Effect of Genetic Modification on the Content and Composition of Bioactive Constituents in Soybean Oil[1]

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ABSTRACT: The content and composition of tocopherols, sterols, and phospholipids in soybean oils derived from genetically-modified soybeans were determined by normal and reverse-phase high-performance liquid chromatography and gas–liquid chromatography. Tocopherol content was lowered in oils from soybeans selected to yield high palmitate and stearate contents. However, β-tocopherol, which amounts to less than 1 ppm in control oils, was increased to 25–53 ppm in these oils. Sterol content was higher in one reduced-linolenate oil, which also had the highest oleate content. The greatest variability was observed in the content of β-sitosterol, which ranged from 46.9–151.6 mg/100/g in the modified oils. Although, in general, there was little impact on the phospholipids, the content of phosphatidic acid was elevated in crude oils from three of the lines. Increases in phosphatidic acid are generally associated with storage deterioration of soybeans. Individual major classes of phospholipid were isolated, and the molecular species composition of each was determined. Compositional variations in molecular species indicated that there was an impact of the genetic modification of soybeans at the molecular level of the phospholipids that are primary plant cell components.

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KEY WORDS: Genetic modification of soybeans, high saturated acids, high-performance liquid chromatography, phospholipid, phospholipid molecular species, phytosterol, reduced linolenate, soybean oil, tocopherol.

Fatty acid composition of soybean oil has been selectively modified by chemical mutagenesis and hybridization of soybeans (1–3). Food oils produced from oils with reduced linolenic acid (18:3) contents are good alternatives to hydrogenated frying oils (4–9). Based on these and other research efforts, some of these oils are being evaluated for commercialization (10). In addition to being the major source of edible oil for the world market, crude soybean oil is also the source of important bioactive constituents that are value-added by-products of the processing industry, such as phospholipids (lecithin), tocopherols (antioxidants, vitamin E), and sterols (feedstock for pharmaceuticals).

Phospholipids are recovered during the degumming step of oil processing. The commercial lecithin products are used as emulsifiers, stabilizers, conditioning and release agents, and as antioxidants (11). Foodstuffs in which lecithin is used include baking products and mixes, candy, chewing gum, chocolate, dehydrated foods, ice cream, and instant foods. It also is used in many industrial products.

The tocopherol and sterol contents of soybean oil are lowered in each processing step and in deodorization. Mixed sterols and mixed tocopherols are recovered from the collected deodorizer distillate. The principal use of mixed tocopherols is for fractional distillation (12) to produce natural vitamin E and, therefore, they are less available for use as antioxidants. The pharmaceutical use of fractionated mixed sterols is in the partial synthesis of sex hormones or other intermediates, the manufacture of progesterone and corticoids, and the production of androstenedione and androstadienedione. The latter compound (after further reaction) gives estrogens, contraceptives, diuretics, and the male anabolic and androgenic hormones (13).

For commercialization of the new oilseeds, it is important that they provide oils as sources of the bioactive constituents, i.e., tocopherols, sterols, and phospholipids. As part of our evaluation of the new modified soybeans, we report the characterization of the bioactive constituent composition of the extracted crude oils.

EXPERIMENTAL PROCEDURES

Materials. Experimental soybean lines, produced by hybridization breeding, were provided by R.W. Wilson (ARS, USDA, North Carolina State University, Raleigh, NC; N 83-375, N 85-2176 and N 89-2009); W.R. Fehr (Iowa State University, Ames, IA; A16, A17, and A87-191039); J.R. Wilcox (ARS, USDA, Purdue University, Lafayette, IN; CX1279 58-3, CX1279 135-5, CX1279 168-3, CX1279 182-3); and C. Jennings (Pioneer Hybrid International, Inc., Waterloo, IA; A90-214040, A90-143073). A standard cultivar (Hardin 91) was provided by W.R. Fehr. Duplicate one-bushel samples of each line were processed as described previously (8).

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Reference standards for peak identification by relative retention time (volume) were obtained from Eastman Kodak Company (Rochester, NY), (tocopherols); Sigma Chemical Co. (St. Louis, MO) (phytosterols); and Avanti Polar Lipids, Inc. (Alabaster, AL) (phospholipids).

Fatty acid composition. Fatty acid composition of the crude oil was determined by capillary gas-chromatographic (GC) analysis with a Varian 3400 GC (Palo Alto, CA), equipped with an SP 2380 column (30 m, 0.25 mm i.d., 0.20-micron film thickness; Supelco, Bellefonte, PA). Column temperature was held at 170°C for 10 min and programmed to 220°C at 3°C/min. Injector temperature was 240°C, and detector temperature was 280°C.

