09 | 23.62 | 76.38 |

| | Peanut oil | | | | | | Cottonseed oil | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Triacylglycerols | 16:0 18:1 18:2 | | | | | | 16:0 18:1 18:2 | | | | | |
| *sn*-1,2(2,3)-diacylglycerols[a] | 18:1-18:2 | | 16:0-18:2 | | 16:0-18:1 | | 18:1-18:2 | | 16:0-18:2 | | 16:0-18:1 | |
| Mole %[b] | 48.65 | | 41.81 | | 9.54 | | 49.43 | | 29.62 | | 20.95 | |
| Enantiomers | *sn*-1,2- | *sn*-2,3- | *sn*-1,2- | *sn*-2,3- | *sn*-1,2- | *sn*-2,3- | *sn*-1,2- | *sn*-2,3- | *sn*-1,2- | *sn*-2,3- | *sn*-1,2- | *sn*-2,3- |
| Mole %[c] | 38.68 | 61.32 | 66.86 | 33.14 | 36.08 | 63.92 | 47.91 | 52.09 | 49.99 | 50.01 | 53.38 | 46.62 |

[a]The *sn*-1,2(2,3)-diacylglycerol fraction was separated from the *sn*-1,3-isomers in total diacylglycerols on borate-impregnated silica-TLC.
[b]Mean of seven values determined in three different ways (8).
[c]Mean of three values determined in two different ways (9).

**TABLE 2**

**Determination of the 1,2,3-Triacyl-*sn*-Glycerol Composition of Oleoyldilinoleoylglycerol Isolated from Peanut Oil**

| TG[a] | *sn*-2-TG[a] | *sn*-1,2,3-TG[a] | Mole% | *sn*-1,2-DG[b] | Mole% | *sn*-2,3-DG[b] | Mole% |
|---|---|---|---|---|---|---|---|
| 18:1 18:2 18:2 | 18:1 18:2 18:2 | 18:1-18:2-18:2 | a | 18:1-18:2 | a/3 | 18:2-18:2 | a/3 |
| | | 18:2-18:2-18:1 | b | 18:2-18:2 | b/3 | 18:2-18:1 | b/3 |
| | 18:2 18:1 18:2 | 18:2-18:1-18:2 | c | 18:2-18:1 | c/3 | 18:1-18:2 | c/3 |

| Equations[c] | | | Solution (A)[d] | Solution (B)[d] | Mean |
|---|---|---|---|---|---|
| (1) $a + b = 44.03 \times 2 = 88.06$ | (3) $\frac{a}{b} = \frac{18.09}{81.91}$ | a | 15.93 | 14.50 | 15.21 |
| | | b | 72.13 | 73.56 | 72.85 |
| (2) $c = 100 - 88.06 = 11.94$ | (4) $\frac{a + c}{b + c} = \frac{23.62}{76.38}$ | c | 11.94 | 11.94 | 11.94 |

[a]The triacylglycerol (TG) comprises two TGs whose fatty acid at the *sn*-2-position (*sn*-2-TG) is known, and three stereoisomers whose fatty acids at the 1,2- and 3-positions (from left to right) are known (*sn*-1,2,3-TG).

[b]*sn*-1,2- and *sn*-2,3-Diacylglycerols generated by chemical partial deacylation.

[c]Equations [1] and [2] are derived from the percentage of the individual *sn*-1,2(2,3)-diacylglycerols (Table 1, fifth line); and equations [3] and [4] from the percentage of the enantiomers (Table 1, last line) as described in the text.

[d]Solution A is derived from equations [2] and [3] and solution B from equations [2] and [4].

partial deacylation is assumed to generate the same proportion of *sn*-1,3-, *sn*-1,2- and *sn*-2,3-diacylglycerols, proportional to a/3, b/3, c/3 for the three TG isomers, respectively.

The percentage of the enantiomeric *sn*-1,2(2,3)-18:2 18:2 (*sn*-1,2-plus *sn*-2,3-18:2-18:2) is proportional to 1/3 (a+b) and equal to 44.03% (Table 1). With this percentage being calculated for only the two *sn*-1,2- and *sn*-2,3-diacylglycerols (excluding the *sn*-1,3-isomer), the following equation can be derived from these data:

$$1/3\ (a+b) = 2/3 \times 44.03$$

which is equation [1] in Table 2. From this follows:

$$a + b = 88.06$$

Since $a + b + c = 100$,

$$c = 100 - 88.06 = 11.94.$$

This is also shown as equation [2] in Table 2.

