Triacylglycerol Composition and Structure in Genetically Modified Sunflower and Soybean Oils

Johanna Reske, Jodi Siebrecht, and Jan Hazebroek*

Pioneer Hi-Bred International, Inc., Johnston, Iowa 50131-1004

ABSTRACT: Changes in composition were examined in oils extracted from genetically modified sunflower and soybean seeds. Improvements were made to the analytical methods to accomplish these analyses successfully. Triacylglycerols (TAG) were separated on two 300 mm × 3.9 mm 4µ Novapak C18 high-performance liquid chromatography (HPLC) columns and detected with a Varex MKIII evaporative light-scattering detector. Peaks were identified by coelution with known standards or by determining fatty acid composition of eluted TAG by capillary gas chromatography (GC). Stereospecific analysis (fatty acid position) was accomplished by partially hydrolyzing TAG with ethyl magnesium bromide and immediately derivatizing the resulting diacylglycerols (DAG) with (*S*)-(+)-1-(1-naphthyl)ethyl isocyanate. The derivatized *sn*-1,2-DAG were completely resolved from the *sn*-2,3-DAG on two 25 mm × 4.6 mm 3 µ silica HPLC columns. The columns were chilled to –20°C to obtain baseline resolution of collected peaks. The distribution of fatty acids on each position of the glycerol backbone was derived from the fatty acid compositions of the two DAG groups and the unhydrolyzed oil. Results for the *sn*-2 position were verified by hydrolyzing oils with porcine pancreatic lipase, isolating the resulting *sn*-2 monoacylglycerols by TLC, and determining the fatty acid compositions by GC. Results demonstrated that alterations in the total fatty acid composition of these seed oils are determined by the concentration of TAG species that contain at least one of the modified acyl groups. As expected, no differences were found in TAG with fatty acid quantities unaffected by the specific mutation. In lieu of direct metabolic or enzymatic assay evidence, the authors' positional data are nevertheless consistent with TAG biosynthesis in these lines being driven by the mass action of available acyl groups and not by altered specificity of the acyltransferases, the compounds responsible for incorporating fatty acids into TAG.

JAOCS 74, 989–998 (1997).

KEY WORDS: Fatty acid composition, genetic modification, sunflower oil, soybean oil, triacylglyceride composition, triacylglyceride stereospecificity.

Modification of seed oil quality in soybean, sunflower, and canola by genetically altering fatty acid composition of storage lipids is a commercial goal of Pioneer Hi-Bred. The rationale behind this line of product development is that many value-added functional and nutritional attributes of a vegetable oil are determined by fatty acid composition. However, fatty acid distribution alone does not fully predict the value of an oil in the marketplace. Proper assessment of a specialty oil requires functional testing that mimics its targeted use. Unfortunately, in the early stages of a breeding program, the amount of oil available for testing is almost always less than that needed for these functional tests. Thus, generating the required amounts usually requires one or more generations of growouts, which are costly in both time and resources.

The functional and nutritional characteristics of an oil are affected by triacylglycerol composition, position of the fatty acids on the glycerol backbone (stereospecificity), and the levels of minor constituents. However, the basis for these relationships is not understood completely (1–6). Nevertheless, the functional and/or nutritional properties, that are determined by these chemical characteristics directly affect the value of specialty oils with genetically altered fatty acid profiles. Thus, to characterize these attributes in an altered oilseed germplasm, the authors analyzed triacylglycerol (TAG) structure in genetically modified sunflower and soybean seed oils produced by Pioneer Hi-Bred. Characterization of tocopherols and phytosterols, two minor constituents known to influence oil stability and nutritional properties, will be reported in subsequent papers.

EXPERIMENTAL PROCEDURES

Materials. TAG composition and stereospecific distribution of fatty acids were determined on crude sunflower and soybean oils with altered fatty acid profiles. Levels of saturated, monounsaturated, and polyunsaturated fatty acids were modified in this germplasm. All profiles were generated through classical mutation/selection breeding techniques.

Ethyl magnesium bromide, 4-pyrrolidinopyridine, (*S*)-(+)-1-(1-naphthyl)ethyl isocyanate and silver oxide were obtained from Aldrich (Milwaukee, WI). Trimethylsulfonium iodide was from ACROS Organics (Fair Lawn, NJ). Hydrochloric acid was from GFS Chemicals (Columbus, OH). 1-Propanol, toluene, and isooctane were from Sigma-Aldrich (Sigma, St. Louis, MO). Hexanes, chloroform, methyl alcohol, acetonitrile, and methylene chloride were from Mallinckrodt (Phillipsburg, NJ). Ethyl ether (without preservative) was from Baxter (Muskegon, MI). All of the previously listed solvents were of high-performance liquid chromatography (HPLC) grade. Ethyl

*To whom correspondence should be addressed.

E-mail: hazebroekjp@phibred. com.

alcohol and hexanes (technical grade) were from Fisher Scientific (Fair Lawn, NJ). Tripalmitin, tristearin, trilinolenin, and pancreatic lipase were from Sigma; triheptadecenoin was from Nu-Chek-Prep (Elysian, MN).

Thin-layer chromatography (TLC) silica gel G plates (20 × 20 cm, 1000 μ) were obtained from Analtech, Inc. (Newark, DE). Sep-pak Plus solid- phase extraction columns, each containing 360 mg C18 (SPE), were obtained from Waters (Milford, MA).

Sample preparation. Crude oils were extracted from sunflower achenes and soybean seeds with HPLC-grade hexane. Approximately 100 sunflower seeds were ground in a mortar and pestle with hexane in a 1:1 (wt/vol) ratio. This slurry stood in a closed container overnight, after which the extracted oils were decanted and centrifuged at 2000 rpm. Soybean seeds were ground to a fine powder in a mill (Regal Ware, Kewaskum, WI). Powder (10 g) was extracted in 40 mL technical-grade hexane over 3 h, occasionally stirred by hand, and passed through 0.2-μm filter. Solvent was removed from all extracts by evaporation at room temperature, and the crude oil was stored at 5°C in the dark.

