Characterization of Oil Exhibiting High γ-Linolenic Acid from a Genetically Transformed Canola Strain

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ABSTRACT: The seed oil from a genetically transformed canola (*Brassica napus*) containing 43% (w/w) of γ-linolenic acid (G, 18:3n-6), 22% linoleic acid (L, 18:2n-6), and 16% oleic acid (O, 18:1n-9) was evaluated. In this high γ-linolenic acid canola oil (HGCO), the predominant 18:3n-6-containing triacylglycerol (TG) molecular species were GGL (23%), GLO (20%), and GGG (11%). In the total TG, approximately 75% of the 18:3n-6 was located at the *sn*-1,3 positions, while only 34% of linoleic acid was at the *sn*-1,3 positions. The GGL molecular species of HGCO contained approximately equal amounts of GLG and GGL positional isomers, while the GLO molecular species had 95% GOL and 5% GLO isomers. The general characteristics and the tocopherol and phytosterol contents were mostly similar between HGCO and nontransformed canola oil. No detectable amounts of amino acids and nucleotides were observed in the HGCO.

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KEY WORDS: Fatty acid composition, genetically transformed canola, high-performance liquid chromatography, γ-linolenic acid, nuclear magnetic resonance, positional distribution, phytosterols, tocopherols, triacylglycerol molecular species.

Dietary supplementation of γ-linolenic acid (G, or Δ6,9,12-18:3, or 18:3n-6) is beneficial in many physiological and pathological conditions such as inflammatory diseases, cancer, and diabetes (1–4). Gamma (γ)-linolenic acid is commercially available mainly from the seed oils of borage, black currant, and evening primrose. It is also found in some fungi and algae such as *Mortierella* spp. and *Spirulina* spp. However, γ-linolenic acid-containing oils are not widely used due to their high production cost, fluctuation in availability, or safety concerns. In order to develop an economical, reliable, and wholesome source of γ-linolenic acid, we have genetically transformed a low α-linolenic acid canola plant into strains that can produce high levels of γ-linolenic acid (5). The nontransformed canola plants produce seed oils with large amounts of oleic (O, 18:1n-9) and linoleic (L, Δ9,12-18:2) acids. By introducing the Δ6-desaturase (converts L to G) and the Δ12-desaturase (converts O to L) genes from the fungus *M. alpina*, within a seed-specific expression vector, into canola plant cells, a high level of 18:3n-6 was produced in seeds of the genetically transformed canola plant. The seeds from the fifth generation field trial were crushed, and the oil was extracted and refined in a commercial facility. The oil is the first kind of genetically transformed canola oil that contains a high level of 18:3n-6. In this study, we evaluated the fatty acid composition, triacylglycerol (TG) molecular species, positional distribution of n-6 fatty acids, unsaponifiable matter (phytosterols and tocopherols), general quality, and nonlipid constituents of this high-18:3n-6 canola oil (HGCO) and compared them with properties of the oils from borage, black currant, evening primrose, and the nontransformed canola.

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EXPERIMENTAL PROCEDURES

Canola oils. In this study, the seed oils from transgenic and nontransformed canola (*Brassica napus*) were analyzed. The method of cloning Δ12- and Δ6-desaturases from *M. alpina* and recombinant production of 18:3n-6 in plants was previously reported (5). Briefly, two cDNA clones with homology to known desaturase genes were isolated from the fungus *M. alpina.* Verification of substrate specificity of desaturase clones was obtained by expression in recombinant *Saccharomyces cerevisiae* and transgenic canola seeds. For expression in canola, the cDNA were inserted into a napin expression cassette (6) and transferred into a binary vector (7) for *Agrobacterium*-mediated transformation (8) of a low α-linolenic variety of *B. napus*. Regenerated plants were grown in the greenhouse, and seeds from different generations were analyzed for fatty acid composition. In 1998 the seeds from the fifth generation field trial were pressed, extracted, and refined by POS Pilot Plant Corporation (Saskatoon, Canada).

Chemical analyses. The TG fraction of HGCO was first purified in an open column packed with silicic acid. The purified TG fraction was then separated into various subfractions (molecular species) using a Hewlett-Packard 1090 high-performance liquid chromatograph (HPLC) (Palo Alto, CA) equipped with two Supelcosil C-18 columns (25 cm × 0.46 mm i.d., 5 μm) (Supelco, Bellefonte, PA) connected in series as described previously (9). The TG subfractions were eluted isocratically with acetonitrile/2-propanol (65:35, vol/vol) at 1 mL/min for 100 min and monitored with a Hewlett-Packard 1090 series II diode array detector at 210 nm. The distribution (% by mass) of each TG molecular species was determined by using an Alltech (Deerfield, IL) model 500 evapo-

rative light scattering detector (ELSD) (10). Based on their fatty acid compositions and retention times, TG molecular species were identified.

