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Enrichment of Tocopherols and Phytosterols in Canola Oil during Seed Germination

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The effect of canola (*Brassica napus* L.) seed germination under illuminated and dark environments on the total concentration and the composition of tocopherols and phytosterols in seedlings and extracted oil were investigated. During the first 10 days of germination, a decrease in γ-tocopherol was offset by an increase in α-tocopherol, indicating the interconversion of these isomers. From day 10 to day 20 under illumination, there was a net increase in α-tocopherol and total tocopherols suggesting the synthesis of new tocopherols, whereas there was no net increase in tocopherols in dark. Tocopherols were mainly concentrated in the leafy seedling tops rather than in the non-photosynthesizing bottoms, whereas phytosterols were equally distributed across both sections. The total tocopherol content of oil extracted from 20-day-old seedlings was 4.3- to 6.5-fold higher than that of intact seeds. On a dry seedling basis, the content and composition of phytosterols did not change significantly ($p > 0.05$) over the sprouting period, but the concentration of total phytosterols in the oil fraction increased 4.2- to 5.2-fold. The concentration of these valuable phytochemicals in the oil fraction is largely due to the depletion of oil reserves during germination, as well as the de novo synthesis of new α-tocopherol stimulated by the presence of light. Germination may represent a viable means to naturally concentrate these high-value constituents in canola oil, offering improvements in oil quality based on the nutritional value and oxidative protection offered by tocopherols and the health benefits provided by both tocopherols and phytosterols.

KEYWORDS: Oilseeds; canola; germination; seedling; tocopherols; phytosterols; minor constituents; sprouts

INTRODUCTION

Tocopherols and phytosterols are important lipid-soluble minor constituents of canola (*Brassica napus* L.) seeds. The content of tocopherols in canola seed oil ranges from 230 to 1000 mg/kg (*1*). In canola oil, distribution of the four known tocopherol isomers is commonly found to be ca. 64% γ-tocopherol, 35% α-tocopherol, <1% δ-tocopherol, and β-tocopherol is absent (*2*, *3*). Among all tocopherol isoforms, α-tocopherol has the highest vitamin E activity (*4*–*6*), whereas γ-tocopherol demonstrates the most potent antioxidant activity (*7*). Changes in isomeric forms of tocopherols during germination of rapeseed (*8*), soybean (*9*), and several cereal grains (*10*–*12*) have been reported.

Phytosterols are ubiquitous in the plant kingdom and play a role in membrane microstructure and cell integrity (*13*). In humans, consumption of plant-derived sterols and their conjugates has been correlated with lowering of blood cholesterol levels and reducing the risk of heart disease and cancer (*14*). Canola oils typically contain 0.5–1.1% phytosterols, of which the total phytosterol fraction is comprised of 45–58% sitosterol, 25–39% campesterol, 5–13% brassicasterol, 3–7% Δ5-avenasterol, <1% stigmasterol, and <1% Δ7-stigmasterol (*15*). During seed germination and subsequent plant growth, phytosterols undergo changes to meet the demands of the developing seedling (*16*). Since membrane formation and transformation is an important early event of seed germination, a number of researchers have studied the biosynthesis and interconversion of sterols during this phase of development and reported that the total sterol content of white mustard remained relatively constant on a dry weight basis but there was a distinct increase (about 2.8-fold) on a per plant basis (*9*, *17*–*20*). An increase in the phytosterol content of germinating alfalfa seeds (*18*) and soybean (*9*) has also been reported in literature. Little is known about changes in the phytosterol profile and concentration in canola seeds during germination.

Due to the nutritional value of α-tocopherol, the antioxidant properties of tocopherols in general, and the beneficial health effects of phytosterols, these constitutents are considered to be value-added compounds in seed oils. Our previous investigation reported the changes in the major nutrient contents (i.e., oil, protein, etc.) of canola seeds during germination (*21*). The objective of this study was to investigate whether germination improves the value of extracted canola oil and canola sprouts

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for food or feed applications via changes in tocopherol and phytosterol content and/or composition. The effect of light and seedling part on these changes was also investigated.