TAG composition analysis. TAG were separated by reversed-phase HPLC with a Waters chromatography system, consisting of a WISP 712 autosampler and a model 680 Automatic Gradient Controller, that directed two model 510 pumps. Twenty-microliter samples (~15 mg oil in 1 mL acetone) were injected into two C18 columns (4 μ Nova-Pak, 300 mm × 3.9 mm i.d.; Waters) in series. The system was maintained at room temperature, and all oils dissolved readily in the initial mobile phase. TAG were eluted with a 1 mL/min acetonitrile/methylene chloride gradient from 25 to 45% methylene chloride in 90 min. This gradient afforded separation of most peaks in the majority of samples. Detection was achieved with a MKIII evaporative light-scattering detector (ELSD; Varex, Burtonsville, MD) at a drift tube temperature of 72°C and a N_2 nebulizer flow rate of 2.0 L/min (sunflower oils) or of 2.5 L/min (soybean oils). A representative TAG profile is shown in Figure 1. Peaks were integrated by Perkin-Elmer (Cupertino, CA) Turbochrom Version 3.2 software.

The identity of individual TAG peaks was determined by passing three 200-μL samples through a semipreparative column (Nova-Pak HR 300 mm × 19 mm i.d.; Waters) at room temperature, and determining the fatty acid composition of each eluted peak combined over the three runs. An adjustable stream splitter between the column and the detector directed 90% of the effluent to collection. The splitter was not used with the analytical column. The gradient profile described previously was used at a flow rate of 10 mL/min.

An external calibration curve was constructed with a triheptadecenoin standard in a high-oleate sunflower oil matrix. Thirteen levels, from 0.02–4.00 mg/mL, were run in duplicate, and mean integrated peak areas were plotted against concentration. Because detector response was not linear over the working range, a quadratic fit was used. TAG compositions are expressed as oil wt%.

TAG stereospecificity determination. The stereospecific distribution of fatty acids of TAG in unfractionated oils was determined with a modified method of Christie *et al.* (5). Crude oil samples (~40 mg) were dissolved in 2 mL dry diethyl ether. Five-hundred microliters of freshly prepared 0.5 M ethyl magnesium bromide (Grignard reagent) was added, and the mixture was shaken for 40 s to partially deacylate the TAG. Acetic acid (50 μL), 5 mL hexane (HPLC-grade), and 2 mL water were added to stop the reactions and extract products.

The organic layers were washed twice with 2 mL water and dried with anhydrous sodium sulfate. In an attempt to de-

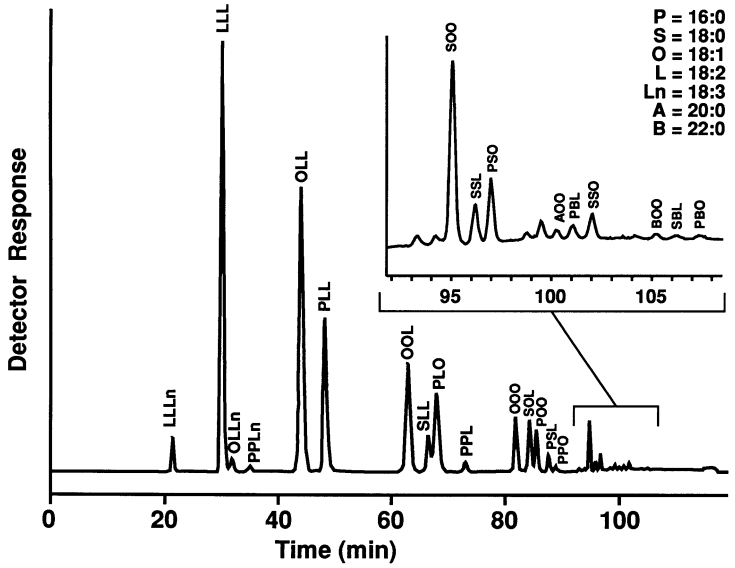

FIG. 1. Representative triacylglycerol profile. P, palmitate; S, stearate; O, oleate; L, linoleate; Ln, linolenate; A, arachidate; B, behenate.

crease acyl migration, hydrolysis and product extraction were performed over a bed of dry ice. The organic layers were dried under N_2 at 45°C and redissolved in 0.5 mL dry toluene.

Derivatizing agent, 12.5 μL (*S*)-(+)-1-(1-naphthyl)ethyl isocyanate, and catalyst, 4 mg 4-pyrrolidinopyridine, were added immediately, and samples were incubated at 50°C. After an overnight incubation, samples were dried under N_2 at 45°C, redissolved in 4 mL 95% aqueous methanol, and warmed to 40°C.

Derivatized diacylglycerols (DAG) were purified in C18 solid-phase extraction columns. Each column was solvated sequentially with 10 mL methanol, 10 mL water, and 10 mL 95% aqueous methanol. The dissolved lipid was applied, and the column was washed with a 10-mL aliquot of 95% aqueous methanol. The reaction products were eluted with 13 mL acetone and concentrated with a stream of N_2 at 45°C.

Diastereomeric *sn*-1,2- and *sn*-2,3-DAG derivatives were separated in the Waters high-performance liquid chromatograph described previously. Samples (20 μL) were injected into two silica columns (3 μ Hypersil, 250 mm × 4.6 mm i.d., Phenomenex; Torrance, CA), placed in series and chilled to −20 + 2°C . This temperature was empirically determined to yield maximal separation between the *sn*-1,2- and *sn*-2,3-DAG enantiomers. Freshly prepared mobile phase, 1-propanol (containing 2% water) in isooctane (0.4%, vol/vol), was delivered at 0.7 mL/min. Detection was achieved with a model 481 UV/VIS detector (Waters) at 280 nm. The *sn*-1,2- and *sn*-2,3-derivatives were collected as a group of unresolved molecular species and analyzed for fatty acid composition.

The effect of column temperature on the chromatographic separation of the DAG derivatives was determined by using homogeneous TAG of different molecular weights. Tripalmitin, tristearin, and trilinolenin standards were partially hydrolyzed by the Grignard reaction, and (*S*)-(+)-1-(1-naphthyl)ethyl urethane derivatives were synthesized by using the previously mentioned procedures. The resulting derivatized DAG were chromatographed individually and as a three-component mixture at room temperature and −20 ± 2°C. Retention times of individual DAG were used to calculate chromatographic selectivity at the two different temperatures.

TAG sn*-2 position determination.* TAG in unfractionated oils were subject to hydrolysis with porcine pancreatic lipase as described by Luddy *et al.* (7). The 2-monoacyl-*sn*-glycerols were isolated by TLC on preparative silica gel plates (20 cm × 20 cm × 1 mm thick), developed in 75:25:2 hexane/diethyl ether/acetic acid (vol/vol/vol). Products from two digestions were chromatographed on each plate. Lipids were visualized by brief exposure to I_2 vapor. Each monoacylglaycerol (MAG) band was transferred to a 20-mL glass scintillation vial. Ten milliliters of chloroform or 95:5 or 2:1 chloroform/methanol (vol/vol) were added, depending on the fatty acid content of the MAG. Increased solvent polarity, achieved by adding methanol, was required to fully dissolve highly unsaturated MAG. The mixture was shaken for 30 min at 320 rpm and decanted. Solvent (5 mL) was added to the silica, mixed vigorously for 5 s, centrifuged at 2000 rpm, and passed through a 0.45-μ polyvinylidene fluoride syringe filter (Gelman Sciences, Ann Arbor, MI). The eluted MAG were then analyzed for fatty acid composition.