Considering the enantiomers *sn*-2,3- and *sn*-1,2-18:2-18:2, their percentage ratio is a/b and equal to 18.09/81.91 (Table 1). This is shown as equation [3] in Table 2:

$$a/b = 18.09/81.91.$$

It was easy to calculate a and b from equations [1] and [3], which were found to be 15.93 and 72.13, respectively.

Considering the enantiomers of diacylglycerol 18:1-18:2, another equation could be written. This is equation [4], as in Table 2:

$$sn\text{-}1,2\text{-}/sn\text{-}2,3\text{-} = a + c \,/\, b + c = 23.62/76.38$$

By combining equations [1], [2] and [4], other values of a and b could be calculated. They were:

$$a = 14.50 \qquad b = 73.56$$

These two series of values for a and b, issued from two independant enantiomer analyses, are close to each other. Means of values obtained by both methods can then be used. This method of calculation also was applied to the other two diacid-triacylglycerols, 16:0 18:1 18:1 and 18:1 18:1 18:2.

The determination of the triacid-triacylglycerols 16:0 18:1 18:2 from peanut oil and cottonseed oil was more complicated, since there were six possible triacylglycerol molecular species. An example is given in Table 3 for peanut oil 16:0 18:1 18:2. Six equations were derived from experimental data as above. The first three were established from the percentage of the three following enantiomeric *sn*-1,2(2,3)-diacylglycerols: 18:1-18:2, 16:0-18:2 and 16:0-18:1 (equations [1], [2] and [3]). Three other equations were written from the enantiomer composition of the same three diacylglycerols (equations [4], [5] and [6]). There are six total equations for six values to be calculated (a through f). Since the six equations were not independent, resolution of the set of equations was not possible. An approximation had to be made which did not greatly decrease the calculation accuracy.

From the first three equations, the values for A = (a+b), C = (c+d) and E = (e+f) could be calculated. These were A = 16.38, C = 80.92, E = 2.70. As expected, E was low, since it represented the percentage of the two molecular species with the saturated fatty acid (16:0) in the internal position.

The approximation concerned the percentage (e and f) of the two molecular species having 16:0 at the *sn*-2-

TABLE 3

**Determination of the 1,2,3-Triacyl-*sn*-Glycerol Composition of Palmitoyloleoyllinoleoylglycerol Isolated from Peanut Oil**

| TG[a] | *sn*-2-TG[a] | *sn*-1,2,3-TG[a] | Mole% | *sn*-1,2-DG[b] | Mole% | *sn*-2,3-DG[b] | Mole% |
|---|---|---|---|---|---|---|---|
| 16:0 18:1 18:2 | 16:0 18:1 18:2 | 16:0-18:1-18:2 | a | 16:0-18:1 | a/3 | 18:1-18:2 | a/3 |
| | | 18:2-18:1-16:0 | b | 18:2-18:1 | b/3 | 18:1-16:0 | b/3 |
| | 16:0 18:2 18:1 | 16:0-18:2-18:1 | c | 16:0-18:2 | c/3 | 18:2-18:1 | c/3 |
| | | 18:1-18:2-16:0 | d | 18:1-18:2 | d/3 | 18:2-16:0 | d/3 |
| | 18:1 16:0 18:2 | 18:1-16:0-18:2 | e | 18:1-16:0 | e/3 | 16:0-18:2 | e/3 |
| | | 18:2-16:018:1 | f | 18:2-16:0 | f/3 | 16:0-18:1 | f/3 |

Equations[c]

(1) $(a+b) + (c+d) = 48.65 \times 2 = 97.30$

(2) $(c+d) + (e+f) = 41.81 \times 2 = 83.62$

(3) $(a+b) + (e+f) = 9.54 \times 2 = 19.08$

(4) $\frac{b + d}{a + c} = \frac{38.68}{61.32}$

(5) $\frac{c + f}{d + e} = \frac{66.86}{33.14}$

(6) $\frac{a + e}{b + f} = \frac{36.08}{63.92}$

Solutions

| | A[d] | B[d] | Mean |
|---|---|---|---|
| a | 5.57 | 6.00 | 5.78 |
| b | 10.81 | 10.38 | 10.59 |
| c | 54.09 | 53.66 | 53.88 |
| d | 26.83 | 27.26 | 27.05 |
| e | 0.88 | 0.88 | 0.88 |
| f | 1.82 | 1.82 | 1.82 |