MATERIALS AND METHODS

Materials. Canola seeds Q2 summer rape (*Brassica napus* L.) were from Agricore United (Calgary, AB). Butylated hydroxytoluene (BHT), potassium hydroxide (KOH), bis(trimethylsily)trifluoroacetamide (BSTFA) + trimethylchlorosilane (TMCS) (99:1), and authentic tocopherol and phytosterol standards were procured from Sigma Scientific Co. (St. Louis, MO). All solvents were from Fisher Scientific Co. (Nepean, ON).

Seed Germination. The methods of germination, harvesting of seedlings, and sample preparation for analysis were the same as that described in our previous work (*21*). A sample (5 g) of canola seeds was soaked in 200 mL of distilled water for 4 h. After draining excess water, the seeds were allowed to germinate in Petri dishes lined with moistened filter paper. The germination was carried out in the presence and absence of light in a greenhouse at 20 °C. For the former, light was provided for 16 h/day, whereas for the latter, the dish was covered with aluminum foil to block the light. The light was supplied with 400 W high-intensity sodium bulbs. The germinating seeds and seedlings were watered twice daily using tap water (distilled) until the seedlings were harvested at intervals of 2, 5, 10, 15, and 20 days of germination, freeze-dried and stored airtight for analysis. In a separate experiment, seedlings were harvested at 10-, 15-, and 20-days time intervals, dissected into two sections, and freeze-dried. The leafy top portion was labeled "seedling tops", whereas the remaining portion (stem, roots, and seed coat) was labeled "seedling bottoms". Both whole seedlings and dissected tops and bottoms were subjected to analysis. The control seed samples were prepared by draining excess water after soaking and subsequently freeze-drying the seeds.

Determination of Tocopherols. Determination of tocopherols was carried out according to a modified method of Goffman and Becker (*22*). Approximately 0.5 g of seed or seedling sample was mixed with 25 mL of isooctane (containing 0.01% BHT) in a 50 mL screw-top plastic tube. The sample was ground using Polytron (PT3000, Brickman Instruments, Westbury, NY) at speed 6 for 1 min, and the tube was capped tightly before placing in a mechanical shaker for 4 h in the dark. The mixture was then centrifuged (Model MP4, International Equipment Company, Needham Heights, MA) at 5000 rpm for 10 min. A 5 mL aliquot of the supernatant was transferred into a glass tube, and the solvent was removed under a stream of nitrogen at room temperature in a fume hood. The residue was redissolved in 1.0 mL of hexane (HPLC grade, Fisher Scientific, Nepean, ON), and the contents were vortexed (Model G-560, Scientific Industries, Inc., Bohemia, NY) for 1 min, transferred into an HPLC vial, and subjected to high-performance liquid chromatography (HPLC).

A Shimadzu HPLC system (Shimadzu Scientific Instruments, Inc., Columbia, MD), loaded with a Shimadzu Class-VP chromatography laboratory automated software system for data handling, was used. The system used a Shimadzu RF535 fluorescence detector set at 295 nm for excitation and 330 nm for emission detections. Resolution of tocopherols was achieved on a Supelco LC-Diol column (25 cm × 4.6 cm, 5 μm, Supelco, Inc., Bellefonte, PA). Samples (20 μL) placed in a Hewlett-Packard 1050 autosampler were injected, and the elution under isocratic conditions was carried out using hexane with 0.6% isopropanol as mobile phase at a flow rate of 1 mL/min by Varian 9010 solvent delivery system (Varian Associates, Sugarland, TX). A calibration curve prepared for external standards of α-, β-, γ-, and δ-tocopherols (5−150 μg/mL) was used for identification and quantification purposes. The linear regression coefficient for each tocopherol standard curve was greater than 0.99. In addition, three sets of standard solutions were placed at the beginning, in the middle, and at the end of each batch.