Fatty acid analysis. Crude oil samples (~15 mg) were dissolved in 1 mL HPLC-grade hexane. Transmethylation was achieved with trimethylsulfonium hydroxide (TMSH) according to the method of Butte *et al.* (8). TMSH (100 μL) was used to transmethylate each crude oil sample. Fatty acid composition was determined by capillary GC in a Hewlett-Packard model 5890 Series II chromatograph (Palo Alto, CA), equipped with a flame-ionization detector and a split inlet with a split ratio of approximately 1:130. Methyl esters were separated on a fused-silica Carbowax capillary column, (15 m, 0.25 mm i.d., 0.25 μm film thickness; Supelco, Bellefonte, PA). Temperature of the inlet was set at 250°C, the detector was set at 300°C, and the oven was maintained at 220°C. The data are expressed as normalized percent of all identified fatty acid methyl esters.

Fatty acid composition of samples from TAG analysis, stereospecific analysis, and lipase hydrolysis were also analyzed by capillary GC. Samples were evaporated to dryness under N_2 at 45°C and redissolved in 1 mL dry toluene. Two milliliters 0.5 M sodium methoxide was added, and the samples were incubated at 50°C for 10 min. Acetic acid (100 μL) and 5 mL H_2O were added to stop the reaction. Fatty acid methyl esters were extracted twice in 3-mL aliquots of HPLC-grade hexane and concentrated under a stream of N_2 at 45°C. GC analysis was performed as described for crude oils.

RESULTS AND DISCUSSION

Fatty acid composition of crude oils. Fatty acid compositions of the genetically modified seed oils used in this study are given in Tables 1 and 2. In sunflower oils, palmitic, oleic, and linoleic acid levels have been manipulated specifically, while in soybean oils, palmitic, stearic, and linolenic acids were the targets.

TABLE 1
Fatty Acid Composition of Sunflower Seed Triacylglycerols (mol%)

	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1	24:0
Commodity	0.1	7.0	0.2	4.8	27.8	58.7	0.1	0.3	0.1	0.7	0.1	0.3
High oleic (HO)	0.0	3.1	0.1	1.5	91.5	2.1	0.0	0.2	0.4	0.7	0.1	0.3
High linoleic (HL)	0.1	7.5	0.1	1.9	13.3	76.0	0.1	0.1	0.2	0.4	0.0	0.2
High stearic/high oleic (HS/HO)	0.0	4.6	0.1	11.0	79.1	2.0	0.1	0.9	0.2	1.8	0.0	0.3
High palmitic/high oleic (HP/HO)	0.0	24.6	6.1	2.9	59.8	3.5	0.1	0.4	0.2	1.8	0.1	0.6
High palmitic/high linoleic (HP/HL)	0.1	27.3	4.4	2.7	17.1	46.8	0.1	0.3	0.1	0.9	0.1	0.3

TABLE 2
Fatty Acid Composition of Soybean Seed Triacylglycerols (mol%)

	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1	24:0
Commodity	0.1	10.2	0.1	4.1	23.4	52.8	8.3	0.3	0.2	0.3	0.0	0.1
Low saturate (Lsat)	0.0	3.5	0.1	2.8	22.7	60.3	9.8	0.2	0.3	0.2	0.0	0.1
High linoleic (LLn)	0.1	10.9	0.1	5.7	27.5	51.5	3.0	0.5	0.2	0.4	0.0	0.1
High palmitic (HP)	0.1	23.8	0.7	3.8	15.4	44.1	11.0	0.4	0.1	0.6	0.0	0.1
High stearic (HS)	0.0	8.0	0.1	24.7	17.2	39.2	8.3	1.5	0.1	0.7	0.0	0.1
High saturate (Hsat)	0.1	21.9	0.3	17.5	9.4	37.5	11.0	1.3	0.1	1.0	0.0	0.2
Lsat/LLn	0.0	4.1	0.1	3.4	28.3	60.6	2.7	0.2	0.3	0.3	0.0	0.1
HP/LLn	0.1	19.2	0.8	4.1	23.2	48.2	3.3	0.4	0.1	0.5	0.0	0.1

Triacylglyceride analysis. TAG were resolved by RP-HPLC with a binary gradient of acetonitrile/dichloromethane as described previously. Complete baseline separations were obtained for all TAG found in the simpler oils, such as high-oleic sunflower oil, as well as for most TAG in the more complex oils. A stable, noise-free baseline was obtained with no gradient drift.

The ELSD provides significant advantages over refractive index detection for this application. The ELSD accommodates gradient elution and greatly enhances the ability to resolve complex TAG mixtures. Furthermore, ELSD affords much greater sensitivity and a more stable baseline than refractive index detection. However, because the ELSD response is not linear over a moderate range of analyte concentrations, a quadratic standard curve was required for quantitation according to the equation $A = a \cdot C^b$, where A is peak area, C the sample concentration, and a and b are constants (9). Concentrations of individual TAG were calculated from peak areas that were interpolated against a quadratic standard curve from triheptadecenoin standards run in an oil matrix. The sunflower oil matrix improved sensitivity of the ELSD at the low end of the standard curve, enabling calibration down to 0.02 mg/mL. The authors found that injecting approximately 0.1-mg samples in 20 μL solvent resulted in reliable quantitation and unambiguous identification of most peaks.