[a,b,c]As in Table 2.
[d]Solution A is derived from equations [2], [5] and [1], [4] and solution B from equations [3], [6] and [1], [4].

position. We can tentatively estimate e and f by the frequency of 18:2 (or that of 18:1) at the *sn*-1- and *sn*-3-positions in the four major molecular species of the triacylglycerol 16:0 18:1 18:2. By taking e = f = 0 and successively using equations [2], [5], [1], [4] and [3], [6], [1], [4] we obtained two series of values for a, b, c, d, whose means are:

$$a = 5.32, b = 11.06, c = 54.35, d = 26.57$$

Considering the concerned four molecular species, the relative frequency of 18:2 for *sn*-1-position (as compared to *sn*-3-) was b/a = 2.08. That of 18:1 for *sn*-3- (as compared to *sn*-1-) was c/d = 2.05. For the two molecular species 18:2-16:0-18:1 and 18:1-16:0-18:2, the ratio determined for 18:2 and 18:1 must be the same. It was taken as the mean of the two values, *i.e.*, 2.065. From the two equations, f/e = 2.065 and e + f = 2.70, it was easy to calculate the values for e and f:

$$e = 0.88 \text{ and } f = 1.82$$

When combining equations [2] and [5], values for c and d were found. When reported in equations [1] and [4], values for a and b were found. These values were referred to as solution A in Table 3. Another series of values (solution B) were found by combining equations [3] and [6] for a and b to be calculated, and equations [1] and [4] for the other values c and d, as done previously. These two series of values are reported in Table 3, together with their mean.

The same procedure was applied to cottonseed oil 16:0 18:1 18:2. The values for e and f were tentatively determined as above. In this case the ratio of the percentages of 18:2 at *sn*-1- and *sn*-3-position was 0.84. That of 18:1 at *sn*-3- and *sn*-1-positions was 1.02. The ratio f/e was taken as the mean, *i.e.*, 0.93. Since e + f = 1.14, the values for e and f were 0.59 and 0.55, respectively. These values are reported in Table 4, together with the average percentage of the four major triacylglycerol species calculated from these figures.

Overall results concerning the four major peanut oil triacylglycerols analyzed in this work are reported in Table 4. The four triacylglycerols together represented 48.6% of the total oil. If we take into account the monoacid-triacylglycerols, *i.e.*, trioleoylglycerol (10.66% of the total) and trilinoleoylglycerol (2.08%), more than 61% of the molecular species of the analyzed peanut oil triacylglycerols were identified and quantitated. The three major molecular species were trioleoylglycerol (10.7%), 1-oleoyl-2-linoleoyl-3-oleoyl-*sn*-glycerol (9.2%) and 1-linoleoyl-2-linoleoyl-3-oleoyl-*sn*-glycerol (8.6%). 1-Palmitoyl-2-linoleoyl-3-oleoyl-*sn*-glycerol, comprising the major saturated fatty acid, was next, and represented 6.3%.

In the latter triacylglycerol, in which each fatty acid presents the same proportion, we can calculate the affinity

**TABLE 4**

**Molecular Species Composition of the Major Peanut Oil Diacid- and Triacid-Triacylglycerols**

| | | Peanut oil | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Triacylglycerol | | 16:0 18:1 18:1 | | | 18:1 18:1 18:2 | | | 18:1 18:2 18:2 | |
| Mole %[a] | | 8.48 | | | 16.55 | | | 11.85 | |
| | | Mole % | | | Mole % | | | Mole % | |
| | | TG[b] | Oil[c] | | TG[b] | Oil[c] | | TG[b] | Oil[c] |
| 1,2,3-Triacyl-*sn*-glycerol | 16:0-18:1-18:1 | 56.88 | 4.82 | 18:1-18:2-18:1 | 55.54 | 9.20 | 18:1-18:2-18:2 | 15.21 | 1.80 |
| | 18:1-18:1-16:0 | 36.16 | 3.07 | 18:1-18:1-18:2 | 11.51 | 1.90 | 18:2-18:2-18:1 | 72.85 | 8.64 |
| | 18:1-16:0-18:1 | 6.96 | 0.59 | 18:2-18:1-18:1 | 32.95 | 5.45 | 18:2-18:1-18:2 | 11.94 | 1.41 |

