Tocopherols in Flaxseed

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The tocopherol content of oil from eight flaxseed cultivars grown at four locations in western Canada for 3 years was determined to examine genotypic and environmental effects. Flaxseed contained an average of 9.3 mg/100 g of total tocopherol in the seed, with γ-tocopherol representing 96−98% of the total tocopherols. The level of tocopherol in flaxseed was cultivar specific and regulated by environmental conditions as indicated by the strong cultivar × location × year interaction. Seasonal differences in total tocopherol content were significant, although the contribution of the δ isomer was constant at 2.5% of the total tocopherol. In flaxseed, tocopherol content was weakly but positively associated with oil content. The ratio of the tocopherol isomers α, γ, and δ in flaxseed hull at 17:61:22 was different from that in the seed.

Keywords: *Flaxseed; tocopherol; tocopherol isomers; cultivar effects; seasonal variations; environment; flaxseed hull; oil; Linum usitatissimum*

INTRODUCTION

Tocopherols (vitamin E) are the most powerful fat-soluble antioxidants. They exist in four homologous isomers: α (5,7,8-trimethyltocol), β (5,8-dimethyltocol), γ (7,8-dimethyltocol), and δ (8-methyltocol), which differ in number or position of methyl groups in the molecules. The various tocopherols differ in their biological activities and their ability to protect fats and oils from oxidative rancidity. Generally, antioxidative and biological activities of the isomers increase and decrease, respectively, in the following order: α, β, γ, and δ (Engberg et al., 1993; Hashim et al., 1993). Tocopherol is important especially when flaxseed is used for animal feed since decrease in both α- and γ-tocopherols in rat tissues with increasing dietary flaxseed has been reported (Ratnayake et al., 1992). Furthermore, tocopherol naturally present in foods has been strongly correlated with the polyunsaturated fatty acid since it counteracts the potential oxidative stress caused by fats in the diet (Anttolainen et al., 1995).

The tocopherol level in plants is governed by the level of unsaturated fatty acids; a simple increase in unsaturation results in the formation of higher amounts of antioxidants to protect the oil (Eskin et al., 1996). Variation in tocopherol levels of sesame seed oil has been ascribed to differences in genotype, maturity level, and environmental temperature during seed development (Kamel-Eldin and Appelqvist, 1994). Other factors, such as seed, oil storage, and processing, are known to affect tocopherol levels in vegetable oils. A commercial flaxseed variety grown in 1994 was reported to contain 0.88, 2.42, 9.2, 0.24, and 12.74 mg/100 g of seed (wb) of α-, β-, γ-, and δ-tocopherols and total tocopherols, respectively, and was essentially devoid of tocotrienols (Budin et al., 1995). Total tocopherol content of 9 flaxseed varieties grown at 13 worldwide geographic locations ranged from 39.5 to 50 mg/100 g of oil (mean value of 43.6 mg/100 g of oil), and these differences in total tocopherol content were primarily due to location (Marquard et al., 1977).

The regulatory mechanism of tocopherol composition in sunflower seed has been identified as two nonallelic unlinked genes designated tph-1 and tph-2 (Demurin, 1993). The tph-1 gene controls the ratio of α- and β-tocopherols, whereas the tph-2 gene affects the α and γ isoforms (Demurin et al., 1996). The synthesis of γ-tocopherol is associated with seed formation or, particularly, seed maturation in peanuts (Hashim et al., 1993). γ-Tocopherol is believed to be formed by the process of demethylation from α-tocopherol in the seeds, to improve seed stability (Demurin, 1993; Hashim et al., 1993). Therefore, changes in the composition of tocopherols would be expected to occur genetically by mutation in the methylation steps. Such genetic manipulation has already been demonstrated in sunflower (Demurin et al., 1996).

The current trend in genetic modification of oil and fatty acids of oilseed crops, especially flax (Thompson et al., 1996; Rowland et al., 1995), further increases the importance of antioxidants such as tocopherols for increased stability of these new oils. The increase in β-, γ-, or δ-tocopherol content as well as of oleic acids has been reported to enhance oil oxidative stability in sunflower (Demurin et al., 1996). Moreover, when a high content of strong antioxidant is combined with high content of oleic acid, synergism is clearly observed. This synergy of tocopherol content and composition with high oleic soybean oil might explain its higher (3−4 times) oxidative stability compared with those of high oleic canola and high oleic sunflower oils, despite their similar fatty acid composition (Kinney, 1996). Hence, to manipulate the level and maybe the composition of tocopherols in flaxseed, the mechanism that governs and regulates that level must be identified. For this purpose, our first step was to investigate the determinants of tocopherols in flaxseed.