Each TG molecular species eluted from the HPLC was collected individually and repeatedly until enough mass was obtained for each sample. The pooled sample of each TG molecular species was transesterified with methanol and analyzed for fatty acid composition using a Hewlett-Packard 5890 II plus gas chromatograph (GC) equipped with a Supelco Omegawax 320 capillary column (30 m × 0.32 mm i.d.) as described previously (11). The purified total TG fraction and the GGL and GLO molecular species were also analyzed by high-resolution proton-decoupled ^{13}C nuclear magnetic resonance (NMR) for positional distribution of fatty acids in TG as previously described (12). Briefly, for the purified total TG fraction, approximately 100 mg of sample in 700 μL deuterochloroform was used. The proton-decoupled ^{13}C NMR data were collected at 30°C utilizing a Varian Inc. 5-mm broadband probe (Palo Alto, CA). The purified GGL and GLO molecular species samples (approximately 1 mg each) were dissolved in approximately 80 μL of deuterochloroform. Owing to the limited sizes of the GGL and GLO samples, the NMR data were collected in 3-mm Shigemi NMR tubes using a 3-mm dual-tuned ($^{13}C/^{31}P$) Nalorac probe (Martinez, CA) at 27°C. All proton-decoupled ^{13}C NMR data were collected on a Varian Unity 500 MHz spectrometer (Palo Alto, CA) operating at 125.728 MHz. The purified GGL and GLO molecular species were also analyzed for the distribution of positional isomers by using the silver-ion high-performance liquid chromatography (HPLC) method of Adlof (13) with a modified mobile phase. For this assay, a mixture of hexane/acetonitrile/2-propanol (99:1:0.2) was used as the mobile phase.

Phytosterol contents were determined by GC (14) with a Supelco SAC column (30 m × 0.25 mm i.d., 0.25 μM film). Tocopherols were analyzed according to the HPLC method of Balz *et al.* (15). The general physical and chemical properties of HGCO were determined by Covance Laboratories (Madison, WI) according to the respective methods of U.S. Pharmacopeia (16) and The American Oil Chemists' Society (17). Amino acids and nucleotides were also determined by Covance Labs using the modified methods of the Association of Official Analytical Chemists (18) and Paubert-Braquet *et al.* (19), respectively.

RESULTS AND DISCUSSION

Introduction of Δ6*-desaturase and* Δ12*-desaturase genes.* In canola, introduction of the *M. alpina* Δ6–desaturase gene alone facilitated the conversion of L (Δ9,12–18:2) to G (Δ6,9,12-18:3). Oleic acid (Δ9-18:1) also was converted to form substantial quantities (4.5%) of Δ6,9-18:2, an uncommon fatty acid (Table 1). This indicated that the activity of the endogenous Δ12-desaturase in the transformed canola plant was not adequate to convert the newly formed Δ6,9-18:2 to Δ6,9,12-18:3. To minimize the accumulation of Δ6,9-18:2, a Δ12-desaturase gene from *M. alpina* also was inserted into the transformed canola cells. As shown in Table 1, simultaneous expression of both Δ6- and Δ12-desaturase genes maximized the conversion of Δ6,9-18:2 to Δ6,9,12-18:3 and produced HGCO with basically no (<0.05% or less) Δ6,9-18:2.

TABLE 1
Fatty Acid Compositions of the Seed Oil from (A) Nontransformed Canola (control), (B) Transgenic Canola with the *Mortierella alpina* Δ6-Desaturase Gene, (C) Transgenic Canola with the *M. alpina* Δ12-Desaturase Gene, and (D) Transgenic Canola with Both Δ6- and Δ12-Desaturase Genes

Fatty acid	A (control)	B (Δ6 only)	C (Δ12 only)	D (both Δ6 and Δ12)
16:0	4.2	5.5	4.7	5.3
18:0	2.7	4.0	1.8	3.0
18:1	71.5	60.3	45.4	20.0
18:2n-6	19.9	11.4	45.8	22.4
18:3n-3	1.6	1.1	2.3	1.4
Δ6,9-18:2	0	4.5	Trace[a]	Trace
18:3n-6	0	13.2	0	43.0
Others	0.1	0	0	4.9

[a]Trace (<0.05%).