Sample preparation. Tocopherol composition was determined from the oil samples directly without isolation or derivatization. Phytosterols were isolated by a modification of published procedures (14–16). Crude oil (0.1 g) was mixed with 20 mL 1 N ethanolic KOH and allowed to react overnight at room temperature, washed with 20 mL 18-ohm water, and extracted three times with fresh ethyl ether. The extracts were pooled and resaponified with 10 mL 0.5 N ethanolic KOH and washed with 20 mL batches of deionized and distilled water until neutral to pH paper. Solvent was evaporated, and the sample was derivatized as follows: 0.25 mL reaction-grade pyridine and 0.25 mL *bis*(trimethylsilyl)-trifluoroacetamide (BSTFA) were added to the residue, the mixture was heated for 10–15 min at 60°C and allowed to set at room temperature overnight. The derivatized sample was dried under N_2, diluted with methylene chloride, and analyzed immediately by GC.

Phospholipids were isolated from crude oils as follows: duplicate samples of approximately 5 g oil were fractionated on a 10-g column of silica gel (60–200 mesh). Sequential elution was by 200 mL chloroform, 100 mL acetone, 100 mL methanol, and 100 mL 0.1% phosphoric acid in methanol. The methanol and phosphoric acid/methanol fractions were combined for recovery of total phospholipids. Solvent was removed by rotary evaporation at room temperature, the residue was redissolved in chloroform, duplicate samples were pooled and washed three times with 1 mL of saturated salt solution, followed by addition of sodium bicarbonate until neutral. The sample was dried with sodium or magnesium sulfate, and filtered, and the solvent was removed under N_2 and frozen until analyzed (17).

Analysis procedures: tocopherols. The content of α-, β-, γ-, and δ-tocopherol was determined directly by analysis of the oils by high-performance liquid chromatography (HPLC) on a Waters μ-BondaPak-NH2 (300 × 3.9 mm i.d.) column (Millipore, Milford, MA) by following modified published procedures (18). A 1:1 dilution of crude soybean oil with hexane gave satisfactory response for analysis. Separations were performed with an SP8700XR ternary solvent delivery system (Thermo-Separation Products Inc., Fremont, CA), an SP8500 dynamic mixer, a Rheodyne 7125 injector (20 μL loop), and a Spectroflow 980 Fluorescence detector (Applied Biosystems, Ramsey, NJ). The solvent system (98:2, hexane/isopropanol) was pumped through the column at a flow rate of 1 mL/min. Detector conditions were: emission wavelength of 345 nm, excitation wavelength of 298 nm, and range of 0.1. The analog signal from the fluorescence detector was interfaced with a real-time computer (ModComp Inc., Fort Lauderdale, FL), programmed to calculate peak areas and component relative percentage composition.

Phytosterols. A Varian 3400 capillary GC (Varian, Palo Alto, CA), equipped with a 30 m × 0.25 mm (0.25 μm coating) PTE5 capillary column (Supelco) and a flame-ionization detector, was used for analysis of BSTFA-derivatized phytosterols. The GC conditions were: initial column temperature at 275°C for 20 min, then ramp to 290°C at 3°C/min; injector temperature of 275°C; detector temperature of 300°C. Quantitative analysis of phytosterols was by regression analysis of peak area. Regression equations were based on analysis of standards.

Phospholipids. Analytical and preparative HPLC separations of phospholipids were performed on a Thermo-Separation Products Model SP 8800 ternary solvent delivery system with an SP 8500 dynamic mixer and a Rheodyne 7125 injector, equipped with 10–100 μL sample loops (Thermo-Separation Products, Fremont, CA). Effluent detection was by a Varex evaporative light scattering detector (ELSD) (Model ELSD II-; Alltech Associates, Inc., Deerfield, IL). The samples were eluted on a Lichrosphere Si 60/II, 3 μ, 250 × 4 mm i.d. column (EM Separations, Gibbstown, NJ) for analytical separations or a Lichrosorb Si-60, 10 μ, 250 × 10 mm i.d. column (Alltech) for preparative separation and collection of fractions. The solvent system was a linear-gradient elution from (A) chloroform/tertiary-butyl-methyl ether (750:150, vol/vol) to (B) methanol/ammonium hydroxide/chloroform (920:70:10, vol/vol/vol) in 30 min and held at (B) for 10 min, followed by a reverse linear-gradient to the starting solvent in 10 min. Flow rates were 0.5 mL/min for analytical and 2 mL/min for preparative analysis (a micro-metric sample splitter was set at a 40:60 ratio to deliver 0.8 mL/min to the ELSD and 1.2 mL/min for collection of peak fractions) (17). The analog signal from the ELSD was interfaced with the PC 1000 computer system *via* the SP 4500 data interface module, programmed to calculate the peak areas and relative percentage composition of the eluted components. Multiple preparative separations were performed to obtain adequate amounts of the phosphatidylcholine (PC) and phosphatidylethanolamine (PE) components for molecular species analyses.

