Triacylglyceride Composition of Cottonseed Oil by HPLC and GC

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The triacylglyceride components of cottonseed oil were isolated and positively identified by a combination of high performance liquid chromatography (HPLC) and gas chromatography (GC). Both reversed-phase HPLC and capillary GC were capable of separating the oil into triacylglyceride peaks. These peaks were isolated by HPLC and their component acyl groups were converted to fatty acid methyl ester derivatives. The acyl constituents for each triacylglyceride were determined by GC analysis, thus positively identifying the triacylglyceride associated with each HPLC peak. The triacylglyceride elution order agreed with predictive methods. HPLC and capillary GC peaks were correlated by peak area, thus identifying the GC peaks. The corresponding GC elution order of triacylglycerides also agreed with predictive methods.

KEY WORDS: Capillary GC, cottonseed oil, HPLC, triacylglyceride, triglyceride.

Our research has been directed toward elucidating the effects of varietal differences, cultural practices and environmental conditions during growth and storage after harvest on cottonseed quality, particularly on the quality of the cottonseed oil. In these studies, it was desirable not only to evaluate the oil by conventional techniques, such as color and free fatty acid content, but also to determine the composition and relative amounts of the component triacylglycerides (TGs). Because large numbers and types of samples were involved and because in some cases limited amounts of oil were available, the method used for TG analysis not only had to be rapid, precise, and accurate, but also applicable to <1.0 g of cottonseed oil, which could range in quality from very good to very poor. Available methods for TG analysis included thin-layer chromatography (TLC), gas chromatography (GC) high-performance liquid chromatography (HPLC), mass spectrometry (MS), low-temperature crystallization or combinations of these techniques (1–6). Among these, HPLC and GC appeared to provide the speed, accuracy and versatility required. However, neither method had positively identified the component TG peaks; instead, they were only tentatively identified by predictive methods. There has been one reported attempt to isolate and identify the TG peaks eluted from HPLC, but the chromatogram obviously had poor resolution (7; chromatogram not shown). Previously, the TG composition of cottonseed oil had been determined by a combination of chemical and enzymatic hydrolysis (8). Unfortunately, there was no correlation between the results from these studies and data from HPLC or capillary GC separation methods. This paper reports the positive identification of the triacylglycerides in cottonseed oil and the applicability of either reversed-phase HPLC or capillary GC for verification of their presence, based on comparative analysis of a standard sample of oil by both methods and the GC analysis of the fatty acid methyl esters derived from each TG peak obtained from the HPLC analysis.

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EXPERIMENTAL PROCEDURES

Chemicals. Cottonseed oil and triacylglyceride (TG) standards were from Sigma Chemical Co. (St. Louis, MO). Fatty acid methyl ester (FAME) standards were from Supelco, Inc. (Bellefonte, PA). HPLC-grade acetone was from J.T. Baker Inc. (Phillipsburg, NJ). B&J-brand, HPLC-grade acetonitrile was from American Scientific Products (McGaw Park, IL). Meth-Prep II was from Alltech/Applied Science (Deerfield, IL).

HPLC. High-performance liquid chromatography was performed on a reversed-phase C-18 column system—two Waters 3.9 × 150 mm, 60Å, 4 micron, Nova Pak columns in series (Waters Associates, Millford, MA) preceded by a Bio-Rad Micro-Guard ODS-10 guard column (Bio-Rad Labs, Richmond, CA). A Bio-Rad HPLC Column Heater maintained the system at 34°C. The Waters Maxima 820 Chromatography software was used for data handling and for control of the Waters 6000A pump and Waters 410 Differential Refractometer. Sensitivity was set at 32 and the scale factor at 20. The eluant was acetone/acetonitrile (60:40) as an isocratic mixture at a flow rate of 1.5 mL/min. It was degassed by sonication and cooled to 4°C.