Determination of Total Phytosterols. Phytosterols in crude oil were determined using a modified method of Mounts et al. (*23*). To approximately 0.05 g of oil placed in a glass test tube, 0.2 mg of dihydrocholesterol internal standard and 5 mL of 2 N alcoholic KOH were added. The contents were vortexed, incubated first at 70 °C for 1 h, and then allowed to stand overnight at room temperature. After adding 5 mL of deionized water and 15 mL of cyclohexane, the mixture was vortexed vigorously and centrifuged at 1000 rpm for 10 min. The cyclohexane fraction was transferred into a glass tube and washed with water until the pH reached neutral. It was then evaporated under a stream of N_2 at room temperature, and the residue was redissolved in 0.25 mL of pyridine and 0.25 mL of BSTFA containing 1% TMCS. This mixture was heated for 30 min and allowed to stand overnight at room temperature. The derivatized sample was dried under N_2, the residue was dissolved in hexane, and it was analyzed immediately by gas chromatography (GC).

TMS solutions (0.5 μL) were injected into a J&W Scientific DB-5HT fused silica capillary column (30 m × 0.25 mm i.d., 0.1 μm film thickness, Agilient Technologies) in a Varian 3400 gas chromatograph (Walnut Greek, CA) equipped with a flame ionization detector and a Varian 8100 autosampler. Helium was the carrier gas used at a head pressure of 25 psi. An initial column temperature of 70 °C was maintained for 0.2 min, ramped to 250 °C at 20 °C min^{-1}, and then to a final temperature of 280 °C at 15 °C min^{-1} where this temperature was maintained for 17 min. The initial injector temperature of 80 °C was maintained for 0.2 min and then ramped to 280 °C at 150 °C min^{-1}. The flame-ionization detector temperature was set at 280 °C. Chromatograms were recorded, and the peak integration was carried out using Shimadzu Class-VP software (version 4.2, Shimadzu Scientific Institutes, Inc., Columbia, MD). All species of sterols were quantified using an internal standard with the same response factor of 1.0. Peak identification was achieved by comparing the retention time of authentic sterols in a mixture containing campesterol, stigmasterol, sitosterol, and brassicasterol.

Statistical Analysis. All germination experiments were carried out in replicates, and analyses were carried out in duplicates. Analysis of variance of the results was performed using the General Linear Model procedure of SAS Statistical Software, Version 8 (SAS Institute Inc., 1999). The effect of germination, light, and seedling part on the content and composition of all components were evaluated at a significance level of 0.05.

RESULTS AND DISCUSSION

Changes in Tocopherols in Seedlings. Changes in the tocopherol concentration and composition in canola seedlings during germination in illuminated and dark conditions are shown in **Figure 1**. During the first 10 days of germination under illumination, levels of γ-tocopherol declined to baseline and levels of α-tocopherol increased such that there was no net change in the total tocopherol content during this period. However, after day 10, there was a further increase in the α-isomer, resulting in a net increase in total tocopherols from 370 mg/kg in dry seeds to 540 mg/kg in 20-day-old sprouts. No β-tocopherol was detected in unsprouted seeds, but detectable amounts (17−37 μg/g) were present after germination. A similar phenomenon has also been observed in sprouted seeds of *Picea abies* (*10*).

In dark conditions, a similar interconversion between the γ- and α-tocopherol isomers was observed, but the transformation occurred more rapidly than under illuminated conditions and was nearly complete only 5 days after germination. Levels of β-tocopherol also became detectable by germination in the dark. However, unlike the illuminated conditions, there was no net increase in tocopherol concentration over the 20-day period when germination took place in the dark. In this case, the concentration of α-tocopherol leveled off after 5 days. These findings suggest that the presence of light during germination is required to stimulate greater net production of tocopherols, α-tocopherol in particular.

Interconversion of the γ- and α-tocopherol isomers may be due to activation of the enzyme γ-methyltransferase (γ-MTF) during germination, which catalyzes the final step of the

