# Phytosterol Accumulation in Canola, Sunflower, and Soybean Oils: Effects of Genetics, Planting Location, and Temperature

**Chris Vlahakis and Jan Hazebroek***

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

**ABSTRACT:** To assess the potential of traditional selection breeding to develop varieties with increased phytosterol content, we determined concentrations of those sterols in canola, sunflower, and soybean seed oils produced from breeding lines of diverse genetic backgrounds. Seed oils were extracted and saponified, and the nonsaponifiable fractions were subjected to silylation. The major phytosterols, brassicasterol, campesterol, stigmasterol and β-sitosterol, were quantified by capillary gas chromatography with flame-ionization detection. Canola contained approximately twice the amount of total phytosterols (4590–8070 μg $g^{-1}$) as sunflower (2100–4540 μg $g^{-1}$) or soybean (2340–4660 μg $g^{-1}$) oils. Phytosterol composition varied among crops as expected, as well as within a crop. Both genetic background and planting location significantly affected total phytosterol concentrations. Soybean plants were maintained from flower initiation to seed maturity under three temperature regimes in growth chambers to determine the effect of temperature during this period on seed oil phytosterol levels. A 2.5-fold variability in total phytosterol content was measured in these oils (3120–7920 μg $g^{-1}$). Total phytosterol levels increased with higher temperatures. Composition also changed, with greater percent campesterol and lower percent stigmasterol and β-sitosterol at higher temperatures. In these soybean oils, total phytosterol accumulation was correlated inversely with total tocopherol levels. Owing to the relatively limited variability in phytosterol levels in seed oils produced under field conditions, it is unlikely that a traditional breeding approach would lead to a dramatic increase in phytosterol content or modified phytosterol composition.

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**KEY WORDS:** Canola oil, fatty acid composition, genetic modification, phytosterols, planting location, soybean oil, sunflower oil, temperature, tocopherols.

Interest in phytochemicals has grown in recent years. For example, sterols derived from plant seed oils represent an important base stock for the health and nutrition industry. Plant-derived sterols have been shown to significantly lower blood serum cholesterol in people with mildly elevated cholesterol levels (1). β-Sitosterol was used at one time to treat hypercholesterolemia in the United States (2). Phytosterols have also been used by the cosmetic industry as emulsifiers (2). Phytosterols are precursors to hormonal sterols, 75% of which worldwide are derived from soybean oil (2). These natural-source phytosterols are recovered as a co-product from the deodorization step during processing of crude soybean and other vegetable oils.

If significant genetically controlled variability in seed phytosterol levels exists in the germplasm base or can be generated by mutation or genetic engineering, classical breeding techniques might be used to develop varieties that produce increased amounts of this value-added co-product. For this reason, we quantified phytosterols in seed oils produced by selected lines of canola, sunflower, and soybean. As with most traits, stability of expression across many growing environments is highly desired. To assess this, we analyzed oils produced by several soybean lines grown at five locations in the midwestern United States. We also investigated the effect of temperature on phytosterol concentration in soybean seeds.

## MATERIALS AND METHODS

*Plant material.* Seeds were produced from commercial and experimental lines developed through mutation and selection breeding. The canola, sunflower, and some of the soybean lines tested produce oils with genetically modified fatty acid compositions, but additional soybean lines with other traits, such as enhanced yield, disease resistance, or herbicide tolerance, were included. To test the effect of planting location, 12 genetically diverse soybean lines that produced commodity (not genetically modified for fatty acid composition) oil types were harvested from selected 1995 yield trial plots located in Johnston, Iowa; LaSalle, Illinois; Napoleon, Ohio; Jasper, Michigan; and Pocahontas, Iowa. Each test plot consisted of four 4.6-m rows planted at a rate of 28 seed $m^{-1}$. Seed from the center two rows were harvested, pooled, and stored at 4°C prior to analysis.