GC (triacylglyceride separation). Gas chromatography of TGs was accomplished with a Hewlett-Packard 1089 GC (Palo Alto, CA) equipped with a splitless, cold-on-column injector (J&W Scientific) and a Chrompack TAP capillary column (triglyceride analysis phase; 25 m × 2.5 mm fused silica column with special 0.1 μm 50% phenol/50% methyl polysiloxane) at a column inlet pressure of 1.0 bar. Hydrogen was the carrier gas. The detector temperature was 400°C. A temperature gradient of 1°/min from 340°C to 360°C was used. The sample used for injection was a 0.1% solution of cottonseed oil in benzene.

HPLC isolation of TGs. For collection of TG fractions the HPLC system was modified as follows: A Waters Associates R401 Differential Refractometer replaced the 410 detector and the columns were kept at ambient temperature. This was necessary because the 410 detector is constructed with several feet of large ID tubing on the outlet side of the detection cell, which dilutes and mixes fractions eluted from the column. From a sample of cottonseed oil in acetone (100 mg/mL), four injections (15 μL each) were employed to obtain usable quantities of each TG. Nine fractions were collected representing peaks with retention times of 12.2, 16, 17, 21.8, 23, 25.3, 30.1, 32 and 34.7 min.

GC (fatty acid determination). Each of the nine TG fractions was transesterified to their component FAMEs with Meth-Prep II (0.2N methanolic solution of m-trifluoromethylphenyl trimethylammonium hydroxide). Gas chromatography of FAMES was performed with a Tracor 540 GC (Tracor, Inc., Austin, TX) in which a 6′ × 1/4″ × 2 mm ID glass column packed with 10% Silar 10CP on Gas

Chrom Q, 100/120 mesh was used. Detector and injection temperature was 250°C. Oven temperature was programmed from 170°C, 3 min initial hold, 3°C/min to 205°C and a final hold of 10 min. Helium was the carrier gas. Data were collected on an HP 3392A integrator. FAME peaks were identified by comparison of retention times to a standard mixture. The retention times of the palmitic, stearic, oleic, linoleic and linolenic FAMEs were 5.0, 7.8, 8.7, 10.3 and 12.2 min, respectively.

RESULTS AND DISCUSSION

The purified cottonseed oil from Sigma was used to establish the HPLC triacylglyceride (TG) profile. Crude oil samples were found to have identical profiles, except that they contained additional earlier-eluting mono- and diacylglyceride peaks. As shown in Figure 1, cottonseed oil as separated by HPLC into nine isolatable peaks. The elution sequence of TGs was predicted based on their theoretical carbon number (TCN) (9). It has been reported that 96% of cottonseed oil is composed of the three fatty acids—palmitic (P), oleic (O) and linoleic (L), at 25%, 20% and 52%, respectively (8). Therefore, the TCN of all combinations of P, O and L were calculated according to the equation TCN = ECN − (.7)L − (.6)O where ECN = CN − 2(ND); ECN is the equivalent carbon number, CN is the number of carbons in the three fatty acids of the triacylglyceride, ND is the number of double bonds, L is the number of linoleic acids, and O is the number of oleic acids. Table 1 shows the possible triacylglycerides and their TCN values.

The groupings of the possible TGs based on their TCN values matches the 1:2:3:3 grouping seen in the tracing from the HPLC (Fig. 1). There are, however, four possible TGs for the last grouping. To correlate the TCN values with the TG retention times, three standard TGs, LLL, OOO and PPP, were used as retention time references. As seen in Figure 2, projections of TCN values of the cottonseed oil TGs onto the line through the three standards give retention time values that closely correlate with experimental values. Also, from this study, it can be seen that PPP is the TG missing from the last set of peaks.

A similar sample of cottonseed oil was analyzed for TG composition by capillary GC. As shown in Figure 3, GC separated the oil into 17 distinguishable peaks. In the predicted order of elution from the GC column, TGs with the smaller CN elute first and the retention time of those with the same CN is proportional to the number of double bonds (ND). Table 2 lists this predicted order of elution of TGs containing P, O and L acids.