Alterations in fatty acid composition in various modified sunflower seed oils (Table 1) were reflected in their TAG profiles (Table 3). High-saturate hybrids produced seed oils with

TABLE 3
Triacylglycerol Composition of Sunflower Seed Oils (wt%)[a,b]

	Commodity	HO	HL	HS/HO	HP/HO	HP/HL
LLL	20.61		37.53			7.64
PoLL						2.65
OLL	25.94		19.05			4.94
PLL	8.86		12.85			17.38
PPoL						3.54
PoOO					2.68	
OOL	11.80	3.92	2.48	3.21	4.43	1.76
SLL	6.44		2.87		3.53	5.06
POL	6.43		3.15		6.57	6.64
PPoO						1.37
PPL	0.77		1.07			11.89
OOO	2.49	73.90	0.67	41.85	12.85	1.30
SOL	4.15		1.00			1.91
POO	1.60	8.64	0.72	10.50	31.19	2.95
PSL	1.19		0.83			2.71
PPO	0.49		0.65		13.80	3.50
SOO	1.19	4.25		25.08	4.97	
SSL	0.69		0.66			1.22
PSO	0.66	1.06	0.64	3.65	3.67	1.36
AOO	0.54	1.15	0.66	3.45	2.56	
PBL	0.49					1.40
SSO				3.64	2.26	
BOO		1.87		3.07	2.88	
TAG	94.34	94.79	84.83	94.46	91.39	79.21
N.I.	3.82	3.73	11.24	2.55	4.74	16.83
Total	98.16	98.52	96.07	97.01	96.13	96.04

[a]Nomenclature given in Table 1.
[b]P, palmitate; Po, palmitoleate; S, stearate; O, oleate; L, linoleate; A, arachidate; B, behenate; TAG, triacylglycerol; N.I., not identified.

elevated levels of TAG that contained palmitic, stearic, arachidic, and/or behenic acids. Low-saturate seed oil, produced by the high-oleic hybrid, contained lower levels of these TAG. This oil was comprised mainly of TAG with two or more oleic acid moieties.

In soybean oils, changes in the fatty acid distribution (Table 2) were also reflected in the TAG profiles (Table 4). High-palmitic and high-stearic acid varieties produced oils with elevated levels of TAG that contained these fatty acids. Oils derived from low saturate varieties contained less saturated fatty acid-enriched TAG. Likewise, low-linolenic acid oils were deficient in linolenate-containing TAG. The distribution of TAG in the Lsat/LLn and HP/LLn oils reflected the combined influence of the individual traits.

Fatty acid composition of each sunflower and soybean oil, calculated from the TAG composition, agrees well with that determined directly by GC/FID (data not shown). Minor differences were most likely due to unidentified TAG and the overlap of identified TAG in the more complex oils. TAG identification was particularly problematic in the high-palmitic oils that contain significant amounts of 16:1 or 16:2. However, the deviations between calculated and actual fatty acid composition are well within the ranges found by other laboratories that use HPLC to determine TAG profiles (10–13). Furthermore, the contribution from this source of error has been minimized by accounting for over 92% of the mass of each oil sample by quantified peaks (Tables 3 and 4). Some of the remaining mass in these crude oils is attributable to unsaponifiable constituents, such as tocopherols and phytosterols, that were at 0.3–0.7% in these samples (data not shown).

This report contributes significantly to what is known about TAG composition in genetically modified oils. These results agree with the full TAG profile of an LLn soybean oil produced by related germplasm (11). There is also agreement with the TAG group analysis reported for several HS soybean oils (14), although the authors found a higher percentage (27.7 vs. 17.2%) of disaturated TAG molecules in HS oil. However, this difference could be due to the 25% stearic acid level in the HS oil sample analyzed for this report, compared to just under 17% in the earlier report. The increased frequency of disaturated TAG should improve the oil's solid fat index profile, rendering it more suitable for a margarine basestock than previously thought (14). As in the HS soybean oil, TAG that contain one or two stearic acid moieties are also more abundant in genetically modified high-stearate canola oil (12).

TAG stereospecificity. Separation of enantiomeric *sn*-1,2

TABLE 4
Triacylglycerol Composition of Soybean Seed Oils (wt%)[a,b]

	Commodity	Lsat	LLn	HP	HS	Hsat	Lsat/LLn	HP/LLn
LLnLn	3.10	3.73		1.97	1.46			
LLLn	7.90	11.31	3.78	4.85	3.55	3.53	4.12	3.23
PLnLn				1.89		3.04		
LLL	17.64	23.24	17.68	6.89	5.92	3.88	24.49	9.40
OLLn	4.83	6.31	3.03	3.39	2.25		3.16	2.86
PLLn	3.67	3.00		8.07	2.32	6.69		3.20
OLL	15.28	17.82	16.14	6.10	6.00	3.34	21.58	9.73
OOLn		3.04						
PLL	10.23	4.46	9.90	14.01	5.00	9.58	5.03	14.23
POLn				2.67	4.44	4.52		
PPLn				2.18		3.17		
OOL	6.30	8.44	8.50	2.14	2.50		11.65	4.86
SLL	4.22	3.67	4.50	4.47	9.67	6.76	4.30	5.00
POL	6.89	3.49	7.31	7.69	3.68	5.07	4.20	9.83
SOLn					1.88			
PPL	3.11		3.01	9.94	1.67	9.56		6.82
OOO	3.28	3.95	4.58		1.41		5.59	2.96
SOL	3.71	3.42	4.54	2.09	9.75	4.46	4.56	3.62
POO	3.39		3.95	2.00	1.45		3.18	4.00
PSL	3.08		3.07	3.33	7.35	13.84		3.70
PPO				2.23		3.09		3.24
SOO		3.04	3.43		3.08		3.50	2.82
SSL				1.62	10.12	8.10		
PSO			2.87	1.62	2.50	3.72		2.88
PBL				1.54	1.96	3.25		
SSO					4.10	3.20		
TAG	96.61	98.90	96.30	90.68	92.32	98.79	95.37	92.37
N.I.	0.00	0.00	0.00	4.72	5.66	0.00	0.00	0.00
Total	96.61	98.90	96.30	95.40	97.98	98.79	95.37	92.37

[a]Nomenclature given in Table 2.
[b]Ln, linolenate. For other abbreviations see Table 3.

and *sn*-2,3-DAG as the diastereomeric (*S*)-(+)-1-(1-naphthyl)ethyl urethanes was accomplished by HPLC with two normal-phase columns in series (5). Good resolution was obtained with pure standards but only partial resolution with more complex oils, thus introducing significant error into the analysis. Incomplete chromatographic separation of DAG derivatives from such oils that contain longer chain fatty acids was probably due to a wider range of molecular weights. These results confirmed earlier reports (15).

Enantiomer separation was significantly improved when the HPLC columns were maintained at −20°C, the temperature at which optimal separation was achieved. Temperatures below −20°C caused the columns to freeze, and abrupt pressure increases interrupted operation of the chromatograph. Decreasing the temperature to −20°C increased the separation of enantiomer groups and decreased the resolution of individual species within those groups. For the present study, this effect was highly beneficial. By condensing the peaks within each enantiomeric group, it was possible to obtain baseline or near-baseline resolution between each (Fig. 2). A similar result was found when the separation was performed on a chiral HPLC column (16). In this case, lowering the temperature had little effect on the resolution of nonchiral compounds (i.e., fatty acyl moieties), but increased separation for chiral species (i.e., DAG enantiomers). Low column temperatures have also been shown to increase the selectivity values of TAG resolved by reversed-phase HPLC (17), indicating that the phenomenon is not specific to a particular analyte or column type.