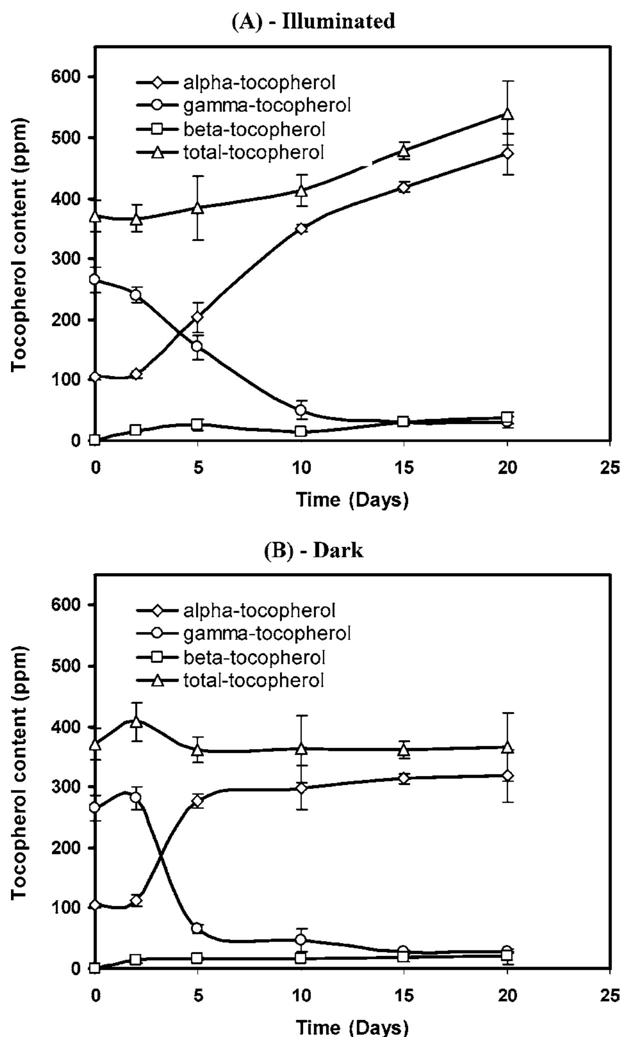

Figure 1. Changes in tocopherol content on seed dry weight basis under (**A**) illuminated and (**B**) dark conditions.

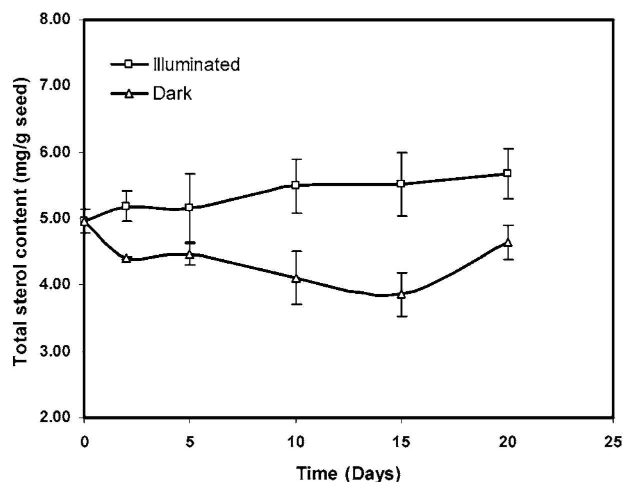

Figure 2. Changes in total sterol content (seed dry weight basis) during canola seed germination.