To determine which of the 17 GC peaks corresponded to the 9 HPLC peaks, the areas of the GC peaks were cross-matched with those of the HPLC peaks. As seen in Table 3, the predicted elution order from the GC (Table 2) is obtained when this operation is performed. GC peaks that do not correspond to HPLC peaks account for 7% of the total GC peak area. Based on previous studies, these peaks are probably TGs containing stearic (18:0), palmitoleic (16:1) and myristic (14:0) acid; cottonseed oil contains about 2% stearate, 1% palmitoleic and 1% myristic acid (8). Also, the area of the predicted peaks should give a fatty acid composition comparable to literature values reported for cottonseed oil. Calculations from the areas of both the GC and the HPLC chromatograms give P, O and L percentages of 24, 19 and 57, respectively. These values match closely with the 25, 20 and 52% reported for cottonseed oil (8).

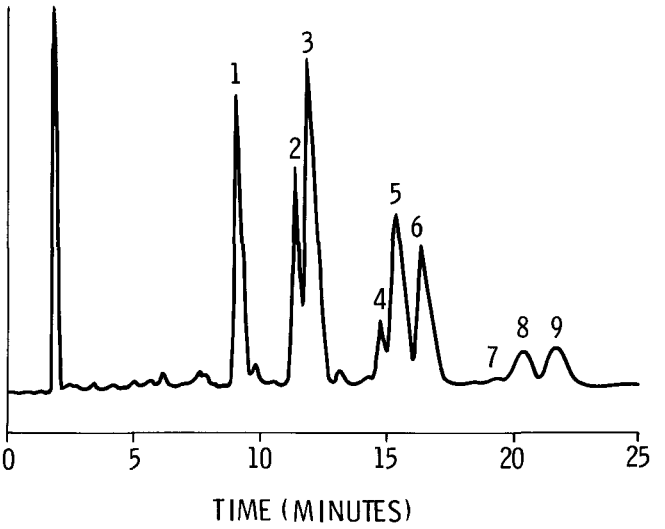

FIG. 1. HPLC of cottonseed oil. Conditions: two Waters 3.9 × 150 mm, 60Å, 4 micron, Nova Pak reversed-phase C18 columns, acetone/acetonitrile (60:40) isocratic mixture at a flow rate of 1.5 mL/min, detection by differential refractometry. Numbered peaks were isolated and used for correlation in Tables 3 and 4.

TABLE 1

Predicted Elution Order of Triacylglycerides from RP-HPLC

TG[a]	TCN[b]	ECN[c]	CN:ND[d]
LLL	39.9	42	54:6
OLL	42.0	44	54:5
PLL	42.6	44	52:4
OOL	44.1	46	54:4
POL	44.7	46	52:3
PPL	45.3	46	50:2
OOO	46.2	48	54:3
POO	46.8	48	52:2
PPO	47.4	48	50:1
PPP	48.0	48	48:0

[a]Triacylglycerides containing P, O and L.
[b]Theoretical carbon number: TCN=ECN-(0.6)O-(0.7)L.
[c]Equivalent carbon number: ECN=CN-(2)ND.
[d]Actual carbon number/number of double bonds in the molecule.

To positively characterize the composition of the TGs of cottonseed oil, the nine peaks separated by HPLC were isolated. Each TG was transesterified to its component fatty acid methyl ester (FAME). The resulting FAMEs were then identified by GC based on comparison of retention time with FAME standards. The calculated TG composition of each peak is given in Table 4. The elution order of the major TG of each peak coincides with the predicted order given in Table 1. Portions of PLL (peak 3) seem to have streaked or have been held up on the column to coelute with the first peak of the following two sets of peaks (peaks 4 and 7). Also, peak 7 was found to overlap slightly with peak 8.