Phospholipid molecular species. Reverse-phase HPLC separations for PC and PE molecular species analyses were performed on a Waters Nova-Pak C 18, 4 μM (300 × 3.9 mm i.d.) column (Millipore). A Waters model M-6000A solvent delivery system (Millipore), a Rheodyne 7125 injector (50-μL sample loop), and a Varex model ELSD MK III (Alltech) were used for separation and analysis of PE and PC (19). The eluting solvent used for PE was 20:1:1 methanol/water/chloroform (vol/vol/vol), and for PC molecular species determination the eluting solvent was 30:1:1 methanol/water/chloro-

form (vol/vol/vol). Flow rate was 0.8 mL/min. The analog signal from the ELSD was interfaced with a real-time computer (ModComp Inc.), which was programmed to calculate peak areas and component relative percentage composition.

Statistical analysis. Data from duplicate analyses were evaluated for standard deviation and reported as the relative standard deviation.

RESULTS AND DISCUSSION

The fatty acid composition of each oil is presented in Table 1. As indicated by the specific composition, genotypes A16, A17, N85-2176, A87-191039, and N89-2009 were developed in programs designed to lower the linolenate content for use as alternative frying oils. Genotypes CX1279 135-5, CX1279 182-3, A90-214040, and A90-143073 were developed to provide increased contents of saturated fatty acids for solid-fat food applications. Lines N83-375, CX1279 58-3, and CX1279 168-3 were high-yielding lines that were mated with those genotypes having the same initial letter.

Tocopherols. The results of tocopherol analyses of each crude oil are presented in Table 2. Except for the A16 oil, all of the reduced linolenate oils had total tocopherol levels lower than that of the Hardin 91 standard. The lower amount of γ-tocopherol accounted for most of the deviation in total tocopherol content in oils N85-2176, A87-191039, and N89-2009. Oil N83-375 also had a lower content of γ-tocopherol and total tocopherol than Hardin 91 even though there was little difference in the fatty acid composition.

Most of the oils with elevated saturated fatty acid contents had contents of individual tocopherols and total tocopherol content that were different from those of the Hardin 91 standard. These variations may be related to the plant's response to changing requirements, based on production site or the genetic modification of the oilseeds.

Reduced linolenate oils generally showed higher total tocopherol contents than the oils with elevated contents of saturated fatty acids. The tocopherol composition was altered in some of the oils. For example, N85-2176 oil had the lowest percentage of γ-tocopherol (60%) and the highest percentage of α-tocopherol (12.2%) of the low-linolenic acid oils. This oil also had the lowest polyunsaturated content of the low-linolenate oils.

Except for A90-214040, all of the oils with elevated saturated fatty acids and samples CX1279 58-3 and CX1279 168-3 showed levels of β-tocopherol ranging between 3 to 9.5%. Little or none of this compound was found in oil from the Hardin 91 standard.

There does not seem to be an appropriate interpretation, based on modification of the fatty acid composition, to explain the substantial decrease in total tocopherol content in oil CX1279 135-5. Except for 9.5% β-tocopherol, the relative percentage composition of the individual tocopherols in this oil was not greatly different from that determined for the Hardin 91 standard oil. There was a substantial change in the γ-/δ- ratio in oils A90-214040 and A90-143073 with the percentage of decreased γ-tocopherol and the increased percentage of δ-tocopherol.

Phytosterols. The results of phytosterol analyses of each crude oil are presented in Table 3. The total content of phytosterols was increased in oils N85-2176, N89-2009, N83-375, and A90-214040, and decreased in oils A16, CX1279 135, CX1279 168-3, and CX1279 182-3, compared to the Hardin 91 standard. In general, the increase or decrease in total content did not alter the relative percentage composition of the individual phytosterols, even though the absolute amounts varied. However, the percentage of β-sitosterol, the primary phytosterol in soybean oil, was lower in the A16 (48.9%) and N85-2176 (47.7%) oils. There was no obvious explanation for this observation.