| | | Peanut oil | | Cottonseed oil | |
|---|---|---|---|---|---|
| Triacylglycerol | | 16:0 18:1 18:2 | | | |
| Mole %[a] | | 11.75 | | 16.40 | |
| | | Mole % | | Mole % | |
| | | TG[b] | Oil[c] | TG[b] | Oil[c] |
| 1,2,3-Triacyl-*sn*-glycerol | 16:0-18:1-18:2 | 5.78 | 0.68 | 22.13 | 3.63 |
| | 18:2-18:1-16:0 | 10.59 | 1.24 | 18.64 | 3.06 |
| | 16:0-18:2-18:1 | 53.88 | 6.34 | 29.37 | 4.82 |
| | 18:1-18:2-16:0 | 27.05 | 3.18 | 28.72 | 4.71 |
| | 18:1-16:0-18:2 | 0.88 | 0.10 | 0.59 | 0.10 |
| | 18:2-16:0-18:1 | 1.82 | 0.21 | 0.55 | 0.08 |

[a]Mole % in total oil triacylglycerols.
[b]Mole % in the triacylglycerols.
[c]Mole % in total oil triacylglycerols.

**TABLE 5**

**Distribution of Fatty Acids in the Three Positions of the Glycerol Moiety of Palmitoyloleoyllinoleoyl-*sn*-Glycerol from Peanut Oil and Cottonseed Oil**

| Fatty acids | Positions 1, 2, 3 | *sn*-1 | | *sn*-2 | | *sn*-3 | | Total oil TGs | |
|---|---|---|---|---|---|---|---|---|---|
| | | Peanut oil | Cottonseed oil | Peanut oil | Cottonseed oil | Peanut oil | Cottonseed oil | Peanut oil | Cottonseed oil |
| 16:0 | 33.3 | 59.7 | 51.5 | 2.7 | 1.1 | 37.6 | 47.4 | 13.1 | 29.5 |
| 18:1 | 33.3 | 27.9 | 29.3 | 16.4 | 40.8 | 55.7 | 29.9 | 47.0 | 18.6 |
| 18:2 | 33.3 | 12.4 | 19.2 | 80.9 | 58.1 | 6.7 | 22.7 | 29.9 | 46.4 |

of the three fatty acids for the *sn*-1-, *sn*-2- and *sn*-3-positions, respectively. It is represented by the percentage of each fatty acid esterified at each position. Results are reported in Table 5. When examined horizontally, data represent affinity of each fatty acid for the three positions. It is as follows:

palmitic acid (16:0) : pos. 1 > pos. 3 >> pos. 2

oleic acid (18:1) : pos. 3 >> pos. 1 > pos. 2

linoleic acid (18:2) : pos. 2 >> pos. 1 > pos. 3

When examined vertically, data represent affinity of the three fatty acids for each position. It is as follows:

position 1 : 16:0 > 18:1 > 18:2

position 2 : 18:2 >> 18:1 >> 16:0

position 3 : 18:1 > 16:0 >> 18:2

Results obtained with the peanut oil triacylglycerol can be compared to those calculated for the same triacylglycerol from cottonseed oil also reported in Table 5.

Curiously, each fatty acid of this same triacylglycerol presents approximately the same affinity for the external *sn*-1- and *sn*-3-positions, contrary to what was observed previously. The order of affinity for the *sn*-3-position was the reverse of that observed in peanut oil, *i.e.*:

16:0 > 18:1 > 18:2.

TABLE 6

**Distribution of the Major Fatty Acids at the *sn*-1,2,3-Positions of Peanut Oil Total Triacylglycerols and Composition in Molecular Species of Peanut Oil Triacylglycerols as Predicted by a 1-Random-2-Random-3-Random Hypothesis and as Experimentally Determined**

| Fatty acid | 16:0 | | | 18:1 | | | 18:2 | | |
|---|---|---|---|---|---|---|---|---|---|
| Position | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| Mole % | 23.36 | 1.28 | 15.78 | 39.02 | 44.48 | 50.78 | 30.32 | 54.06 | 10.58 |