Although peaks 5, 8 and 9 were predominantly POL,

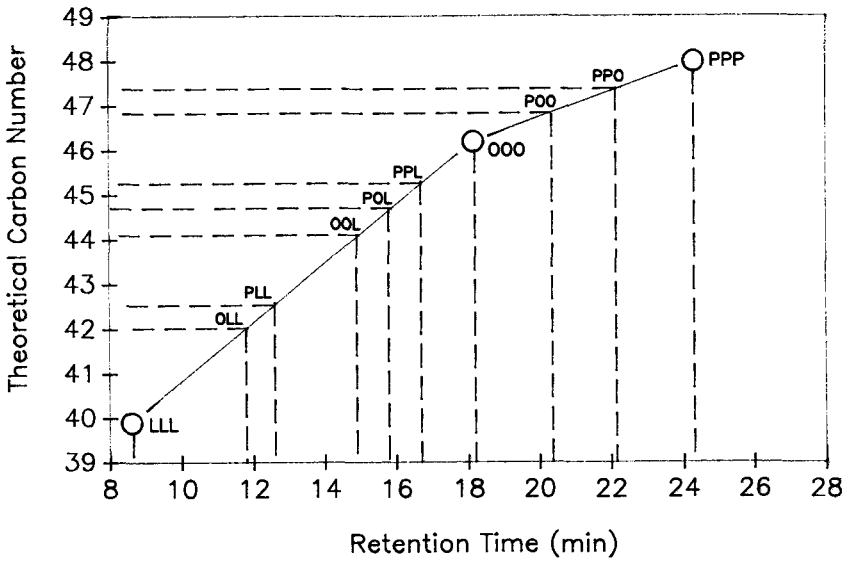

FIG. 2. Relationship between theoretical carbon number (TCN) and HPLC retention time based on the retention time of the standard triacylglycerides LLL, OOO and PPP.

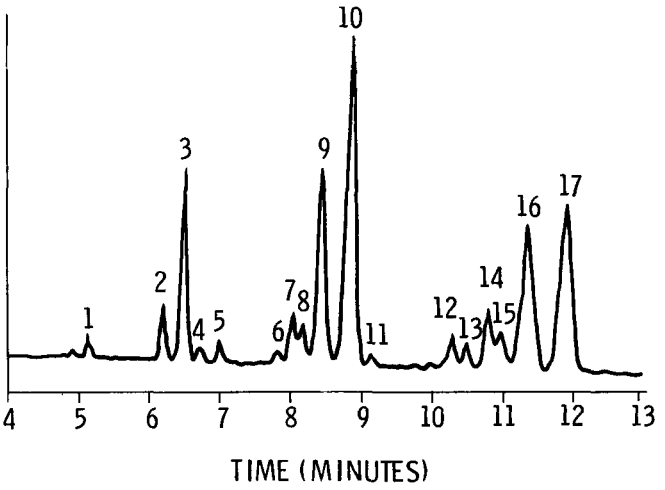

FIG. 3. Capillary GC of cottonseed oil. Conditions: Chrompack TAP capillary column with a temperature gradient of 1°C/min from 340°C to 360°C. Numbered peaks were used for correlation in Tables 3 and 4.

TABLE 2

Predicted Elution Order of Triacylglycerides from GC

TG[a]	CN[b]	ND[c]
PPO	50	1
PPL	50	2
POO	52	2
POL	52	3
PLL	52	4
OOO	54	3
OOL	54	4
OLL	54	5
LLL	54	6

[a]Triacylglycerides containing P, O and L.
[b]Number of carbon atoms in the molecule.
[c]Number of double bonds in the molecule.