Fatty acid composition. HGCO contained 43% 18:3n-6, which was much higher than other 18:3n-6-containing oils such as evening primrose, black currant, and borage oils (Fig. 1). The acids 18:2n-6 and 18:1n-9 were also present in substantial amounts in the HGCO. The 18:2n-6 (23%) of HGCO was similar to the 21% of the nontransformed canola oil (NTCO), but lower than the other 18:3n-6-containing oils. The 18:1n-9 content (16%) of HGCO was considerably lower than the 60% of the NTCO, although it was comparable to those of the other 18:3n-6 containing oils.

Similar to NTCO, HGCO contained the following minor fatty acids: palmitic (P, 16:0), palmitoleic (Po, 16:1n-7), stearic (S, 18:0), α-linolenic (Ln, 18:3n-3), gadoleic (Eo, 20:1n-9), and erucic (Do, 22:1n-9) acids. In addition, HGCO contained trace amounts of stearidonic acid (Sd, 18:4n-3), a desaturation product of 18:3n-3. In comparison with other 18:3n-6-containing oils (Fig. 1), HGCO had similar amounts of 18:3n-3 and 18:4n-3 to evening primrose and borage oils. HGCO had a very low amount (0.02%) of erucic acid while NTCO and borage oil had 0.7 and 2.5%, respectively.

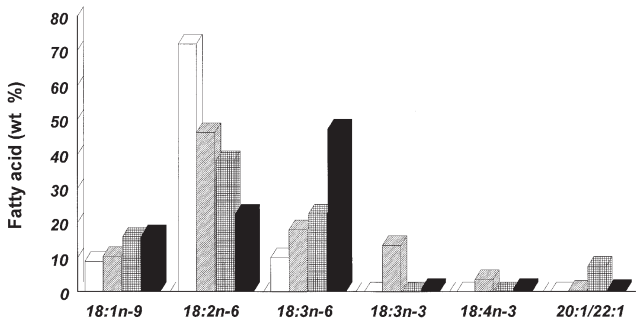

FIG. 1. Comparison of fatty acid composition in evening primrose oil (open blocks), black currant oil (diagonally lined), borage oil (cross-hatched) and high γ-linolenic acid canola oil (HGCO) (solid).

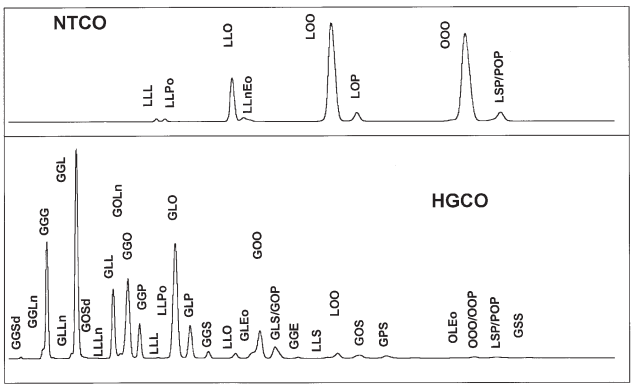

FIG. 2. A typical reversed-phase high-performance liquid chromatography (HPLC) chromatogram of triacylglycerol (TG) molecular species in nontransformed canola oil (NTCO) and HGCO. G, γ-linolenic acid, 18:3n-6; L, linoleic acid, 18:2n-6; O, oleic acid, 18:1n-9; P, palmitic acid, 16:0; Po, palmitoleic acid, 16:1n-7; S, stearic acid; 18:0; Ln, α-linolenic acid, 18:3n-3; Eo, gadoleic acid, 20:1n-9; Sd, stearidonic acid, 18:4n-3. For other abbreviation see Figure 1.

TG molecular species analysis. Figure 2 shows a typical reversed-phase HPLC separation of the individual TG molecular species in NTCO and HGCO. Each TG molecular species was arbitrarily labeled according to its three fatty acid components without defining their stereospecific positions in the molecule.

There were three major TG molecular species in NTCO, i.e., LLO, LOO, and OOO, as reported previously by Prevot *et al.* (20), in rapeseed oils with low 18:3n-3 content. As a result of 18:3n-6 synthesis, HGCO contained a total of 33 TG molecular species (Fig. 2). These included the 18:3n-6-containing TG molecules, GGG, GGX, and GXX, where X represents fatty acids other than18:3n-6. The distribution of 18:3n-6-containing TG molecular species in HGCO and NTCO is shown in Table 2. The predominant 18:3n-6-containing TG molecular species in borage oil were: GLL (15%), GLO (12%), GGL (10%), and GLP (10%), as reported by Huang *et al.* (9). Evening primrose and blackcurrant oils contained 17.6 and 12.5% GLL, respectively, as the most abundant 18:3n-6-containing TG molecular species (9,21). Among the four oils, HGCO had the highest levels of GGG (11%), GGL (23%), GGO (12%), GLO (20%), and GOO (5.5%) but the lowest level of GLL (7.7%) (Table 2).