MATERIALS AND METHODS

Samples of eight oil-type flaxseed cultivars were obtained from standardized cooperative tests conducted at four locations

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(Brandon and Portage la Prairie in Manitoba, Elrose and Melfort in Saskatchewan) during 1991−1993 growing seasons. The tests were standardized as a randomized complete block experimental design with four replications according to procedures established by the Western Expert Committee on Grain (Anonymous, 1992) for official registration of flax in Canada.

Oil was obtained by defatting flaxseed according to the multisequential method of Appelqvist (1967) using petroleum ether. The tocopherols were extracted in duplicate from the seed or oil in minimal light essentially as described by Budin et al. (1996). Briefly, flaxseed oil (250 mg) or seed (1 g) was homogenized for 1 min in HPLC grade methanol (20 mL) with a polytron (Model 10/35; Brinkman Instruments, Mississauga, ON) on the no. 4 setting. The samples were centrifuged (Sorvall Model RC2-B; DuPont Co., Wilmington, DE) at 3600*g* for 10 min. The supernatant was removed, placed in a 25 mL glass vial, and dried under nitrogen. The pellet was resuspended in HPLC grade methanol (15 mL), and the homogenization and centrifugation steps were repeated. The supernatant was removed, added to the first extract, and dried under nitrogen. The dried extract was resuspended in HPLC grade hexane (20 mL), mixed briefly in a vortex mixer (Model S8220; Scientific Products, McGaw Park, IL), and dried under nitrogen. The dried extract was then resuspended in 2 mL of eluent (HPLC grade acetonitrile/methanol, 85:15 v/v) and an aliquot microfuged at 13 000 rpm for 5 min (Biofuge, Baxter Diagnostic Corp., Mississauga, ON). The supernatant was placed in a 2 mL amber crimp vial and stored under nitrogen at −20 °C until analysis.

Samples (100 μL) were analyzed by an HPLC system (LKB Produkter, Bromme, Sweden) consisting of two pumps (Model 2150), a controller (Model 2152), a Rheodyne 7125 injector valve with a 100 μL loop, and a Hewlett-Packard multiple-wavelength UV detector 1050 series (Hewlett-Packard [Canada] Ltd., Mississauga, ON) interfaced through a PE Nelson 900 series with a personal computer. An analytical prepacked column (4.6 × 150 mm), Prodigy 5μ ODS (2) (Phenomenex, Torrance, CA), was used with 85% CH_3CN and 15% MeOH as mobile phase. The system was operated isocratically at a flow rate of 1 mL/min. Separations were carried out at 21 ± 1 °C, and detection was at 298 nm. Typically, a 10-min equilibration period was used between samples, requiring about 40 min/sample. Quantitation was based on an external standard method; the calibration curves ranged from 3.75 to 30, from 5 to 40, and from 8.875 to 71 μg/mL of reference compounds α-, γ-, and δ-tocopherol, respectively (Sigma Chemical Co., St. Louis, MO), using Model 2600 chromatography software, revision 3.1 (Nelson Analytical, Inc., Cupertino, CA).

Oil content was measured gravimetrically after the seed was defatted. Dehulling of flaxseed was performed as described by Oomah et al. (1996) using the tangential dehulling device except that the sample was not heat treated prior to dehulling.

Analysis of variance by the general linear model (GLM) procedure, means comparison by Duncan's test, and Pearson correlation were performed according to the Statistical Analysis System (SAS Institute, Inc., 1990). The variance components were estimated with PROC VARCOMP of SAS with the maximum-likelihood method.