To test the effect of temperature from flowering to seed maturation on phytosterol accumulation, seeds from each of nine soybean lines were sown in 1-gal pots in Universal Mix potting soil (Strong Lite, Seneca, IL). Each pot contained one plant and was kept in a greenhouse maintained between 18 and 35°C. Plants were top watered and fertilized periodically with 20N-20P-20K commercial fertilizer. Upon flowering, two plants from each line were placed in each of three growth chambers (Controlled Environments, Winnipeg, Canada). Pots were posi-

*To whom correspondence should be addressed at Pioneer Hi-Bred International, Inc., 7300 NW 62nd Ave., P.O. Box 1004, Johnston, IA 50131-1004. E-mail: hazebroekjp@phibred.com

tioned at the same location within each growth chamber. A nonrandom experimental design was used to reduce the effect of position within the growth chamber on the temperature response. Although this design compromises the significance of differences across lines, quantifying these differences was not a primary goal of the experiment. Three daily temperature regimes were used: 35, 27, and 20°C for the 12-h light cycle alternating with 25, 17, and 10°C, respectively, for the 12-h dark cycle. Light, provided by a combination of incandescent and fluorescent lamps, was at an intensity of 220 μEi $m^{-2}$ $s^{-1}$ in all three chambers. Plants were allowed to mature, at which time seeds were harvested. Seeds from individual plants were pooled and stored at 4°C until analyzed.

*Crude oil extraction.* For each sample, 10 g of seed was ground to a powder in a small mill (Regal Ware, Kewaskum, WI). Forty milliliters of hexane (technical grade, Fisher Scientific, Fair Lawn, NJ) was added, and crude oil was extracted over 4 h with occasional stirring. Extracts were decanted through 0.2-μm cellulose acetate filters (Nalge, Rochester, NY), and the hexane was removed by evaporation at room temperature under reduced light. Crude oil extracts were transferred to amber vials and stored at –20°C in the dark. Extracts were prepared from duplicate seed subsamples, except for soybeans raised in growth chambers. In this case, single extracts were made from seeds from each of two duplicate plants.

*Saponification.* Phytosterols in the extracted oils were isolated by a modification of the procedure of Mounts and coworkers (3). Since this procedure includes an alkaline hydrolysis step, both free and bound (fatty acid esters, glucosides, and glucoside esters) sterols were quantified. Approximately 0.1 g crude oil was placed into a 16 × 125 mm glass test tube and 40 μg cholestane internal standard (Aldrich, Milwaukee, WI) was added. Two milliliters of 2 N KOH in ethanol was added, and the samples were vortexed briefly and then sonicated for 10 min to dissolve the oil. Samples were incubated at 60°C for 1 h, then at room temperature overnight. Two milliliters of 18-Ω water was added, and the nonsaponifiable matter was extracted with three 2-mL aliquots of ethyl ether. The pooled ether extracts were washed with 4-mL portions of 18-Ω water until pH paper yielded a neutral result. Ether fractions were evaporated under a stream of $N_2$ at room temperature and redissolved in 1.0 mL bis(trimethylsilyl)trifluoroacetamide (BSTFA, Supelco, Bellefonte, PA). Silylation was achieved after incubation at 60°C for 1 h. Samples were stored at 4°C overnight prior to analysis.

*Gas chromatography.* Brassicasterol, campesterol, stigmasterol, and β-sitosterol were isolated by capillary gas chromatography using a Hewlett-Packard 5890 gas chromatograph (Palo Alto, CA). Samples (1 μL) were introduced by splitless injection onto a 30 m × 0.25 mm i.d. × 0.25 μm film thickness SAC$^{TM}$-5 column (Supelco). An initial oven temperature of 50°C was held for 2 min, increased at 20°C $min^{-1}$ to 250°C, then at 1°C $min^{-1}$ to 280°C and held for 3 min, and finally increased at 20°C $min^{-1}$ to 305°C where it was held for 10 min. Inlet and flame-ionization detector temperatures were 300°C. Helium carrier gas flow was 2 mL $min^{-1}$ at 50°C. Detector signal was recorded and peaks integrated using PerkinElmer Turbochrom v. 3.2 software (Cupertino, CA). A six-point internal standard calibration curve was constructed between 5 and 300 μg $mL^{-1}$ of each phytosterol (Matreya, Pleasant Gap, PA, except for β-sitosterol, Sigma, St. Louis, MO) with 40 μg $mL^{-1}$ cholestane in BSTFA as trimethylsilyl ethers. The coefficient of linearity for each phytosterol was greater than 0.999. Phytosterols were quantified using a standard calibration curve created at the same time that the samples were run. Results are reported as a mean of the two replicate extracts. Fatty acid and tocopherol compositions in these oils have been reported previously (4).