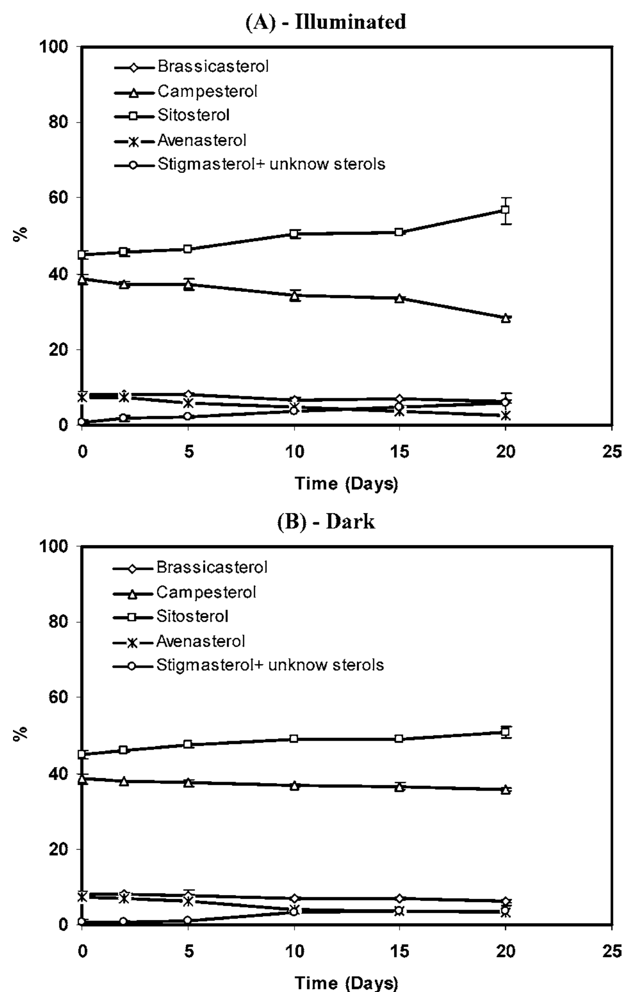

Figure 3. Changes in sterol composition in seedlings during canola seed germination: (**A**) illuminated and (**B**) dark conditions.

α-tocopherol synthesis pathway for which γ-tocopherol is a direct precursor (*24*). The decrease in γ-tocopherol was of a magnitude similar to the combined increase in the α- and β-isomers. γ-MTF is also responsible for the conversion of δ-tocopherol to the β-isoform. Thus an increase in its activity may also be responsible for the observed absence of δ-tocopherol postgermination (which is present in trace quantities in canola oil from intact seeds (*25*)) and the emergence of trace quantities of the β-isomer after day 2.

We were interested to see whether these changes in tocopherols occurred preferentially in the green leafy parts of the seedlings as compared to the bottom parts. In both lighting conditions, the leafy top parts of the seedlings contained significantly higher α-tocopherol, and consequently, higher total tocopherol levels than the seedling bottoms ($p < 0.05$) (data not shown). This result was expected because previous studies have shown that tocopherols accumulate in photosynthetically active tissues which are exposed to greater oxidative stress than other tissues (*6*, *10*). The γ- and β-tocopherol levels remained constant during this period under all conditions, and no significant changes in tocopherol levels occurred in the seedling bottoms.

Changes in Sterols in Seedlings. The total phytosterol content in canola seedlings measured over the course of 20-day germination is illustrated in **Figure 2**. No significant changes occurred in the total phytosterol content of the seedlings during sprouting, although the sprouts grown under illumination had significantly higher levels of phytosterols than those grown in the dark ($p < 0.05$). **Figure 3** shows the breakdown of changes in the phytosterol composition during germination. β-Sitosterol was the predominant phytosterol in unsprouted canola seeds, followed by campesterol, brassicasterol, avenasterol, and stigmasterol. Differences in phytosterol composition at the end of the germination period compared to the beginning were significant for all analytes ($p < 0.05$), whereas light was