TABLE 1
Fatty Acid Composition (%)[a]

Variety[b]	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$
A-16[b]	12.3	4.9	29.6	51.6	1.7
A-17[b]	17.3	5.3	25.2	50.3	1.9
N 85-2176[b]	10.5	3.1	49.5	35.0	1.9
A 87-19103[b]	10.6	4.3	26.7	55.9	2.5
N 89-2009[b]	11.5	4.5	28.8	52.3	2.9
N 83-375[b]	12.8	4.1	26.0	51.6	5.5
Hardin 91[b]	10.9	3.8	23.9	55.0	6.5
CX1279-3[d]	10.4	4.1	21.9	53.6	9.0
CX1279 168-3[d]	10.7	3.7	21.3	58.5	5.1
A 90-214040[e]	25.8	3.3	14.0	42.9	13.4
A 90-143073[e]	8.0	17.2	16.7	47.0	10.1
CX1279 182-3[d]	14.4	4.2	18.9	54.5	5.5
CX1279 135-5[d]	15.3	3.8	19.8	50.7	8.8

[a]% Total lipid.
[b]From Iowa State University (Ames, IA).
[c]Varieties from ARS, USDA, North Carolina State University (Raleigh, NC).
[d]From ARS, USDA, Purdue University (Lafayette, IN).

TABLE 2
Tocopherol Analysis—Crude Oils (ppm)[a]

Variety[b]	α	β	γ	δ	Σ
A16	141.0	<1	1257.0	323.0	1721
A17	70.0	<1	1210.0	290.0	1570
N85-2176	179.0	<1	884.0	406.0	1468
A87-19103	79.0	<1	840.0	274.0	1193
N89-2009	116.0	<1	962.0	365.0	1455
N83-375	132.0	<1	972.0	310.0	1414
Hardin 91	135.0	<1	1247.0	338.0	1739
A90-214040	47.4	<1	734.6	403.3	1184
A90-143073	54.0	87.3	762.1	390.6	1294
CX1279 58-3	74.6	53.1	571.3	192.9	792
CX1279	26.9	35.9	253.3	62.3	378
CX1279 168-3	92.9	33.0	670.3	221.3	1017
CX1279 182-3	60.6	24.8	549.4	183.8	819
RSD[c]	2.1–11	2.4–5.7	1.0–15.4	1.0–7.9	1.0–10.0

[a]Duplicate analysis by high-performance liquid chromatography.
[b]See Table 1 for source information.
[c]Relative standard deviation.

TABLE 3
Phytosterol Composition—Crude Oils (mg/100/g)[a]

Variety[b]	Campesterol	Stigmasterol	β-Sitosterol	Σ
A16	48.3	48.4	92.8	189.5
A17	50.3	52.3	113.1	215.7
N85-2176	89.5	92.2	166.0	347.7
A87-191039	65.1	47.7	114.4	227.2
N89-2009	95.3	64.5	171.3	331.1
N83-375	63.1	50.8	151.6	265.5
Hardin 91	62.3	44.7	122.1	229.1
A90-214040	66.8	50.4	145.0	262.2
A90-143073	52.4	51.7	114.4	218.5
CX1279 58-3	49.6	56.1	117.3	223.0
CX1279 135-5	43.8	48.3	97.7	189.8
CX1279 168-3	38.5	44.3	92.7	175.5
CX1279 182-3	40.1	48.5	106.9	195.5
RSD	1.0–6.1	1.0–10.7	2.9–8.9	1.0–6.0

[a]Duplicate analysis by gas–liquid chromatography.
[b]See Tables 1 and 2 for source information and abbreviation.

Phospholipids. The phospholipid major class composition is presented in Table 4 and indicates some variability between the phospholipid composition of the standard variety and some of the oils from modified oilseeds. Low-linolenate oils, A16 and A17, had elevated contents of PE and depressed contents of PC. Other low-linolenate oils showed phospholipid compositions to be fairly consistent with the standard variety. The phosphatidic acid (PA) content of three of the Purdue oils, CX1279 58-3, CX1279 168-3, and CX1279 182-3, was substantially higher than the standard variety, as well as all of the other test oils. Because increased levels of PA in crude oils have been associated with pre-harvest and post-harvest damage to soybeans (20–24), this observation is probably not related to the genetic modification of soybeans.

Peaks eluted during the HPLC analysis of the phospholipid major classes were collected and subsequently analyzed to determine the molecular species distribution of PE and PC. Results of these analyses are presented in Tables 5 (PE) and 6 (PC). There is substantial variability in the molecular species composition of both the PE and PC fractions of the phospholipids. Although there is no consistent pattern to the compositional variation, it does indicate that the phospholipids, which are constituents of cell membranes, are altered by the biomolecular changes occurring during the breeding of soybeans.