*Statistical analysis.* A two-factor with replication analysis of variance (ANOVA) was performed on data from soybean lines from the 1995 yield trial and from those raised in the growth chambers to describe the relationships between total tocopherol concentration, phytosterol concentration and composition, genetic background, planting location, and temperature. Comparisons of means were made among lines. Least significant differences were calculated for each line grown in growth chambers under the three temperature regimes. Correlation coefficients were calculated for all crops to describe the relationships between phytosterol amount and tocopherol amount, and phytosterol amount and fatty acid composition expressed as calculated iodine value.

## RESULTS AND DISCUSSION

*Phytosterols in selected germplasm.* Phytosterol concentrations in the canola oils analyzed are shown in Table 1. Total phytosterol concentrations in these oils ranged between 4590 and 8070 μg $g^{-1}$. Total phytosterol concentrations in sunflower seed oils ranged between 2100 and 4540 μg $g^{-1}$ (Table 2). Among soybean oils with genetically modified fatty acid compositions, total phytosterol concentrations were between 2350 and 4050 μg $g^{-1}$ (Table 3). Single-factor ANOVA revealed that phytosterol concentration was affected significantly by the genetics of each line at the 99% confidence level.

The range of either individual or total phytosterols found was similar to those reported previously in oils of these crops (3,5–8). As expected, brassicasterol was found only in the canola oils. Phytosterol concentration ranged approximately twofold within each crop. Among individual lines of each species, composition also varied. Based on our findings, a selection breeding strategy designed to raise total phytosterol content beyond this level would probably require additional resources. Alteration of gene expression by mutation or introgression of new genes through transformation would be necessary to generate such plants. The result would be more of the value-added co-product as well as an increased likelihood of the crop meeting a commercial specification.

*Effect of planting location on phytosterol accumulation.* Total phytosterol amounts in soybean oils with commodity-type (not genetically modified) fatty acid compositions produced by plants grown at five locations are presented in Table 4. ANOVA results revealed that both genotype and planting location significantly ($P > 0.01$) affected total phytosterol levels in these oils.

**TABLE 1**
**Mean Phytosterol and Total Tocopherol Amounts (μg g$^{-1}$) in Canola Oils**

| Line | Brassicasterol | Campesterol | Stigmasterol | β-Sitosterol | Total sterols | Total tocopherols[a] |
|---|---|---|---|---|---|---|
| NS2309 | 590 c[b] | 1740 b | ND[c] | 2860 d | 5190 b | 570 b |
| 46A12 | 530 a | 1520 a | ND | 2590 b | 4640 a | 640 c |
| 45A37 | 540 b | 1750 b | ND | 3060 e | 5350 c | 500 a |
| 46A16 | 820 g | 2050 c | ND | 3050 e | 5920 e | 590 b |
| NC2304 | 790 f | 1500 a | ND | 2310 a | 4590 a | 560 b |
| NS2290 | 670 e | 2520 e | ND | 2770 c | 5960 e | 690 d |
| 46A65 | 910 h | 2800 f | ND | 3780 f | 7480 f | 560 b |
| 46A05 | 660 d | 2210 d | ND | 2610 b | 5490 d | 570 b |
| Goldrush | 1060 i | 3080 g | ND | 3920 g | 8070 g | 630 c |

[a]Data from Reference 4.
[b]Values in the same column with the same letter are not statistically different at the 0.05 significance level.
[c]ND, not detected.