Improved enantiomer resolution at low temperatures was demonstrated by running individual DAG species, produced by hydrolysis, at the two different temperatures. Selectivity of distearin, dipalmitin, and dilinolenin increased from 1.10–1.12 at room temperature to 1.19–1.23 at −20°C (Fig. 3). The beneficial effect of low temperature was not due to a reduced flow rate of the mobile phase, which was maintained at 0.7 mL/min at both temperatures.

Stereospecific analysis of crude sunflower oils revealed that saturated fatty acids were located principally at the *sn*-1 and *sn*-3 positions of the glycerol (Table 5). However, acyl groups were not arranged in a 1,3-random, 2-random manner. Both palmitic and stearic acids showed preferences for the *sn*-3 over the *sn*-1 position. This specificity was most evident in oils with elevated palmitate and stearate, revealing acyltransferase specificities less apparent at lower saturate levels. Oleic and linoleic acids were distributed over all three positions, their compositions apparently determined by the abundance of palmitic and stearic acids at *sn*-1 and *sn*-3.

This is the first report of stereospecific analysis of genetically modified sunflower oils. Therefore, the results can only be compared to those of an earlier report on a commodity oil type (5). Although the two analyses are in general agreement, significantly higher levels of oleic acid and lower levels of linoleic acid were found at the *sn*-2 position. However, this discrepancy can be attributed to more oleate (27.8 vs. 13.7%) and less linoleate (58.7 vs. 76.3%) in the authors' original oil.

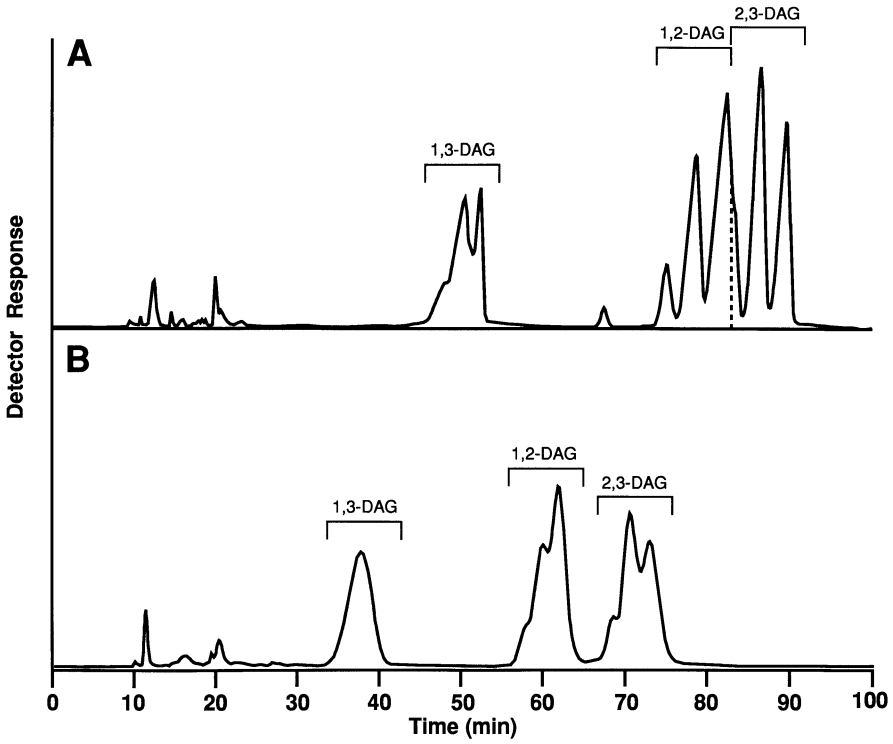

FIG. 2. Effect of column temperature on selectivity of sunflower seed oil DAG. A: Room temperature. B: −20°; DAG, diacylglycerol.

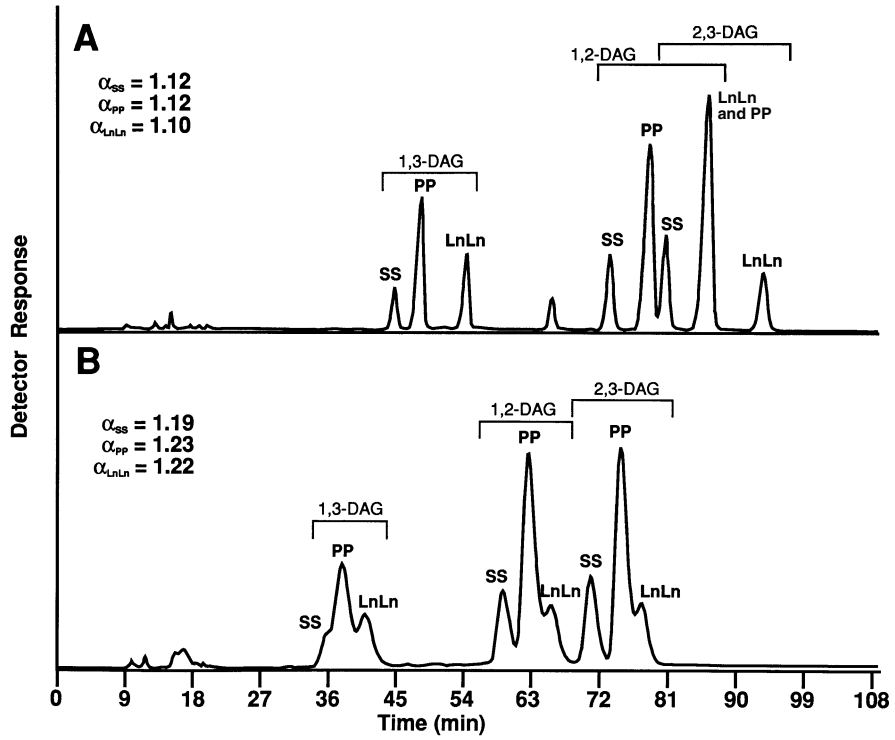

FIG. 3. Effect of column temperature on selectivity of homogenous DAG. A: Room temperature. B: –20°; DAG, diacylglycerol; P, palmitate; S, stearate; Ln, linolenate; α, selectivity.