Although these studies have indicated alteration of each of the bioactive constituents of soybean oil that may be a result of the breeding of the oilseed, previous researchers have reported a range of tocopherol (25,26) and phytosterol (27–29) contents in crude soybean oils. Marquard (25) reported that, as the linolenate content of the oil decreased due to genotype variation or climatically differentiated location of soybean cultivation, the total tocopherol content also decreased. The analyses reported here are not in total agreement with this observation in that in many of the low-linolenate oils the toco-

TABLE 4
Phospholipid Composition—Crude Oils (%)[a]

Variety[b]	Phosphatidylethanolamine	Phosphatidylinositol	Phosphatidic acid	Phosphatidylcholine
A16	35.8	22.8	5.3	36.1
A17	34.3	22.3	4.0	39.4
N85-2176	29.7	19.8	4.5	46.0
A87-191039	26.6	21.4	5.9	46.1
N89-2009	27.5	20.0	4.2	48.3
N83-375	28.7	19.8	5.6	42.9
Hardin 91	26.8	23.6	6.1	45.5
A90-214040	28.0	19.4	2.9	49.7
A90-143073	25.8	27.9	2.1	44.1
CX1279 58-3	38.1	20.5	15.2	26.1
CX1279 135-5	24.9	23.2	5.3	46.7
CX1279 168-3	23.6	18.2	27.9	30.3
CX1279 182-3	20.7	18.9	35.0	25.4
RSD	1.8–8.6	1.0–10.3	1.0–7.2	1.0–8.0

[a]Duplicate analysis by high-performance liquid chromatography.
[b]See Tables 1 and 2 for source information and abbreviation.

TABLE 5
Phospholipid Molecular Species Composition (%) (phosphatidylethanolamine)[a]

Variety[b]	$C_{16:0/18:0}$	$C_{18:2/18:2}$	$C_{16:0/18:2}$	$C_{16:0/18:1}$	$C_{18:0/18:1}$
A16	<1.0	21.7	77.1	<1.0	—
A17	4.6	62.4	31.8	<1.0	trace
N85-2176	1.8	19.5	77.3	trace	1.5
A87-191039	6.1	33.3	56.5	<1.0	—
N89-2009	<1.0	39.0	59.0	1.4	<1.0
N83-375	8.9	45.9	41.7	1.5	2.0
Hardin 91	1.3	4.3	73.8	19.4	1.3
A90-214040	15.5	67.6	13.9	2.9	—
A90-143073	19.6	52.8	14.8	11.2	1.7
CX1279 58-3	12.4	15.7	69.8	<1.0	2.0
CX1279 135-5	4.9	3.0	75.6	16.2	trace
CX1279 168-3	2.2	63.7	32.4	1.4	—
CX1279 182-3	3.3	90.6	5.7	<1.0	—
RSD	2.7–10.0	0.9–10.0	0.2–10.0	2.5–10.0	10.0

[a]Duplicate analysis by reverse-phase high-performance liquid chromatography.
[b]See Tables 1 and 2 for source information and abbreviation.

TABLE 6
Phospholipid Molecular Species Composition (%) (phosphatidylcholine)[a]

Variety[b]	$C_{16:0/18:0}$	$C_{18:2/18:2}$	$C_{16:0/18:2}$	$C_{16:0/18:1}$	$C_{18:0/18:1}$
A16	—	20.3	70.8	8.8	—
A17	—	14.2	74.2	8.3	3.3
N85-2176	—	24.1	64.5	10.6	<1.0
A87-191039	2.4	26.2	68.1	3.2	trace
N89-2009	9.4	70.2	19.7	<1.0	—
N83-375	1.5	46.6	50.0	1.0	1.0
Hardin 91	2.0	42.0	52.1	1.9	2.0
A90-214040	9.9	48.9	40.2	<1.0	—
A90-143073	—	54.0	46.0	—	—
CX1279 58-3	5.5	57.9	35.7	<1.0	<1.0
CX1279 135-5	—	77.7	22.3	—	—
CX1279 168-3	<1.0	59.8	36.4	<1.0	2.0
CX1279 182-3	1.2	47.4	49.2	<1.0	1.4
RSD	4.7–10.0	0.6–10.0	0.4–10.0	5.0–10.0	0.4–10.0

[a]Duplicate analysis by reverse-phase high-performance liquid chromatography.
[b]See Tables 1 and 2 for source information and abbreviation.

pherol content remained high. Also, tocopherol contents were decreased in high-saturated oils, which also contained appreciable amounts of linolenate. In addition, the presence of β-tocopherol in soybean oil has not been reported by any previous investigators. Because the techniques of HPLC analysis of molecular species of phospholipids have only recently been developed, there have been no comparable analyses reported in the literature.

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