| 1,2,3-Triacyl-*sn*-glycerol | Random[a] mole % | Exp. mole % | Difference (%) | 1,2,3-Triacyl-*sn*-glycerol | Random mole % | Exp. mole% | Difference (%) |
|---|---|---|---|---|---|---|---|
| 18:1-18:1-18:1 | 8.81 | 10.66 | +21 | 18:1-18:1-18:2 | 1.84 | 1.90 | + 3.3 |
| 18:1-18:2-18:1 | 10.71 | 9.20 | −14 | 18:1-18:2-18:2 | 2.23 | 1.80 | − 19 |
| 18:2-18:2-18:1 | 8.40 | 8.64 | + 2.9 | 18:2-18:1-18:2 | 1.43 | 1.41 | − 1.4 |
| 16:0-18:2-18:1 | 6.41 | 6.34 | − 1.1 | 18:2-18:1-16:0 | 2.13 | 1.24 | − 42 |
| 18:2-18:1-18:1 | 6.85 | 5.45 | −20 | 16:0-18:1-18:2 | 1.10 | 0.68 | − 38 |
| 16:0-18:1-18:1 | 5.28 | 4.82 | − 8.7 | 18:1-16:0-18:1 | 0.25 | 0.59 | +136 |
| 18:1-18:2-16:0 | 3.33 | 3.18 | − 4.5 | 18:2-16:0-18:1 | 0.20 | 0.21 | + 5.0 |
| 18:1-18:1-16:0 | 2.74 | 3.07 | +12 | 18:1-16:0-18:2 | 0.05 | 0.10 | +100 |
| 18:2-18:2-18:2 | 1.73 | 2.08 | +20 | | | | |

[a]% *sn*--XYZ = [mole % X at *sn*-1-position × [mole % Y at *sn*-2-position] × [mole % Z at *sn*-3-position] × ($10^{-4}$).

The affinity for the *sn*-2-position was still 18:2 > 18:1, but to a much lesser extent than in the previous case.

The order of affinity observed in the triacid-triacylglycerol of peanut oil also can be observed in the diacid-triacylglycerols (upper part of Table 4). In the first one, 16:0 18:1 18:1, the proportion of molecular species reflects affinity of 16:0 for the *sn*-1-position and of 18:1 for the *sn*-3-position. In the second one, 18:1 18:1 18:2, the high affinity of 18:2 for the *sn*-2-position is striking, even when competing with two molecules of 18:1. The preference of 18:1 for the *sn*-3-position is also clearly confirmed in this triacylglycerol, as well as in the next one (18:1 18:2 18:2).

In Table 6 the experimentally determined percentages of the 17 identified and quantified peanut oil triacylglycerol molecular species can be compared to the percentages predicted by a 1-random-2-random-3-random hypothesis (1). They were calculated from the positional distribution in the total oil triacylglycerols of the three fatty acids present in the 17 triacylglycerols.

For the major triacylglycerols, the difference between the two values lay between +21 and −20% (13% average for the first nine triacylglycerols in Table 6). For the two monoacid-triacylglycerols (trioleoyl- and trilinoleoylglycerol) the found percentages were 21 and 20%, respectively, higher than the value calculated according to the random distribution.

## DISCUSSION

Two main points in this study deserve to be discussed. The first point pertains to the method used to determine the molecular species composition of triacylglycerols. The method is quite suitable for diacid-triacylglycerols. For triacid-triacylglycerols an approximation had to be made to solve the equation set. In the case of peanut and cottonseed oil (generally in vegetable oils) the approximation was of little importance, because the proportion of the two molecular species with the long-chain saturated fatty acid in the internal position is low. The problem would be larger with other vegetable oils, such as coconut oil (15,16) or palm-kernel oil (15,17), both of which are rich in medium-chain saturated fatty acids. It would also be difficult with several animal fats, such as lard (7,15), in which palmitic acid is frequently encountered at the *sn*-2-position, or bovine milk fat (15,18), which is rich in short- and medium-chain saturated fatty acids, or even the highly unsaturated fish oils (7,15). For these oils and fats there is no reason that a family of two molecular species comprising a given saturated or unsaturated fatty acid at the *sn*-2-position would occur in low proportion. In those cases, additional independent experimental data have to be obtained to determine an accurate triacylglycerol composition. Several possibilities can be considered: Fractionation can be attempted for the three pairs of triacylglycerols characterized by the fatty acid at the *sn*-2-position. Argentation-HPLC could be a suitable method (19). In this case, direct enantiomer analysis by chiral-phase HPLC of the *sn*-1,2(2,3)-diacylglycerol mixture derived from each pair of molecular species by partial chemical deacylation would be possible because each enantiomer would comprise only two species of different equivalent carbon number, easily resolved by chiral-phase HPLC (7,8). Another way to know which fatty acid occupies one or the other position in the enantiomer would be to collect each enantiomer for analysis of the fatty acid distribution between the internal (*sn*-2-) and the external (*sn*-1- or *sn*-3-) position. The stereospecific deacylation by an appropriate enzyme could be considered.