RESULTS AND DISCUSSION

A typical separation of tocopherol isomers is shown in Figure 1. Flaxseed was extracted in quadruplicate with duplicate injections of each extract. Replicate extractions had a relative standard deviation of 1.2% or less for γ-tocopheral and 2.3% or less for δ-tocopherol. The contents of the three tocopherol isomers, α, γ, and δ, present in flaxseed grown at four locations for 3 years differed significantly among cultivars ($P = 0.05$) (Table 1). The α- and δ-tocopherols were found at relatively low concentrations, which varied between 0.02 and 0.1 and between 0.17 and 0.3 mg/100 g of seed, respectively. The major tocopherol in flaxseed was in the γ form at

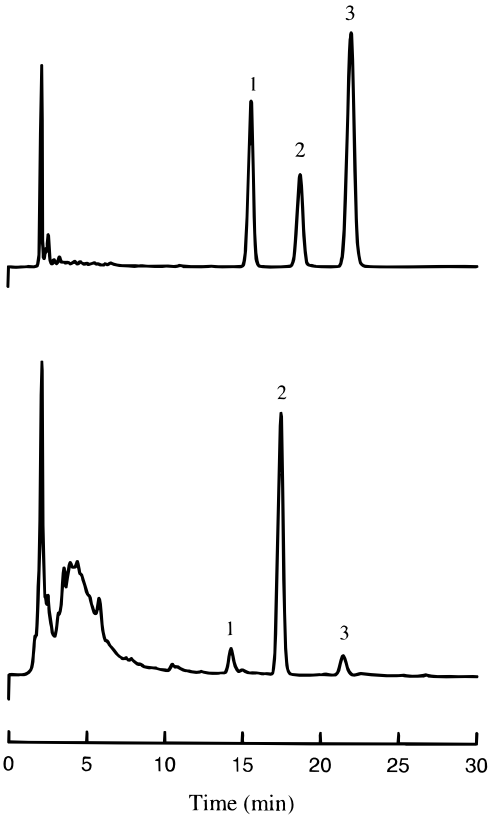

Figure 1. Typical reversed-phase high-performance liquid chromatography profiles of a mixture of the tocopherol standard solution (top) and of a diluted oil extracted from the flaxseed cultivar NorLin grown at Elrose in 1992 (bottom): peak 1, δ-tocopherol; peak 2, γ-tocopherol; peak 3, α-tocopherol. Detection was performed at 298 nm.

Table 1. Tocopherol and Vitamin E Contents (Milligrams per 100 g of Seed) of Flaxseed Cultivars[a]

	tocopherol				
cultivar	α	γ	δ	total	vitamin E
AC Emerson	0.05^{b}	8.80^{de}	0.22^{abc}	9.08^{cd}	0.93^{ef}
AC Linora	0.07^{b}	9.72^{a}	0.22^{abc}	10.01^{a}	1.05^{a}
Flanders	0.10^{a}	8.96^{cd}	0.30^{a}	9.35^{bc}	1.00^{b}
Linola 947	0.06^{b}	9.28^{b}	0.18^{bc}	9.52^{b}	0.99^{bc}
McGregor	0.02^{c}	9.37^{b}	0.17^{c}	9.56^{b}	0.96^{cde}
NorLin	0.10^{a}	8.45^{f}	0.29^{a}	8.84^{d}	0.95^{def}
Somme	0.05^{b}	8.63^{ef}	0.22^{abc}	8.90^{d}	0.92^{f}
Vimy	0.06^{b}	9.12^{bc}	0.26^{ab}	9.44^{b}	0.98^{bcd}
mean	0.06	9.04	0.23	9.34	0.97
SD	0.10	1.66	0.19	1.70	0.18

[a] Means in a column followed by the same letter are not significantly different by Duncan's multiple range test at the 5% level.

96−98% of the total tocopherol. γ-Tocopherol and total tocopherols ranged from 8.5 and 8.8 mg/100 g for NorLin to 9.7 and 10 mg/100 g seed for cultivar AC Linora, respectively. The biologically active vitamin E content relative to that of α-tocopherol, calculated by using the formula $\alpha + 0.1\gamma + 0.01\delta$, proposed by McLaughlin and Weihrauch (1979), ranged from 0.92 to 1.05 mg/100 g of seed for cultivars Somme and AC Linora, respectively. Average values for α-, γ-, and δ-tocopherols, total tocopherol, and vitamin E contents of flaxseed oil from the eight cultivars were 0.15, 21.5, 0.56, 22.19, and 2.32, respectively. These results are consistent with those of Budin et al. (1995), who reported values of 0.88, 9.2, 0.24 and 12.74 mg/100 g seed for α-, γ-, and δ-tocopherols and total tocopherol contents, respectively, of a commercial flaxseed grown at Rosemount, MN, Experi-