TABLE 5
Stereospecific Analysis of Sunflower Seed Triacylglycerol (average fatty acid composition in mol% ± SD)

		Position			
		sn-1[a]	*sn*-2[b]	*sn*-3[c]	*sn*-2[d]
Commodity	16:0	10.9 ± 0.1	1.7 ± 0.1	9.0 ± 0.0	1.4 ± 0.3
	16:1	0.6 ± 0.0	–0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.5
	18:0	1.9 ± 0.6	4.1 ± 0.3	8.7 ± 0.3	1.2 ± 0.4
	18:1	17.1 ± 1.8	36.3 ± 4.1	31.2 ± 2.3	31.5 ± 0.5
	18:2	70.0 ± 1.3	57.8 ± 3.8	51.3 ± 2.5	65.7 ± 0.5
HO	16:0	3.5 ± 0.3	0.3 ± 0.3	5.5 ± 0.0	1.0 ± 0.4
	16:1	0.3 ± 0.0	–0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.1
	18:0	0.4 ± 0.4	1.2 ± 0.7	2.9 ± 0.3	0.8 ± 0.4
	18:1	93.2 ± 1.0	97.3 ± 1.4	88.8 ± 0.4	96.6 ± 1.8
	18:2	2.6 ± 0.3	1.5 ± 0.4	2.5 ± 0.1	1.4 ± 0.8
HL	16:0	10.9 ± 0.1	1.9 ± 0.1	10.0 ± 0.3	2.1 ± 0.2
	16:1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.0 ± 0.2
	18:0	1.4 ± 0.1	1.4 ± 0.1	2.9 ± 0.0	1.2 ± 0.1
	18:1	7.9 ± 0.1	17.3 ± 0.7	15.0 ± 0.6	18.9 ± 0.4
	18:2	79.0 ± 0.4	80.4 ± 0.7	71.3 ± 0.3	76.9 ± 0.3
HS/HO	16:0	6.3 ± 0.7	0.2 ± 0.8	7.9 ± 0.1	1.3 ± 0.2
	16:1	0.3 ± 0.0	–0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.1
	18:0	6.9 ± 0.1	2.3 ± 0.1	25.0 ± 0.0	2.2 ± 0.9
	18:1	84.0 ± 1.0	96.1 ± 1.1	65.0 ± 0.1	95.9 ± 1.2
	18:2	2.8 ± 0.1	1.4 ± 0.1	2.1 ± 0.0	0.6 ± 0.1
HP/HO	16:0	27.4 ± 0.7	–1.0 ± 1.3	49.5 ± 0.6	1.2 ± 0.4
	16:1	13.6 ± 0.3	–3.4 ± 0.8	10.2 ± 0.6	2.3 ± 0.0
	18:0	1.1 ± 0.0	0.7 ± 0.0	6.3 ± 0.0	0.3 ± 0.1
	18:1	51.5 ± 0.8	102.0 ± 1.8	29.8 ± 1.0	93.4 ± 0.6
	18:2	6.1 ± 0.0	1.9 ± 0.0	3.7 ± 0.0	2.8 ± 0.2
HP/HL	16:0	35.9 ± 1.3	2.6 ± 2.0	44.9 ± 0.7	4.0 ± 0.3
	16:1	6.2 ± 0.0	1.5 ± 0.1	5.5 ± 0.1	2.6 ± 0.0
	18:0	0.8 ± 0.1	2.3 ± 0.0	5.0 ± 0.1	0.8 ± 0.3
	18:1	12.1 ± 1.0	25.9 ± 2.1	14.2 ± 1.1	24.9 ± 1.0
	18:2	44.6 ± 0.0	68.1 ± 0.4	30.1 ± 0.4	67.8 ± 1.6

[a]3 × TAG – 2 × 2,3 DAG; [b]3 × TAG – (a + c); [c]3 × TAG - 2 × 1,2 DAG; [d]Via pancreatic lipase hydrolysis. DAG: diacylglycerol; for other abbreviations see Tables 1 and 3.

TABLE 6
Stereospecific Analysis of Soybean Seed Triacylglycerols (average fatty acid composition in mol% ± SD)