Although this would add a supplementary fractionation step to a method that is already somewhat laborious and time-consuming, the number of fractionations, first by argentation-TLC and then by reversed-phase HPLC, could be highly reduced by using preparative materials and methods. Nevertheless, the most suitable method would probably be a combination of chiral-phase HPLC and mass spectrometry, or capillary-GC analysis of the enantiomers, such as those developed by Kuksis' group (20).

Another point in the method we propose in this paper is that the proportion of the molecular species couples presenting a given fatty acid esterified at the *sn*-2-position was determined from the *sn*-1,2(2,3)-diacylglycerol species composition. In Brockerhoff's (2) or Kuksis' method (3),

**TABLE 7**

**Composition in 1,2,3-Triacyl-*sn*-Glycerols Determined from Partial Acylglycerols Formed by Chemical or Enzymatic Partial Deacylation**

| Peanut oil | 16:0 18:1 18:1 | | 18:1 18:1 18:2 | | 18:1 18:2 18:2 | |
|---|---|---|---|---|---|---|
| Isomers | 16:0-18:1-18:1<br>18:1-18:1-16:0 | 18:1-16:0-18:1 | 18:1-18:1-18:2<br>18:2-18:1-18:1 | 18:1-18:2-18:1 | 18:1-18:2-18:2<br>18:2-18:2-18:1 | 18:2-18:1-18:2 |
| *sn*-1,2(2,3)-Diacylglycerols[a] | 93.04 | 6.96 | 44.46 | 55.54 | 88.06 | 11.94 |
| *sn*-2-Monoacylglycerols[b] | 96.71 | 3.29 | 43.40 | 56.60 | 88.70 | 11.30 |
| Difference[c] | 3.8% | | 1.9% | | 0.72% | |

| | Peanut oil 16:0 18:1 18:2 | | | Cottonseed oil 16:0 18:1 18:2 | | |
|---|---|---|---|---|---|---|
| Isomers | 16:0-18:1-18:2<br>18:2-18:1-16:0 | 16:0-18:2-18:1<br>18:1-18:2-16:0 | 18:1-16:018:2<br>18:2-16:0-18:1 | 16:0-18:1-18:2<br>18:2-18:1-16:0 | 16:0-18:2-18:1<br>18:1-18:2-16:0 | 18:1-16:0-18:2<br>18:2-16:0-18:1 |
| *sn*-1,2(2,3)-Diacylglycerols[a] | 16.38 | 80.91 | 2.71 | 40.77 | 58.09 | 1.14 |
| *sn*-2-Monoacylglycerols[b] | 19.57 | 79.89 | 0.54 | 39.32 | 58.62 | 2.06 |
| Difference[c] | | 1.3% | | | 0.90% | |

[a]Calculated from the percentages of the *sn*-1,2(2,3)-diacylglycerols formed by partial chemical degradation of triacylglycerols.

[b]Calculated from the fatty acid composition of the *sn*-2-monoacylglycerols formed by enzymatic (pancreatic lipase) hydrolysis of triacylglycerols.

[c]Calculated, as %, from the higher values.

fatty acids esterifying the *sn*-2-position of glycerol were deduced from the composition of the *sn*-2-monoacylglycerols issued from pancreatic lipase hydrolysis. Data reported in Table 7 show that values obtained from *sn*-1,2(2,3)-diacylglycerols issued from chemical deacylation are close to those obtained from *sn*-2-monoacylglycerols issued from enzymatic deacylation for the major triacylglycerol couples. The difference was from 0.7 to 3.8% between the two values. However, for the minor couples comprising saturated fatty acid in the internal position, differences between the two low values were relatively high. We cannot propose any convincing explanation of this discrepancy. Because hydrolysis by pancreatic lipase also generates representative *sn*-1,2(2,3)-diacylglycerols (1,21), stereospecific analysis of triacylglycerols could also start with enzymatic hydrolysis. However, due to its simplicity, chemical deacylation is the most widely used method.