Table 2. Analysis of Variance for Tocopherols from Flaxseed Grown at Four Locations for 3 Years

		mean square[a] × 100				
source	df	α	γ	δ	total	vitamin E
cultivar (C)	7	1.66 (0.12)	433.95 (0.94)	4.92[d] (0)	379.38 (0.09)	4.26 (0)
location (L)	3	0.96[c] (0)	426.24 (0)	7.12[d] (0)	557.46 (0)	7.46 (0)
year (Y)	2	5.66 (5.33)	4057.97 (14.78)	3.88[e] (0)	4542.77 (15.85)	68.67 (23.62)
C × L	21	1.69 (0)	295.90 (0)	5.01[b] (0)	274.13 (0)	2.94 (0)
C × Y	14	0.92 (0)	267.82 (0)	3.79[d] (0)	303.24 (0)	4.53 (0.05)
L × Y	6	2.76 (3.48)	1655.64 (18.45)	10.27 (5.32)	1783.45 (20.01)	13.62 (11.74)
C × L × Y	42	1.83 (72.84)	443.58 (58.41)	5.69 (46.16)	451.98 (55.93)	5.20 (52.96)
error	98	0.18 (18.04)	21.87 (7.43)	1.76 (48.52)	25.13 (8.12)	0.44 (11.62)

[a] All mean squares are significant at 0.0001 probability levels, except those followed by superscript b, c, d, and e, which are significant at 0.001, 0.002, 0.02, and 0.2 levels, respectively. Values in parentheses are percent variance components.

Table 3. Total Tocopherol Content (Milligrams per 100 g of Seed) of Flaxseed Cultivars in Different Environments[a]

	location				year		
cultivar	Portage	Brandon	Elrose	Melfort	1991	1992	1993
AC Emerson	9.18[a]	9.22[a]	9.37[a]	8.54[b]	8.70[b]	8.76[b]	9.77[a]
AC Linora	10.86[a]	9.98[b]	9.97[b]	9.23[b]	10.25[b]	8.46[c]	11.32[a]
Flanders	8.80[b]	9.13[b]	10.02[a]	9.36[ab]	8.41[b]	8.78[b]	10.95[a]
Linola 947	9.49[b]	8.68[c]	10.33[a]	9.57[b]	9.39[b]	8.93[b]	10.23[a]
McGregor	10.10[a]	9.16[b]	9.00[b]	9.90[ab]	9.36[b]	9.12[b]	10.25[a]
NorLin	9.91[a]	8.47[bc]	7.86[c]	9.11[b]	9.24[a]	8.01[b]	9.16[a]
Somme	8.45[c]	8.34[c]	9.90[a]	8.90[b]	8.79[b]	8.58[b]	9.32[a]
Vimy	10.12[a]	8.03[c]	9.86[ab]	9.74[b]	9.82[a]	7.66[b]	10.83[a]
mean of cultivars	9.61[x]	8.88[z]	9.55[x]	9.30[y]	9.25[y]	8.55[z]	10.24[x]

[a] Means in a row for locations or years and overall means, respectively, followed by the same letter are not significantly different by Duncan's multiple range test at the 5% level.

mental Station. However, our values are lower than those reported for nine flaxseed cultivars grown at Morden in 1972 (average of total tocopherol content of 44.4 ± 6.7 mg/100 g of oil) (Marquard et al., 1977) and for commercially refined, bleached, and deodorized flaxseed oil (Yoshida et al., 1990). These differences could be due to methods of separation and detection of tocopherols. The tocopherol content of flaxseed is similar to those of raw poppy seed (at 1.8, 9.2, and 11.0 mg/100 g of seed of α- and γ-tocopherols and total tocopherol, respectively) (McLaughlin and Weihrauch, 1979). The γ isomer at 95% of the total tocopherol in flaxseed resembles those present in oil of perilla seed at 92% (Shin and Kim, 1994) and sesame seed at 96−99% of the total tocopherol (Kamal-Eldin and Appelqvist, 1994).