**TABLE 2**
**Mean Phytosterol and Total Tocopherol Amounts (μg g$^{-1}$) in Sunflower Oils**

| Line | Brassicasterol | Campesterol | Stigmasterol | β-Sitosterol | Total sterols | Total tocopherols[a] |
|---|---|---|---|---|---|---|
| Sun #1 | ND | 370 c[b] | 620 h | 2480 d | 3470 d | 1300 f |
| Sun #2 | ND | 350 c | 320 c | 2040 b | 2710 b | 900 d |
| Sun #3 | ND | 290 b | 220 a | 2170 c | 2690 b | 710 a |
| Sun #4 | ND | 210 a | 220 a | 1670 a | 2100 a | 840 c |
| Sun #5 | ND | 550 e | 550 g | 2620 e | 3720 e,f | 1270 f |
| Sun #6 | ND | 480 d | 500 f | 2890 f | 3870 f | 730 a,b |
| Sun #7 | ND | 580 e | 220 a | 2040 b,c | 2840 b | 920 d |
| Sun #8 | ND | 360 c | 450 e | 2210 c | 3020 c | 940 d |
| Sun #9 | ND | 500 d | 500 f | 3080 g | 4080 g | 1860 h |
| Sun #10 | ND | 360 c | 460 e | 2820 f | 3640 e | 1200 e |
| Sun #11 | ND | 500 d | 420 d | 3620 h | 4540 h | 1390 g |
| Sun #12 | ND | 200 a | 290 b | 2160 c | 2650 b | 780 b |

[a]Data from Reference 4.
[b]Values in the same column with the same letter are not statistically different at the 0.05 significance level. For abbreviations see Table 1.

There was also a significant ($P > 0.01$) interaction between the two main effects. This result implies that a classical breeding approach designed to develop soybean germplasm with altered phytosterol seed oil contents would likely be complicated by significant instability of the trait across growing locations. Furthermore, it is left to speculation what actual environmental parameter(s) affect phytosterol levels. In an initial effort to investigate this, we produced soybean seeds under controlled temperatures in order to elucidate the effect of this specific environmental parameter on phytosterol quantity and quality.

*Effect of temperature on phytosterol accumulation.* Total phytosterol concentrations in soybean seeds developed under

**TABLE 3**
**Mean Total Phytosterol and Total Tocopherol Amounts (μg g$^{-1}$) in Soybean Oils with Genetic Modification for Fatty Acid Composition**

| Line | Brassicasterol | Campesterol | Stigmasterol | β-Sitosterol | Total sterols | Total tocopherols[a] |
|---|---|---|---|---|---|---|
| BULK ABC | ND[b] | 1170 f[c] | 690 d | 1690 e | 3540 e | 2150 e |
| A91-194022 | ND | 720 b | 590 c | 1350 b | 2660 b | 1510 a,b |
| A91-200049 | ND | 950 d,e | 680 d | 1610 c,e | 3250 d | 1750 b |
| BULK DE | ND | 970 d,e | 660 d | 1570 c,e | 3200 d | 1360 a |
| A92-215069 | ND | 620 a | 470 a | 1250 a | 2350 a | 1410 a |
| YB24ZA | ND | 1310 g | 740 e | 2000 g | 4050 f | 1690 b,c |
| 9253[b] | ND | 790 c | 540 b | 1600 c,e | 2930 c | 1210 a |
| A92-216030 | ND | 620 a | 500 a,b | 1350 b | 2460 a | 1900 c,e |
| YA7777J09 | ND | 910 d | 770 e | 1800 f | 3480 e | 1290 a |
| 9243c | ND | 800 c | 530 b | 1530 c | 2860 c | 1820 c |
| WA7343Z007 | ND | 990 e | 660 d | 2360 h | 4010 f | 2090 e |

[a]Data from Reference 4.
[b]Pioneer® (Johnston, IA) brand.
[c]Values in the same column with the same letter are not statistically different at the 0.05 significance level. For abbreviations see Table 1.