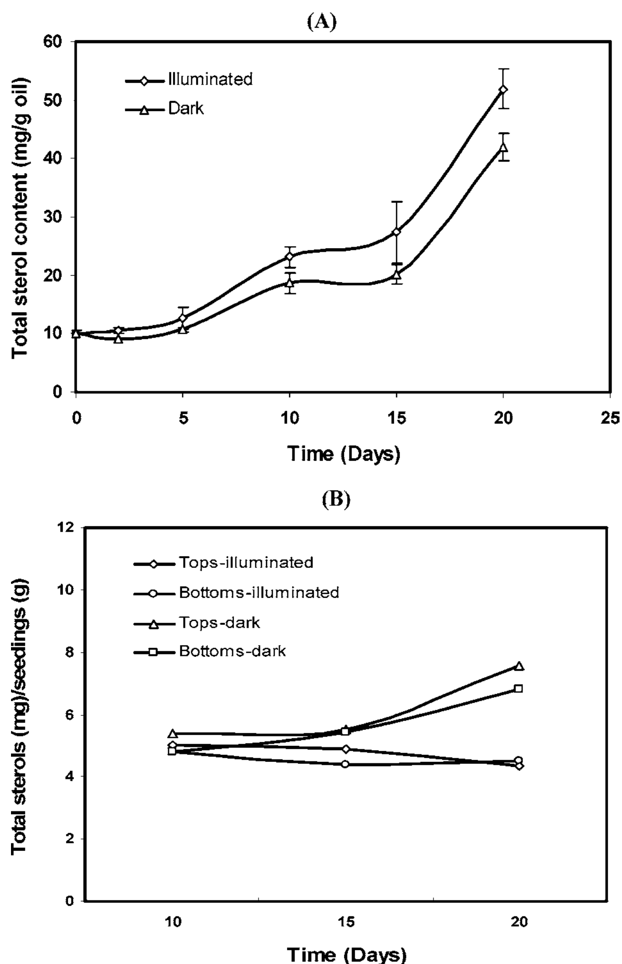

Figure 4. Changes in total sterol content during canola seed germination: (**A**) seedling extracted oil basis and (**B**) seedling tops and bottoms dry weight basis.

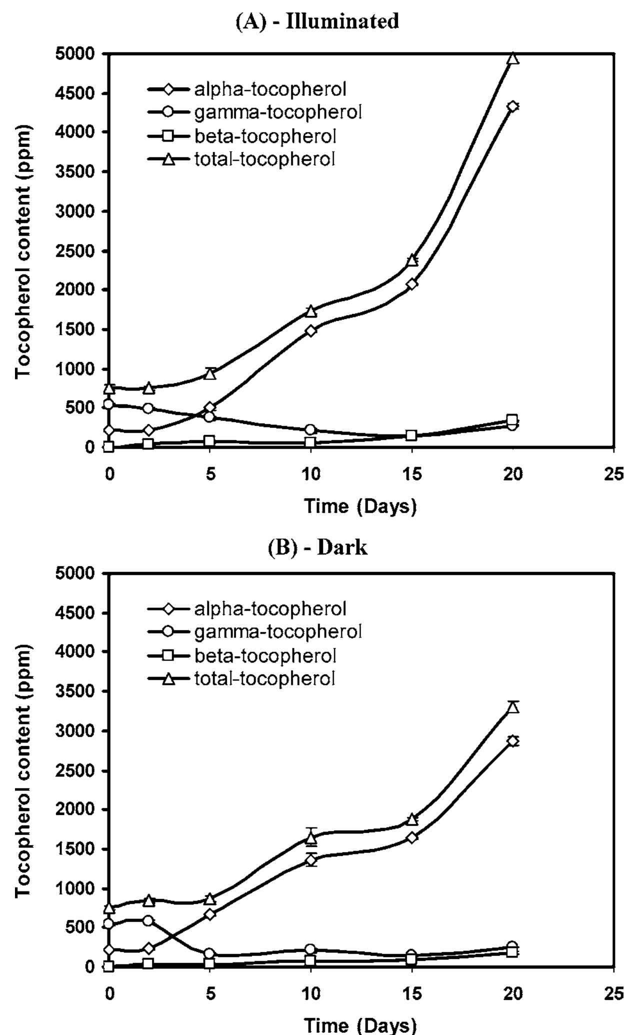

Figure 5. Changes in tocopherol content of canola seedling oil at different stages of germination under (**A**) illuminated and (**B**) dark conditions.

not a critical factor in the phytosterol compositional changes ($p > 0.05$). Other studies have shown no change in the phytosterol composition during germination of alfalfa (*18*) and mustard seeds (*16*), whereas in other crops such as soybean (*9*) and tobacco (*26*), the composition of phytosterols did change during germination.

Changes in Tocopherols and Phytosterols in Extracted Oil. It was expected that the bulk of tocopherols and free phytosterols present in seeds and seedlings would occur in the oil fraction. For this reason, oil was extracted from seeds/seedlings at various stages of sprouting. Changes in the total phytosterol content in oil extracted from seedlings germinated with or without light are shown in **Figure 4A**. The concentration of total phytosterols in the oil fraction increased 4.2- to 5.2-fold over the sprouting period. However, due to proportional loss of oil reserve during germination, no significant ($p > 0.05$) change of total phytostrol content was observed. Furthermore, unlike the tocopherols, total phytosterols were found to be equally distributed across seedling tops and bottoms (**Figure 4B**).