The results of analysis of variance for tocopherol contents of flaxseed grown at four locations for 3 years (Table 2) showed that the tocopherol contents were dependent on cultivar, location, year, and their interactions. All main effects and their interactions were highly significant ($P < 0.0001$) for α-tocopherol (except for location), γ-tocopherol, total tocopherol, and vitamin E. Only the L × Y and C × L × Y interactions were highly significant for δ-tocopherol. Results of analysis for γ-tocopherol parallelled those of total tocopherol. The variation had large components due to year and location × year interaction for all tocopherols, except the δ isomer. Seasonal effects, i.e. year and location × year interactions, explained approximately 15 and 20% of the variation in both γ-tocopherol and total tocopherol contents in flaxseed (Table 2). Cultivar, and its interactions, C × L and C × Y, do not play a significant role in the variability of tocopherol contents, since their variance components were nil. The variance was predominantly associated with the C × L × Y interaction since it accounted for 73, 58, 56, and 53% of the total variation in α- and γ-tocopherols, total tocopherol, and vitamin E contents, respectively. Although the C × L × Y interaction was high for δ-tocopherol, it had a very insignificant effect on the variation of this isomer since its variance (46%) was smaller than that of experimental error (49%). This could be due to the differences in δ-tocopherol contents, which were relatively small in absolute terms. The high variance of the C × L × Y interaction regarding tocopherol contents indicates that cultivar responded differently to each environment, i.e. year and location, resulting in large fluctuations in tocopherol contents within environments.

Generally, flaxseed grown in Elrose and Portage had significantly higher levels of γ-tocopherol and total tocopherol than that grown in Brandon and Melfort (Table 3). Total tocopherol content of flaxseed grown in Portage was 8% higher than that grown in Brandon. Total tocopherol content at the four locations was highly dependent on cultivar. Thus, cultivar AC Linora had the highest average total tocopherol content at Portage and Brandon (10.9 and 9.9 mg/100 g of seed, respectively), while NorLin, Somme, and Vimy had the lowest total tocopherol contents of 7.8, 8.5, and 8.0 at Elrose, Portage, and Brandon, respectively. Similarities in accumulations of tocopherols among cultivars were also observed. For example, cultivars AC Emerson, Flanders, and Somme had similar total tocopherol contents at Elrose and Melfort (9.4−10.0 and 8.5−9.4 mg/100 g of seed, respectively).

Seasonal effects had a greater impact on tocopherol content than location (Figure 2). Typically, flaxseed grown in 1993 had higher α-, γ-, and δ-tocopherol and total tocopherol than that grown in previous years. Flaxseed grown in 1992 accumulated the lowest concentration of γ-tocopherol and total tocopherol (8.3 and 8.6 mg/100 g of seed, respectively) relative to that grown in 1991 and 1993. Although the total tocopherol differed significantly form year to year, the contribution of the δ-tocopherol was fairly constant at 2.5% of the total tocopherol. Total tocopherol content of flaxseed grown in 1991 was not significantly different from that grown in 1992 except for cultivars AC Linora, NorLin, and Vimy (Table 3). Interestingly, Linola 947 and one of

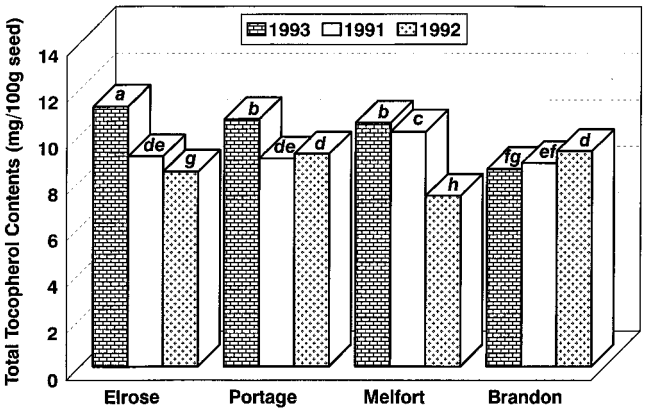

Figure 2. Total tocopherol content of flaxseed grown in three different years at four locations. Bar graphs with the same letters are not significantly different by Duncan's multiple range test at 5% level.