**TABLE 4**
**Mean Phytosterol Amounts(μg $g^{-1}$) in Soybean Oils with Commodity-Type Fatty Acid Composition Produced by Lines Grown in the Midwestern United States**

| Line | Jasper, MI | Johnston, IA | LaSalle, IL | Napoleon, OH | Pocahontas, IA |
|---|---|---|---|---|---|
| 2396[a] | 3680 c[f] | 3900 d | 4430 f | 3930 d | 3740 g |
| 2506[a] | 2980 a | 3450 b | 3950 d,e | 3750 c | 3140 e |
| 2835[a] | 4110 f | 4010 d | 4660 g | 4360 f | 2990 d,e |
| 2990[b] | 3150 b | 3600 c | 4030 d,e | 3620 c | 2340 a |
| JACK | 3610 d | 3900 d | 4330 f | 3320 b | 2560 b |
| 9255[c] | 3500 c,d | 3280 a | 3730 b,c | 3590 c | 3040 d,e |
| 9281[c] | 3650 d | 3690 c,d | 4030 d,e | 4070 d,e | 2890 c,d |
| 1990[d] | 2960 a | 3220 a | 3380 a | 3020 a | 3560 f |
| 2918[d] | 3100 a,b | 4020 d | 3570 b | 3570 c | 2850 c,d |
| 2621[e] | 3360 c | 3230 a | 3680 b | 3540 c | 3700 f,g |
| 2660 | 3840 e | 3860 d | 3870 c,d | 4000 d,e | 2990 d,e |
| YB30M | 3640 d | 3810 d | 4130 e | 4170 e | 2770 c |

[a]Asgrow® (Urbandale, IA) brand.
[b]Agripro® (Ames, IA) brand.
[c]Pioneer® (Des Moines, IA) brand.
[d]Novartis® (Minneapolis, MN) brand.
[e]Stine® (Adel, IA) brand.
[f]Values in the same column with the same letter are not statistically different at the 0.05 significance level.

three controlled temperature regimes are presented in Table 5. Total phytosterol concentrations varied 2.5-fold in these samples. Total phytosterol levels were consistently higher at the elevated temperatures. Composition of the sterols also changed significantly, with proportionally more campesterol as temperature increased at the expense of stigmasterol and β-sitosterol (data not shown). Brassicasterol was either not detected or below limits of quantitation in these samples, as expected for soybean oils. Tocopherol levels in these same oils decreased at elevated temperatures (4), resulting in a negative correlation between total phytosterols and total tocopherols (Fig. 1). This observation is consistent with temperature-regulating flux through these two related biosynthetic pathways. The inverse relationship between phytosterol and tocopherol accumulation in soybean oils was not observed when all plants presumably experienced similar temperature regimes (i.e., had similar planting locations) during development (Table 6). Thus, temperature regulation of the isoprenoid biosynthetic pathway may only be manifested under more extreme temperature regimes.

Little is known about the influence of temperature on accumulation of phytosterols in seeds. However, there is considerable literature on the effect of temperature on phytosterol content in both vegetative (9–11) and reproductive (12–15) tissues of a number of species. The β-sitosterol/stigmasterol ratio is usually elevated in tissues exposed to low temperatures. Apparently, β-sitosterol synthesis continues at a reduced rate at low temperatures (i.e., 2°C), at which stigmasterol synthesis is halted (13). Since stigmasterol is not derived from β-sitosterol (14), temperature regulation is exerted earlier in the biosynthetic pathway. The β-sitosterol/stigmasterol ratio drops upon re-

**TABLE 5**
**Mean Total Phytosterol Amount (μg $g^{-1}$) in Soybean Oils Produced by Lines Grown Under Three Temperature Regimes**

| | Temperature regime (day/night) | | | |
|---|---|---|---|---|
| Line | 20/10°C | 27/17°C | 35/25°C | LSD[a] |
| 2396[b] | 3350 a[g] | 3800 a | 5910 a | 1540 |
| 2835[b] | 3600 a | 4660 a | 6830 a,b | 940 |
| 2990[c] | 3450 a | 4230 a | 6690 a,b | 770 |
| 9255[d] | 3450 a | 3880 a | 5720 a | 1510 |
| 1990[e] | 3120 a | 3640 a | 6320 a | 1520 |
| 2918[e] | 3360 a | 4650 a | 7960 b | 3010 |
| 2621[f] | 3460 a | 3580 a | 6360 a | 1050 |
| 2660[f] | 3270 a | 3900 a | 6460 a | 820 |
| YB30M | 3150 a | 3680 a | 6970 a,b | 880 |

[a]LSD, least significant difference.
[b]Asgrow® brand.
[c]Agripro® brand.
[d]Pioneer® brand.
[e]Novartis® brand.
[f]Stine® brand.
[g]Values in the same column with the same letter are not statistically different at the 0.05 significance level.