		Position			
		sn-1[a]	*sn*-2[b]	*sn*-3[c]	*sn*-2[d]
Commodity	16:0	18.2 ± 0.7	1.9 ± 1.1	12.0 ± 0.4	3.7 ± 0.6
	16:1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.3
	18:0	5.4 ± 1.1	2.7 ± 1.6	4.5 ± 0.4	3.4 ± 0.4
	18:1	15.9 ± 0.0	28.4 ± 0.4	25.0 ± 0.4	21.4 ± 1.6
	18:2	47.8 ± 1.1	64.1 ± 2.1	48.3 ± 1.0	65.0 ± 0.0
	18:3	12.9 ± 0.7	2.6 ± 0.8	10.3 ± 0.,1	6.4 ± 0.3
Lsat	16:0	8.4 ± 0.0	–0.2 ± 0.0	4.4 ± 0.0	3.1 ± 0.3
	16:1	–0.2 ± 0.0	0.4 ± 0.0	–0.2 ± 0.0	0.3 ± 0.0
	18:0	5.9 ± 0.1	0.4 ± 0.0	3.3 ± 0.1	2.8 ± 0.0
	18:1	16.3 ± 0.1	31.5 ± 0.1	17.6 ± 0.0	26.8 ± 1.2
	18:2	53.9 ± 0.0	66.9 ± 0.0	60.7 ± 0.0	60.1 ± 0.5
	18:3	15.5 ± 0.3	1.2 ± 0.1	14.2 ± 0.1	7.1 ± 0.4
LLn	16:0	18.9 ± 0.1	1.0 ± 0.0	14.3 ± 0.1	3.0 ± 0.9
	16:1	–0.1 ± 0.1	0.2 ± 0.3	–0.1 ± 0.1	0.3 ± 0.4
	18:0	8.6 ± 0.0	1.3 ± 0.1	7.5 ± 0.1	2.9 ± 1.1
	18:1	18.6 ± 0.4	33.8 ± 0.4	28.9 ± 0.0	26.5 ± 0.4
	18:2	49.8 ± 0.7	62.3 ± 0.8	46.0 ± 0.1	65.1 ± 1.8
	18:3	4.4 ± 0.1	1.3 ± 0.3	3.6 ± 0.1	2.4 ± 0.1
HP	16:0	32.1 ± 0.3	3.4 ± 0.7	38.6 ± 0.4	3.9 ± 1.0
	16:1	1.0 ± 0.0	0.4 ± 0.0	1.0 ± 0.0	1.1 ± 0.1
	18:0	4.4 ± 0.0	2.0 ± 0.3	5.0 ± 0.3	1.8 ± 0.4
	18:1	11.9 ± 0.1	17.3 ± 0.4	15.8 ± 0.3	15.9 ± 1.3
	18:2	35.4 ± 0.3	70.5 ± 0.4	26.7 ± 0.1	66.4 ± 2.1
	18:3	15.2 ± 0.1	6.4 ± 0.4	12.9 ± 0.3	11.1 ± 0.6
HS	16:0	12.8 ± 0.8	0.8 ± 2.3	13.4 ± 1.4	0.0 ± 0.0
	16:1	–0.2 ± 0.0	0.4 ± 0.0	–0.2 ± 0.0	0.1 ± 0.0
	18:0	35.3 ± 3.3	5.1 ± 1.6	39.4 ± 1.7	2.5 ± 0.3
	18:1	11.3 ± 2.4	25.6 ± 1.7	14.1 ± 0.7	24.8 ± 0.1
	18:2	30.3 ± 3.4	62.4 ± 0.4	24.0 ± 3.8	62.8 ± 0.1
	18:3	10.5 ± 1.4	5.8 ± 1.6	9.2 ± 0.1	9.9 ± 0.2
Hsat	16:0	29.0 ± 0.1	1.1 ± 0.6	38.6 ± 0.4	1.2 ± 0.1
	16:1	0.3 ± 0.0	0.4 ± 0.1	0.2 ± 0.1	0.4 ± 0.0
	18:0	19.4 ± 0.0	5.8 ± 0.3	27.6 ± 0.3	1.1 ± 0.1
	18:1	7.9 ± 0.1	11.9 ± 0.1	9.0 ± 0.0	11.5 ± 0.0
	18:2	29.4 ± 0.1	69.9 ± 0.6	15.0 ± 0.7	70.6 ± 0.1
	18:3	13.9 ± 0.0	11.2 ± 0.1	9.4 ± 0.1	15.5 ± 0.1
Lsat/LLn	16:0	8.1 ± 0.1	1.1 ± 0.1	3.4 ± 0.0	2.8 ± 0.4
	16:1	0.3 ± 0.0	–0.3 ± 0.0	0.3 ± 0.0	0.0 ± 0.0
	18:0	5.6 ± 0.1	2.2 ± 0.1	2.1 ± 0.0	2.8 ± 0.2
	18:1	21.8 ± 0.1	36.6 ± 0.7	25.3 ± 0.6	29.7 ± 0.4
	18:2	60.7 ± 0.4	59.6 ± 0.0	65.1 ± 0.4	63.1 ± 0.2
	18:3	4.0 ± 0.0	0.5 ± 0.1	3.9 ± 0.1	1.9 ± 0.0
HP/LLn	16:0	31.5 ± 1.4	–1.1 ± 2.3	31.1 ± 0.8	0.1 ± 0.0
	16:1	0.9 ± 0.1	0.6 ± 0.3	0.9 ± 0.1	1.1 ± 0.0
	18:0	4.9 ± 0.1	2.7 ± 0.1	5.6 ± 0.0	2.7 ± 0.9
	18:1	14.7 ± 1.8	30.6 ± 2.8	23.7 ± 1.0	27.0 ± 0.2
	18:2	43.1 ± 0.1	66.0 ± 0.3	34.3 ± 0.1	66.6 ± 0.4
	18:3	4.8 ± 0.1	1.4 ± 0.1	4.3 ± 0.0	2.7 ± 0.2

[a]3 × TAG – 2 × 2,3 DAG; [b]3 × TAG – (a + c); [c]3 × TAG - 2 × 1,2 DAG; [d]*Via* pancreatic lipase hydrolysis. For abbreviations see Tables 1–3 and 5.

In the soybean oils, as in the sunflower oils, saturated fatty acids were generally restricted to the *sn*-1 and *sn*-3 positions of the glycerol (Table 6). These findings agree with the analyses of commodity soybean oils by other laboratories (18–20), as well as with one report on regiospecific analysis of HS oil (14). Again, acyl groups were not arranged in a 1,3-random, 2-random manner in these soybean oils. However, neither palmitic nor stearic acids had consistent preferences for either the *sn*-1 or the *sn*-3 position. Oleic and linoleic acids were distributed over all positions, with *sn*-2 preferred. Linolenic acid was largely absent from the *sn*-2 position, except in oils with high levels of saturates that occupied the external (α) positions.

In performing acyl positional analysis, a minor amount of isomerization was expected (5). An equilibrium between the α and β positions is established, with movement to the α position fa-

vored. Acyl migration is likely to account for most, if not all, of palmitate and stearate at the *sn*-2 position (Tables 5 and 6). This phenomenon was controlled by synthesizing the naphylethyl derivatives immediately after minimal hydrolysis time on ice, thus reducing errors. An indication that this was so was that the fatty acid composition at the *sn*-2 position, determined by stereospecific analysis, agreed with that determined by pancreatic lipase hydrolysis for all oils analyzed (Tables 5 and 6).

The present results are consistent with TAG synthesis being driven by mass action from available fatty acids. However, as shown in the high-saturate sunflower and soybean oils examined, the distribution of palmitic and/or stearic acid at the three positions was influenced by enzyme specificities. Glycerol-3-phosphate acyltransferase, the compound that catalyzes the esterification of fatty acids at the *sn*-1 position of TAG, has been shown to prefer 16:0-CoA over 18:0-CoA as an acyl substrate in maturing safflower embryos (21). The effect of this substrate specificity in the author's sunflower oils is reflected in a consistent enrichment of stearate at the *sn*-3 compared to the *sn*-1 position (Table 5). This enrichment occurs in spite of a reported preference in developing sunflower embryos of 16:0-CoA over 18:0-CoA as an acyl donor for diacylglycerol acyltransferase, the enzyme that catalyzes the esterification of fatty acids at the *sn*-3 position (22). In the authors' soybean oils, however, stearate is not always more abundant at the *sn*-3 position (Table 6), possibly indicating a different balance between these two enzymatic processes. Saturated fatty acids were in low abundance in the *sn*-2 position (Tables 5 and 6), indicating that these high-saturate phenotypes are not the result of altered substrate specificity of acyl-CoA:lysophosphatidic acid acyltransferase. This finding was expected because this enzyme almost completely excludes saturated fatty acyl groups in safflower microsomes (23).