TABLE 3

GC Elution Order by Comparison of HPLC and GC Peak Areas

GC peak no.[a]	Area no.[b]	HPLC peak no.[c]	GC TG order[d]
1	15	—	—
2	8	9	PPO
3	5	6	PPL
4	13	—	—
5	14	—	—
6	16	—	—
7	7	8	POO
8	11	—	—
9	3	5	POL
10	1	3	PLL
11	17	—	—
12	9	7	OOO
13	12	—	—
14	6	4	OOL
15	10	—	—
16	4	2	OLL
17	2	1	LLL

[a]Elution order of TGs from the GC; numbers correspond to Figure 3.
[b]Area of GC peaks listed numerically: 1, largest; 15, smallest.
[c]HPLC peak (numbered as in Fig. 1) with peak area number corresponding to column 2.
[d]TG corresponding to HPLC peak number in column 3, giving GC TG order.

POO and PPO, respectively, they also contained TGs that included stearate. The TCN values of SLL, SOL and SPL are 44.6, 46.7 and 47.3, respectively. These TCN values coincide with those of POL, POO and PPO (Table 1), the peaks in which they coelute. By using the percentages of stearate-containing TGs from Table 4 and the area of the HPLC peak it coelutes with, the percentage of stearate in cottonseed oil is calculated to be 1.4%. This value concurs with the reported value of 2% (8). Based on Table 2, the three stearate TGs should elute from the GC in the order SPL, SOL and SLL. The GC peaks in Table 3 that

TABLE 4

HPLC TG Composition as Determined by GC Analysis of FAMEs

Peak no.[a]	TG composition (area %)[b]
1	LLL (90)
2	OLL (97)
3	PLL (94)
4	OOL (70), PLL (30)
5	POL (87), SLL (9)
6	PPL (97)
7	OOO (81), PLL (17)
8	POO (63, SOL (26), OOO (10)
9	PPO (54), SPL (38)

[a]HPLC peak number; numbers correspond to Figure 1.
[b]TG determined from GC FAME peaks/GC peak area % of TG.

TABLE 5

Comparison of GC and HPLC TG Peak Area Percentage

TG	(PI)[a]	GC area %	HPLC area %[b]
PLL	(LLP)	25.7	27.5
LLL	(LLL)	16.1	19.0
POL	(OLP)	14.0	14.0
OLL	(OLL)	12.9	12.5
PPL	(PLP)	8.7	7.1
OOL	(OOL)	4.4	3.1
POO	(OOP)	3.3	3.1
PPO	(POP)	2.5	2.2
OOO	(OOO)	2.4	1.6
SLL	(SLL)	2.4	1.4
SPL	(SLP)	2.1	1.5
SOL	(SOL)	1.5	1.3

[a]Major positional isomer as determined by Bezard *et al.* (8,12).
[b]Corrected with percentages listed in Table 4.

correspond to these TGs are numbers 8, 13 and 15, respectively.

Thus, the triacylglycerides of cottonseed oil have been identified by HPLC and GC and their relative ratios have been measured. Predictive methods proved to be both accurate and useful. From the combination of calculated and analytical data, the TGs of cottonseed oil, in decreasing order of relative amounts, are listed in Table 5. The areas from GC contain a smaller degree of error because the correction computed into the HPLC area is based only on the portion of the peak that was collected for the fatty acid analysis. The separating power of the GC was excellent for verification of and ascertaining the proportion of TGs, especially minor constituents. However, the information obtained from the HPLC analysis was required before positive identification of the GC peaks could be achieved. Previously, GC had been used for the tentative identification of TGs by correlation of their retention time with that of standards. However, this was possible only if standards were available from commercial sources or through the process of enzymatic interesterification (10).

By using multiple techniques, this work provides evidence for conclusive identification of cottonseed oil TGs by HPLC or GC. Previous literature reports identified only a few of the TGs (7) or based identification only on predictive methods (11). Recent studies by Bezard *et al.* (8,12) stereospecifically identified the TGs of African cottonseed oil from enzymatic hydrolysis, but did not correlate the data with any chromatographic method. However, the predominant positional isomer of each TG as determined in these studies is included in Table 5 for completeness. Further studies on cottonseed oil TGs should be enhanced by the analytical methods developed in this study.

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