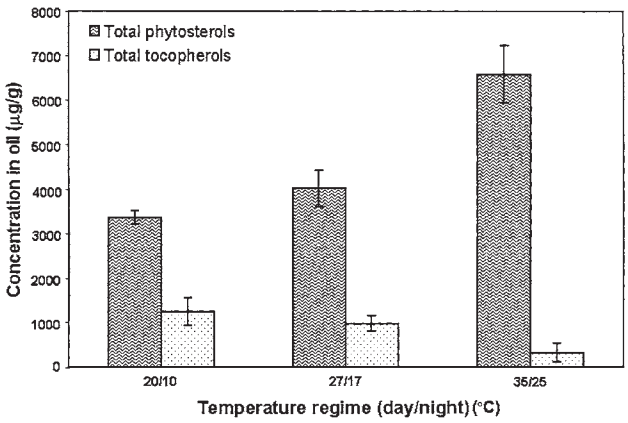


**FIG. 1.** Effect of temperature on total phytosterol and total tocopherol accumulation in soybean seed oils. Shown are means ± SD for all lines in Table 5.

**TABLE 6**
**Relationships Between Total Phytosterols and Total Tocopherols and Fatty Acid Composition (expressed as calculated iodine value) in Soybean, Sunflower, and Canola Oils**

| | | Correlation coefficient between total phytosterols[a] and | |
|---|---|---|---|
| Oils | *n* | Total tocopherols | Calculated iodine value |
| Soybean | | | |
| Genetically modified | 13 | 0.325$^{ns}$ | 0.156$^{ns}$ |
| Commodity-type | 60 | 0.097$^{ns}$ | –0.147$^{ns}$ |
| Sunflower | 12 | 0.690** | 0.164$^{ns}$ |
| Canola | 9 | 0.164$^{ns}$ | 0.766** |

[a]ns, not significant; $^{**}P < 0.05$.

warming, at least in tomato and green bell pepper fruit (15,16). Since the chemical structure of β-sitosterol is more planar than that of stigmasterol, the former has a greater capacity to reduce membrane water permeability (17). Thus, an elevated β-sitosterol/stimasterol ratio should enhance chilling survival by minimizing water loss upon cold treatment (18). Although we also found lower β-sitosterol levels at lower temperatures, we worked with a different tissue and at a different temperature range from those used in these studies. Thus, alterations of membrane constituents in response to cold stress might have little relevance to temperature regulation of phytosterol biosynthesis in oilseeds.

*Relationships between phytosterol accumulation and tocopherol accumulation and fatty acid composition.* Total phytosterols was not correlated significantly to total tocopherols in the canola oils or in genetically modified or commodity-type soybean oils (Table 6). This finding agrees with earlier work with genetically modified soybeans (3). However, in the sunflower oils, total phytosterols and total tocopherols were positively correlated. Whether this relationship is due to natural selection or caused directly by environmental factors such as temperature, light, etc., is not known.

The amount of total phytosterols was not correlated significantly with fatty acid composition, expressed as calculated iodine value, in the sunflower oils or in either type of soybean oils (Table 6). Our observations agree with earlier reports that phytosterol compositions were for the most part conserved in high oleic genotypes (19,20). However, in the canola oils analyzed, total phytosterols and calculated iodine value were significantly correlated. As there is no direct biochemical link between the synthesis of phytosterols and storage lipids, the exact nature of this relationship is not obvious. Proving the universality of the significant relationships among these traits found in the canola and sunflower oils examined would require the analysis of a larger group of oils.

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