In oils with elevated unsaturated fatty acids, oleic, linoleic, and linolenic acids were incorporated into TAG across all three positions according to their abundance. Thus, specificity of the acyltransferases is not a likely mechanism for these altered fatty acid profiles. This result is in agreement with acyl specificities reported from *in vitro* enzyme assays (21,22,24).

Characterization of TAG structure in these genetically modified sunflower and soybean oils suggests that altered TAG fatty acid composition probably results from mutations in the fatty acid biosynthetic pathway. The acyl groups generated by fatty acid synthesis appear to be accommodated by the native TAG assembly enzymatic machinery. Altered specificities of enzymes of the Kennedy pathway do not appear to be involved. A direct test of this hypothesis would of course require metabolic or enzymatic assays.

ACKNOWLEDGMENTS

Glenn Cole provided sunflower achenes. Daria Schmidt provided soybean seeds. Barry Martin provided a critical review of the manuscript.

REFERENCES

1. Neff, W., E. Selke, T. Mounts, W. Rinsch, E. Frankel, and M. Zeitoun, Effect of Triacylglycerol Composition and Structures on Oxidative Stability of Oils from Selected Soybean Germplasm, *J. Am. Oil Chem. Soc. 69*:111–118 (1992).
2. Neff, W., T. Mounts, W. Rinsch, and H. Konishi, Photooxidation of Soybean Oils as Affected by Triacylglycerol Composition and Structure, *Ibid. 70*:163–166 (1993).
3. Neff, W., T. Mounts, W. Rinsch, H. Konishi, and M. El-Agaimy, Oxidative Stability of Purified Canola Oil Triacylglycerides with Altered Fatty Acid Compositions as Affected by Triacylglycerol Composition and Structure, *Ibid. 71*:1101–1109 (1994).
4. Neff, W., M. El-Agaimy, and T. Mounts, Oxidative Stability of Blends and Interesterified Blends of Soybean Oils and Palm Olein, *Ibid. 71*:1111–1116 (1994).
5. Christie, W., B. Nikolova-Damyanova, P. Laakso, and B. Herslof, Stereospecific Analysis of Triacyl-*sn*-glycerols via Resolution of Diastereomeric Diacylglycerol Derivatives by High-Performance Liquid Chromatography on Silica, *Ibid. 68*:695–701 (1991).
6. Gunstone, F., *Fatty Acid and Lipid Chemistry*, Blackie Academic & Professional, Glasgow, 1996, 252 pp.
7. Luddy, F., R. Barford, S. Herb, P. Magidman, and R. Riemenschneider, Pancreatic Lipase Hydrolysis of Triglycerides by a Semimicro Technique, *J. Am. Oil Chem. Soc. 41*:693–696 (1964).
8. Butte, W., J. Eilers, and M. Kirsch, Trialkylsulfonium- and Trialkylselenoniumhydroxides for the Pyrolytic Alkylation of Acidic Compounds, *Anal. Lett. 15(A10)*:841–850 (1982).
9. Christie, W., Evaporative Light-Scattering Detectors in Lipid Analysis, *Lipid Technology May/June*:68–70 (1993).
10. Neff, W., R. Adlof, G. List, and M. El-Agaimy, Analysis of Vegetable Oil Triacylglycerides by Silver Ion High-Performance Liquid Chromatography with Flame Ionization Detection, *J. Liq. Chromatogr. 17*:3951–3968 (1994).
11. Konishi, H., W. Neff, and T. Mounts, Correlation of Reverse-Phase High-Performance Liquid Chromatography and Gas-Liquid Chromatography for Fatty Acid Composition of Some Vegetable Oils, *J. Chromatogr. 629*:237–241 (1993).
12. Byrdwell, W., and W. Neff, Analysis of Genetically Modified Canola Varieties by Atmospheric Pressure Chemical Ionization Mass Spectrometric and Flame Ionization Detection, *J. Liq. Chromatogr. Related Technol. 19*:2203–2225 (1996).
13. Byrdwell, W., E. Emken, W. Neff, and R. Adlof, Quantitative Analysis of Triacylglycerides Using Atmospheric Pressure Chemical Ionization–Mass Spectrometry, *Lipids 31*:919–935 (1996).
14. List, G., T. Mounts, F. Orthoefer, and W. Neff, Potential Margarine Oils from Genetically Modified Soybeans, *J. Am. Oil Chem. Soc. 73*:729–732 (1996).
15. Kuksis, A., Analysis of Positional Isomers of Glycerolipids by Nonenzymatic Methods, in *Advances in Lipid Methodology—Three*, The Oily Press, Dundee, Scotland, pp. 1–36, 1996.
16. Takagi, T., and T. Suzuki, Effect of Temperature on Chiral and Achiral Separations of Diacylglycerol Derivatives by High-Performance Liquid Chromatography on a Chiral Stationary Phase, *J. Chromatogr. 625*:163–168 (1992).
17. Hirano, T., and K. Takahashi, Improvement in Resolution of Low Melting Point Triglyceride Molecular Species on Chilled Column Isocratic Liquid Chromatography, *J. Chromatogr. Sci. 34*:341–348 (1996).
18. Brockerhoff, H., and M. Yurkowski, Stereospecific Analyses of Several Vegetable Fats, *J. Lipid Res. 7*:62–64 (1966).
19. Fatemi, S., and E. Hammond, Glyceride Structure Variation in Soybean Varieties: I. Stereospecific Analysis, *Lipids 12*:1032–1036 (1977).

20. Takagi, T., and Y. Ando, Stereospecific Analysis of Triacyl-*sn*-Glycerols by Chiral High-Performance Liquid Chromatography, *Ibid. 26*:542–547 (1991).
21. Ichihara, K., *Sn*-Glycerol-3-phosphate, Acyltransferase in a Particulate Fraction from Maturing Safflower Seeds, *Arch. Biochem. Biophy. 232*:685–698 (1984).
22. Wiberg, E., E. Tillberg, and S. Stymne, Substrates of Diacylglycerol Acyltransferase from Developing Oil Seeds, *Phytochemistry 36*:573–577 (1994).
23. Griffiths, G., A. Stobart, and S. Stymne, The Acylation of *sn*-Glycerol-3-phosphate and the Metabolism of Phosphatidate in Microsomal Preparations from the Developing Cotyledons of Safflower (*Carthamus tinctorius* L.) Seed, *Biochem. J. 230*:379–388 (1985).
24. Vogel, G., and J. Browse, Cholinephosphotransferase and Diacylglycerol Acyltransferase: Substrate Specificities at a Key Branch Point in Seed Lipid Metabolism, *Plant Physiol. 110*:923–931 (1996).

[Received October 15, 1996; accepted April 29, 1997]