Table 4. Correlation Coefficients for Tocopherol of Flaxseed

	tocopherol				
	α	γ	δ	total	vitamin E
oil	0.190[b]	0.424[c]	0.036	0.408[c]	0.283[c]
α-tocopherol		−0.124	0.468[c]	−0.010	0.435[c]
γ-tocopherol			0.165[a]	0.989[c]	0.839[c]
δ-tocopherol				0.300[c]	0.429[c]
total tocopherols					0.894[c]

[a] $P < 0.05$. [b] $P < 0.01$. [c] $P < 0.0001$ ($n = 194$).

its parents, McGregor, showed similar levels of total tocopherol for the years 1991−1993, suggesting that the stability of total tocopherol (predominantly γ-tocopherol) may be genetically controlled. Variations in tocopherol levels due to differences in cultivar and environmental temperature during seed development have also been observed in sesame seeds (Kamel-Eldin and Appelqvist, 1994). This variability in tocopherol content of flaxseed across locations and years further illustrates the strong cultivar × environment interaction (Table 2).

γ-Tocopherol, total tocopherols, and vitamin E contents showed some association with oil content in flaxseed (data for oil not presented). The Pearson correlation coefficients of γ-tocopherol, total tocopherols, and vitamin E were 0.424, 0.408, and 0.283 for oil contents, respectively (Table 4). α- and δ-tocopherols showed poor correlations with oil content of flaxseed. The weak association between both α- and δ- tocopherols and oil suggests that in flaxseed changes or modifications in these isomers should have very little effect, or at best a slight increase, on oil content. Nonsignificant correlation coefficients between α- and both γ-tocopherol and total tocopherols indicates that the concentrations of these tocopherols are independent of one another. This analysis also showed a highly significant positive correlation between γ-tocopherol, total tocopherols, and vitamin E ($r = 0.99$ and 0.89, respectively) such that the cultivar with the highest total tocopherol content showed the highest concentration of the γ isomer and vitamin E content. As expected, the vitamin E values were strongly correlated with γ-tocopherol and total tocopherols, moderately correlated with the α and δ isomers, and very weakly correlated with the oil content. The high negative correlation between tocopherol and oil content reported for flaxseed grown in phytotron (Marquard et al., 1979) was not observed in our experiments.

Dehulling and sieve separation of a flaxseed sample (Linola 947 grown in Morden in 1993) using the Tangential Abrasive Dehulling device (Oomah et al., 1996) yielded a bran fraction enriched in dietary fiber and a dehulled fraction enriched in oil. The dehulled seed exhibited a tocopherol profile close to that of the whole seed (Table 5), but contained more α- and γ-tocopherols and, hence, total tocopherols than the whole seed. Fractionated hull contained approximately 26% of the total tocopherols found in the whole seed. However, the ratio of α-, γ-, and δ-tocopherol isomers of the hull (17:61:22) was different from that of the seed (1:94:5). The proportionately high ratio of α-tocopherol in the hull suggests that it might not have the same level of antioxidant protection as the whole seed. Similar changes in α- to γ-tocopherol ratio (from 96:4 to 58:42) have been observed in whole seed and seed coat of safflower, respectively (Kajmoto and Hasebe, 1982). This could be due to the preponderance of γ-tocopherol in embryo lipids and the presence of δ- and γ-tocopherol in seed coat lipids.

Table 5. Tocopherol Composition (Milligrams per 100 g) of Various Fractions of Flaxseed[a]

	tocopherol			
fraction	α	γ	δ	total
hull	0.322[a]	1.155[c]	0.417	1.894[c]
seed	0.075[b]	6.912[b]	0.378	7.365[b]
dehulled	0.097[b]	8.409[a]	0.264	8.770[a]

[a] Means in a column followed by different letters are significantly different by Duncan's multiple range test at the 5% level.

The data presented indicate that flaxseed cultivars differ in tocopherol content, although its composition is consistent among cultivars. The findings suggest that it might be possible to breed flax cultivars high in tocopherol content for improved oil stability for the increased utilization of flaxseed in food, feed, pharmaceutical, and cosmetic industries. High levels of γ-tocopherol (a natural antioxidant) are especially important for the genetically modified "Solin" type flax varieties destined for the edible oil and food markets. [The Flax Council of Canada has adopted the generic term "Solin" for low (≤5% by weight) linolenic acid varieties of flaxseed.] For industrial uses of flaxseed oil, especially for oil-based coating materials, a very low or negligible level of tocopherol (i.e. a natural antioxidant that inhibits the induction period for film drying) is desirable to accelerate polymerization of high molecular weight cross-linked products. Flaxseed cultivars with low tocopherol content would be important for this particular industrial use. Since the interaction of cultivars with environments was large, breeding of stable cultivars that interact less with the environment in which they grow should be emphasized to develop cultivars with modified levels of tocopherol.

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