Positional distribution of 18:3n-6 in TG molecules. The positional distribution of 18:3n-6 was determined by the ^{13}C NMR spectrum of the purified total TG fraction of HGCO (Fig. 3). Overall, the NMR determination of fatty acid subclasses was comparable to results of GC analysis of the same purified total TG fraction. The NMR spectrum indicated that approximately 75% of 18:3n-6 was located at the *sn*-1 and *sn*-3 positions, and the remaining 25% was at the *sn*-2 position. These data also showed approximately twice as much (15.68%) Δ9:2,3 fatty acids (L and Ln) at the *sn*-2 position than (7.70%) at the *sn*-1,3 positions. However, there was very little (1.4%) Δ9:3 (Ln) present in HGCO. Thus, 18:2n-6 was distributed preferentially at the *sn*-2 position.

When the positional distributions of L and G in HGCO were compared with other 18:3n-6-containing oils, it was noted that the distribution pattern of linoleic acid in HGCO was different from those of the other three oils as reported by Lawson and Hughes (22). The distribution pattern of 18:3n-6 in HGCO was also different from that of borage oil but similar to those of evening primrose and black currant oils.

The result from silver-ion HPLC analysis showed that the GGL molecular species of HGCO contained 50.2% GGL and 49.8% GLG positional isomers (Fig. 4A). The NMR analysis of the GGL molecular species showed a relatively similar result. Silver-ion HPLC analysis of the GLO molecular species (Fig. 4B) indicates that it consisted of 94.5% GOL and 5.5% GLO positional isomers. Analyses by NMR showed that the GLO molecular species contained no isomers with 18:3n-6 located at the *sn*-2 position. In other words, only GOL and GLO positional isomers were present in the GLO molecular species.

Composition of the unsaponifiable fraction. The unsaponifiable fractions determined in this study were phytosterols and tocopherols. HGCO contained similar amounts of campesterol and stigmasterol (Table 3) as compared with

TABLE 2
Distribution of Triacylglycerol (TG) Molecular Species in High γ-Linolenic Acid Canola Oil Nontransformed Canola Oil (HGCO) and Nontransformed Canola Oil (NTCO)

TG Species[b]	HGCO	NTCO
GGSd	0.2	—[a]
GGLn	0.7	—
GGG	11	—
GLLn	0.4	—
GGL	23	—
GOSd	0.3	—
LLLn	0.1	—
GLL	7.7	—
GOLn	0.6	—
GGO	12	—
GGP	4.1	—
LLL	0.1	0.5
LLPo	0.1	0.5
GLO	20	—
GLP	4.7	—
GGS	0.9	—
LLO	0.8	11
LLnEo	—	1.5
GLEo	0.9	—
GOO	5.5	—
GLS/GOP	2.8	—
GGEo	0.2	—
LLS	0.2	—
LOO	1.1	38
LOP	0.9	2.9
GOS	0.7	—
GPS	0.1	—
OLEo	0.1	—
OOO/OOP	0.3	41
LSP/POP	0.5	3.9
GSS	0.1	—

[a]Not present.

[b]G, γ-linolenic acid, 18:3n-6; L, linoleic acid, 18:2n-6; O, oleic acid, 18:1n-9; P, palmitic acid, 16:0; Po, palmitoleic acid, 16:1n-7; S, stearic acid; 18:0; Ln, α-linolenic acid, 18:3n-3; Eo, gadoleic acid, 20:1n-9; Sd, stearidonic acid, 18:4n-3.