Changes in total tocopherol content and composition in oil extracted from seedlings germinated with or without light are shown in **Figure 5**. The compositional trends of tocopherols observed in the oil fraction extracted at different stages of germination paralleled those in the whole seedling. However, the concentration of both phytosterols and tocopherols in the oil fraction increased substantially in a sigmoidal fashion over the course of germination. Oil from ungerminated canola seeds contained phytosterols at a concentration of about 10 mg/g, whereas oil extracted from 20-day-old seedlings contained phytosterols at concentrations of 52 mg/g and 42 mg/g for illuminated and dark growth conditions, respectively. This 4- to 5-fold increase in concentration corresponds to the 2.5- to 5-fold decrease in extractable oil volume which occurs as oil reserves are depleted during germination, as described in our previous work (*21*). An increase in the concentration of phytosterols in the diminishing lipid fraction during germination of both barley and canola seeds was also observed by Chung et al. (*27*). In a trend similar to the phytosterols, tocopherol concentration in extracted oil increased from 797 mg/kg in ungerminated seeds to 4944 mg/kg in 20-day old seedlings sprouted under illumination, representing a 6.5-fold increase in concentration, resulting from the depletion of lipid and the increased production of α-tocopherol. A comparable 4.3-fold increase in tocopherol concentration was observed in oil extracted from 20-day-old seedlings grown in the dark, compared to oil extracted from ungerminated seeds.

Conclusions. The enrichment of α-tocopherol, total tocopherols, and phytosterols in canola oil extracted from sprouted seeds represents an improvement in the nutritional value and health benefits of canola oil produced in this way. The results of this study suggest that germination of canola seeds may be a viable and natural means of concentrating these valuable phytochemicals in the oil fraction. This concentration effect is largely due to oil depletion during germination and seedling growth. Also, in the presence of light, the net α-tocopherol and

total tocopherol content increased in seedlings and the extracted oil. From a food processing perspective, the improved nutritional quality of the oil extracted from sprouts rather than intact seeds may compensate for the decrease in lipid yield that occurs as germination progresses. Moreover, this research best presents an opportunity to add value to low-grade canola grains (>300K Mt per year) produced in the Canadian prairies.

LITERATURE CITED

(1) Marwede, V.; Mollers, C.; Olejniczak, J.; Becker, H. C. Genetic variation, genotype × environment interactions and heritability of tocopherol content in winter oilseed rape (*Brassica napus* L.). In Proc. 11th Int. Rapeseed Congr., KVL Copenhagen, 6−10 July 2003, pp 212−214.

(2) Marwede, V.; Shierholt, A.; Moellers, C.; Becker, H. C. Genotype × environment interactions and heritability of tocopherol contents in canola. *Crop Sci.* **2004**, *44*, 728−731.

(3) Goffman, F. D.; Velasco, L.; Becker, H. C. Tocopherols accumulation in developing seeds and pods of rapeseed (*Brassica napus* L.). *Fett/Lipid.* **1999**, *101*, 400−403.

(4) Packer L. and Fuchs, J. *Vitamin E in Health and Disease*; Marcel-Dekker Inc.: New York, 1993.

(5) Traber, M. G.; Sies, H. Vitamin E in humans: Demand and Delivery. *Annu. Rev. Nutr.* **1996**, *16*, 321−347.

(6) Bramley, P. M.; Elmadfa, I.; Kafatos, A.; Kelly, F. J.; Manios, Y.; Roxborough, H. E.; Schuch, W.; Sheehy, P. J. A.; Wagner, K.-H. Review: Vitamin E. *J. Sci. Food Agric.* **2000**, *80*, 913−938.

(7) Kamal-Eldin, A.; Appelqvist, L. A. The chemistry and antioxidant properties of tocopherols and tocotrienols. *Lipids* **1996**, *31*, 671−701.

(8) Kim, I. S.; Han, S. H.; Han, K. W. Study on the chemical change of amino acid and vitamin of rapeseed during germination. *J. Korean Soc. Food Sci. Nutr.* **1997**, *26*, 1058−1062.

(9) Mostafa, M. M.; Rahma, E. H. Chemical and nutritional changes in soybean during germination. *Food Chem.* **1987**, *23*, 257−275.