The second point that deserves discussion is the stereospecific distribution of fatty acids in peanut oil and cottonseed oil triacylglycerol molecules. The high affinity of linoleic acid for the *sn*-2-position (Table 5) confirms a property that has been known for a long time in vegetable oils in general (22), and in peanut oil in particular (10,23). This preference is more highly expressed in peanut oil (81% in the internal position in the triacylglycerol 16:0 18:1 18:2) than in cottonseed oil (58%). This peculiarity is related to the percentage of linoleic acid in total triacylglycerols of both oils. When this percentage is lower than 33.3% (peanut oil) the affinity can be expressed to a greater extent than when the percentage is superior to the maximum possibility of esterification at the *sn*-2-position. In that case the fatty acid must necessarily esterify external positions, decreasing the percentage at the internal position. The same reason can explain the relative high percentage of oleic acid at the *sn*-2-position in cottonseed oil 16:0 18:1 18:2. The distribution of fatty acids at the *sn*-2-position corresponds to what we previously observed in peanut oil (10) and cottonseed oil (24).

The *sn*-1-position was preferentially occupied by palmitic acid both in peanut and cottonseed oils (Table 5), whereas the *sn*-3-position was peferentially esterified by oleic acid in peanut oil and by palmitic acid in cottonseed oil. Indeed, in cottonseed oil 16:0 18:1 18:2 the three component fatty acids indifferently occupied one or the other external *sn*-1- or *sn*-3-position. The preference of 18:1 or 16:0 for the *sn*-3-position must be related to the fatty acid composition of the two oils. As demonstrated by De La Roche *et al.* (25) on 12 genotypes of maize seed of widely varying fatty acid composition and as illustrated by Litchfield (1), the frequency of fatty acids at the *sn*-3-position was more highly influenced by their respective concentration in total triacylglycerols than for the other two positions. As far as this property can be generalized, the more elevated frequency of 18:1 at the *sn*-3-position in peanut oil is most likely due to its high percentage in the oil (47%), whereas the percentage of 16:0 is higher in cottonseed oil (29%) than in peanut oil (13%).

In a previous study on another sample of cottonseed oil (26), based on Brockerhoff's method the 16:0 preference for the *sn*-3-position was still more pronounced.

The affinity of 16:0, 18:1, 18:2 for the *sn*-1-, *sn*-2- and *sn*-3-positions observed for peanut oil palmitoyloleoyl-

linoleoyl-*sn*-glycerol (Table 5) is also that observed in total triacylglycerols (upper part of Table 6). These latter values are in close agreement with results reported by Manganaro *et al.* (23) for North American peanut oil, because of close similarity of fatty acid compositions of both oils.

Comparison between the percentile proportion of the major molecular species of peanut oil triacylglycerols with those predicted by a 1-random-2-random-3-random distribution (Table 6) showed that the positional distribution of fatty acids was rather markedly nonrandom. In particular, the percentage of trioleoylglycerol (10.7%), which is the major triacylglycerol of peanut oil, was found to be 21% higher than that predicted by a random distribution (8.8%). The same was true for trilinoleoylglycerol.

It would be interesting to compare the stereospecific distribution of fatty acids in triacylglycerols of different vegetable oils to see whether species differences exist in the triacylglycerol biosynthesis in seeds. Recent results were published by Itabashi *et al.* (20) on the composition of enantiomeric diacylglycerols generated by chemical degradation of corn oil triacylglycerols and separated by chiral-phase HPLC. This type of oil presents the same three major fatty acids (16:0, 18:1, and 18:2) as peanut and cottonseed oil, although most likely in different proportions. If we assume that 18:2 is preferentially esterified at the *sn*-2-position, according to these results 16:0 would be more frequently found at the *sn*-1-position when compared to the *sn*-3-position, whereas 18:1 would prefer the *sn*-3-position, but to a lesser extent. This tentatively determined stereospecific distribution of 18:1 and 16:0 would be rather close to that observed in this work with peanut oil triacylglycerols. However, more detailed results are needed for an appropriate comparison to be made.

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