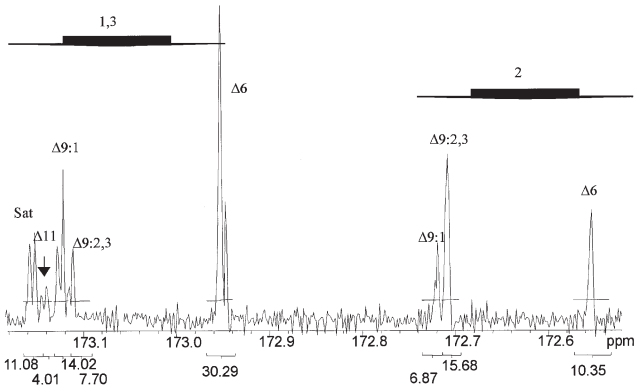

FIG. 3. The ^{13}C nuclear magnetic resonance spectrum of the purified total TG fraction from HGCO. Notation of fatty acid subclasses: Sat = 16:0, 18:0, 20:0, and 22:0; Δ11 = 18:1n-7 and 20:1n-9; Δ9:1 = 16:1n-7 and 18:1n-9; Δ9:2,3 = 18:2n-6 and 18:3n-3; and Δ6 = 18:3n-6. For abbreviations see Figures 1 and 2.

NTCO. However, the concentration of β-sitosterol was higher and that of brassicasterol was lower than NTCO. The total amount of phytosterols was higher in HGCO than in NTCO. Previously, it was reported that genetic modification could increase or decrease phytosterol contents in canola oils (23), depending on the variety.

HGCO contained significantly higher α- and γ-tocopherol but lower amounts of δ-tocopherol as compared to NTCO. However Abidi *et al.* (21) compared the oils from 12 varieties of genetically modified canola and concluded that the genetically modified canola had either higher or lower levels of α-, γ-, and δ-tocopherols than the controls, and there was not a distinct pattern of influence from genetic modification on tocopherol contents.

General quality of HGCO. The general physical and chemical characteristics of HGCO are not different from those of NTCO. The specific gravity (0.923 at 25°C), saponification value (188 mg KOH/g), peroxide value (1.2 meq/kg), and unsaponifiable content (1.6%) of HGCO are similar to NTCO. The only notable difference was that, due to its high degree of unsaturation, HGCO had a much higher iodine value (174 g/100 g) than the 120 g/100 g of NTCO. In HGCO, the impurities such as benzene, chloroform, 1,4-dioxane, methylene chloride, and trichloroethene were all below the detection limit (50–500 ppm) of the method. There were no detectable amounts (less than 5 ppm) of heavy metals (as lead) present in HGCO.

TABLE 3
Phytosterol and Tocopherol Contents of High 18:3n-6 and Conventional Canola Oils

	High 18:3n-6	Conventional
Phytosterol (g/100 g oil)		
β-Sitosterol	1.09	0.63
Campesterol	0.46	0.45
Stigmasterol	0.03	0.03
Brassicasterol	0.07	0.11
Tocopherol (mg/kg oil)		
α	347	175
γ	404	362
δ	12	25

Nonlipid constituents in HGCO. One of the concerns about genetically modified foods is the presence of proteins and nucleic acids. This analysis showed that there were no detectable amounts (less than 100 ppm) of protein or amino acids in HGCO. There were also no detectable amounts of nucleotides found in HGCO. All five nucleotides (cytidine-5′-monophosphate, uridine-5′-monophosphate, guanosine-5′-monophosphate, inosine-5′-monophosphate, and adenosine-5′-monophosphate) were below the detection limits of 1 to 4.6 ppm.

HGCO had a desirable fatty acid composition, which is beneficial for wider nutritional applications. The general quality and unsaponifiable matters of HGCO were not substandard to NTCO. No detectable amounts of proteins, nucleotides, organic volatile impurities, and heavy metals (as lead) were observed in HGCO.

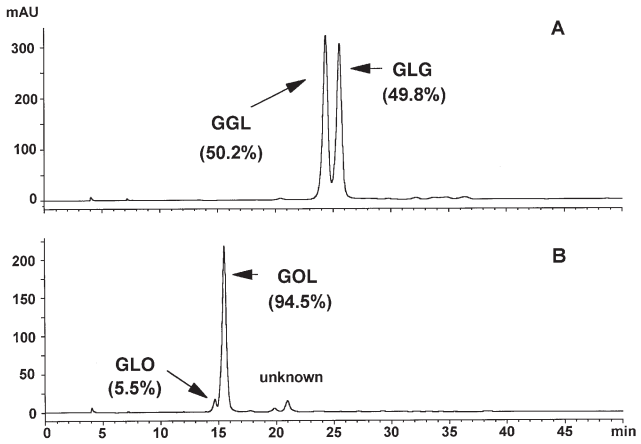

FIG. 4. Silver-ion HPLC separations of positional isomers in the two most predominant TG molecular species of HGCO: GGL (A) and GLO (B). For abbreviations see Figures 1 and 2.

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