(10) Franzen, J.; Haass, M. M. Vitamin E content during development of some seedlings. *Phytochemistry* **1991**, *30*, 2911−2913.

(11) Yang, F.; Basu, T. K.; Ooraikul, B. Studies on germination conditions and antioxidant contents of wheat grain. *J. Food Sci. Nutr.* **2001**, *52*, 319−331.

(12) Liukkonen, K. H.; Katina, K.; Wilhelmsson, A.; Myllymaki, O.; Lampi, A. M.; Kariluoto, S.; Piironen, V.; Heinonen, S. M.; Nurmi, T.; Adlercreutz, H.; Peltoketo, A.; Pihlava, J. M.; Hietaniemi, V.; Poutanen, K. Process-induced changes on bioactive compounds in whole grain rye. *Proc. Nutr. Soc.* **2003**, *62*, 117−122.

(13) Piironen, V.; Lindsay, D. G.; Miettinen, T. A.; Toivo, J.; Lampi, A. M. Plant sterols: biosynthesis, biological function and their importance to human nutrition. *J. Sci. Food Agric.* **2000**, *80*, 939−966.

(14) Moreau, R. A.; Whitaker, B. D.; Hicks, K. B. Phytosterols, phytostanols, and their conjugates in foods: structural diversity, quantitative analysis and health-promoting uses. *Prog. Lipid Res.* **2002**, *41*, 457−500.

(15) Hamama, A. A.; Bhardwaj, H. L.; Starner, D. E. Genotype and growing location effects on phytosterols in canola oil. *J. Am. Oil. Chem. Soc.* **2003**, *80*, 1121−1126.

(16) Kalinowska, M.; Wojciechowski, Z. A. Sterol conjugate interconversions during germination of white mustard (*Sinapis alba*). *Phytochemistry* **1984**, *23*, 2485−2488.

(17) Holmer, G.; Ory, R. L.; Hoy, C. E. Changes in lipid composition of germinating barley embryo. *Lipids* **1973**, *8* (5), 277−283.

(18) *(18) Huang, L. S.; Grunwald, C. Sterol and phospholipid changes during alfalfa seed germination. Phytochemistry* **1988**, *27*, 2049−2053.

(19) Horbowicz, M.; Obendorf, R. L. Changes in sterols and fatty acids of buckwheat endosperm and embryo during seed development. *J. Agric. Food Chem.* **1992**, *40*, 745−750.

(20) Malgorzata, K.; Zdzislaw, A. W. Sterol conjugate interconversions during germination of white mustard (*Sinapis alba*). *Phytochemistry* **1984**, *23*, 2485−2488.

(21) Zhang, H.; Vasanthan, T.; Wettasinghe, M. Dry matter, lipids, and proteins of canola seeds as affected by germination and seedling growth under illuminated and dark environments. *J. Agric. Food Chem.* **2004**, *56*, 8001−8005.

(22) Goffman, F. G.; Becker, H. C. Genetic analysis of tocopherol content and composition in winter rapeseed. *Plant Breeding* **2001**, *120*, 182−184.

(23) Mounts, T. L.; Abidi, S. L.; Rennick, K. A. Effect of genetic modification on the content and composition of bioactive constituents in soybean oil. *J. Am. Oil Chem. Soc.* **1996**, *73*, 581−586.

(24) Shintani, D.; Dean, D. Elevating the vitamin E content of plants through metabolic engineering. *Science* **1998**, *282*, 2098−2100.

(25) Abidi, S. L.; List, G. R.; Rennick, K. A. Effect of genetic modification on the distribution of minor constituents in canola. *J. Am. Oil Chem. Soc.* **1999**, *76*, 463−467.

(26) Bush, P. B.; Grunwald, C. Sterol changes during germination of *Nicotiana tabacum* seeds. *Plant Physiol.* **1972**, *50*, 69−72.

(27) Chung, T. Y.; Nwololo, E. N.; Sim, J. S. Compositional and digestibility changes in sprounted barley and canola seeds. *Plant Foods Hum. Nutr.* **1989**, *39*, 267−278.

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