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ORIGINAL PAPER

Oxidation of Crude Corn Oil with and without Elevated Tocotrienols

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Abstract Kernels and oil from corn with increased concentrations of tocotrienols (T3) due to the transgenic expression of a homogentisic acid geranylgeranyl transferase (HGGT) gene from two growing years were characterized for tocol and fatty acid compositions. The crude extracted oil was analyzed for oxidative properties and compared to non-transformed material derived from the plants grown at the same location and during the same year. No significant difference was observed in composition of major fatty acids. Both the seed (kernel) and extracted crude oil from the HGGT expressing corn had a 18-fold increase in tocotrienol content from 300 to 5,400 ppm in oil. There was a concurrent 18% decrease in tocopherol content, 1,150 ppm total tocopherols in control oil declining to 940 ppm in HGGT oil. Although tocopherols and tocotrienols are generally considered antioxidants, they may exhibit prooxidant effects at higher concentrations and they should be tested. Crude oil was extracted from control and transformed corn produced during 2005 and 2006 yielding four oil samples that were evaluated for their oxidative properties. The formation of lipid hydroperoxides, a primary oxidation product, was evaluated at 60 °C over 9 days by measuring the peroxide value (PV). Resistance to oxidation or induction period (IP) was measured using an Oxidative Stability Instrument. There was a slight decrease in hydroperoxide formation in the HGGT oil compared to the corresponding control but was less than the year to year differences. The induction period was the same for the 2005 oils, with or without the increased tocotrienol content, but the crude oil with enhanced tocotrienol had a longer IP than the control crude oil in the 2006 samples.

Keywords Antioxidants · Corn oil · Homogentisic acid geranylgeranyl transferase · Oxidative stability index · Peroxide value · Tocotrienol · Tocopherol

Introduction

Tocotrienols and tocopherols are lipophilic, plant-produced antioxidants exhibiting vitamin E activity. The composition of the eight tocol homologues differs considerably in different plant species. Common sources of tocopherols, such as nuts and oils of soy, corn, canola and sunflower, contain little or no tocotrienols. Because of their similar phenolic structure, all tocols exhibit antioxidative properties at parts per million (ppm) concentrations in oil or lipid systems. Recent research suggests individual tocotrienols provide benefits beyond these antioxidation properties [1, 2].

The reported benefits of tocotrienols include inhibiting cholesterogenesis and the coronary heart disease [1–3]. By lowering plasma triacylglycerides, tocotrienols may reduce the effects of metabolic syndrome. Tocotrienols have shown promise to control tumor growth and suppress growth of specific melanoma cells in vitro and in animal studies. There may also be synergistic advantages to using tocotrienols with other chemotherapies [4].

Using molecular biology to manipulate metabolic pathways in plants for increased tocol production and varying their compositions were reviewed and accomplished by Cahoon and others in 2003 [5–7]. The

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assumption is that genetic modification of the tocopherol and tocotrienol biosynthetic pathways can reduce oxidative stress in plants, increase crop productivity, increase storage duration of seed and plant material, improve shelf life of vegetable oils, and meet increasing demands for nutraceutical or therapeutic markets [7]. As research continues on the genetics and biochemistry of tocol biosynthesis, further enhancements to tocol-fortified seeds are expected.

Tocols at higher concentrations, however, may act as prooxidants [8]. As little as 250 ppm of α-tocopherol has been reported as having prooxidative effects in the formation of lipid hydroperoxides [9]. In tocotrienol-spiked coconut fat at 60 °C, the addition of α- and β- tocotrienols at 100, 500 and 1,000 ppm increased fat oxidation as measured by PV compared to the control. This prooxidant effect increased with increased tocotrienol concentration [10]. Both γ- and δ-tocotrienols had the opposite effect and they reduced lipid hydroperoxide formation, although the effect was not correlated with γ-tocotrienol concentration [10]. The primary goal of this research was to determine the oxidative effects of very high levels of tocotrienols (>5,000 ppm) in crude oil from corn genetically transformed with a homogentisate geranylgeranyl transferase (HGGT) gene expressing increased tocotrienols.

Materials and Methods

Material

Four bulk samples of approximately 8 kg of corn each were provided by Pioneer Hi-Bred International, Inc. (Johnston, IA, USA). Two samples from each of 2005 and 2006 were provided. One sample was genetically transformed with the barley HGGT gene to express increased amounts of tocotrienols, and the second was a sample control with a similar pedigree but lacking the transgene. No field or yield data was provided. The samples were stored in the laboratory as whole kernels in the dark at ambient temperature. The moisture content of all samples was below 10%.

α-, γ- and δ-Tocotrienol standards were obtained from Davos Life Sciences, PTE Ltd (Singapore). α- and δ-Tocopherol were purchased from MP Biomedicals, LLC (Solon, OH, USA). Acetic acid, chloroform, hexanes, potassium iodide, sodium thiosulfate, potato starch, salicylic acid were all purchased through VWR International, LLC (West Chester, PA, USA).

Oil Extraction Sample Preparation

The corn was ground with a Thomas Wiley lab mill with a 1-mm screen. Sixty gram batches were extracted with hexanes by using a Buchi B811 (New Castle, DE), an automated Soxhlet extraction system. The extraction efficiency was 97% by using a minimum of 10 cycles and 1-h extraction. The mean percentages of oil recovered was 2.63, 2.66, 2.99 and 2.91 for the 2005 control, 2005 HGGT, 2006 control and 2006 HGGT material, respectively. The crude extracted oils were stored under argon in amber glass at −23 °C until tested.

Three 1-mL aliquots of the crude oils were each placed in 1.8-mL uncapped amber vials for each of the 9 days of accelerated oxidation. Twelve vials were removed from a 60 °C oven each day at the same time over nine subsequent days. The samples were blanketed with argon, capped and stored at −23 °C until analyzed for peroxide value. Day zero samples were also placed in amber vials and stored under argon at −23 °C.

Tocopherol and Tocotrienol Content

The tocotrienols and tocopherols were determined by using a modification of AOCS Official Method Ce 8-89 [11]. Tocols were separated by using a Waters HPLC Alliance 2695 (Milford, MA, USA) with a 3 μ NH_2 100A, 150 mm × 3.0 mm column and detected by fluorescence (Waters 2475) with EXλ = 292 nm and EMλ = 335 nm. An external calibration curve of 0.05, 0.1, 0.2, 0.5, 1.0, 2.5 and 5 ppm of each tocol was used for quantification. For single seed tocol analysis, individual kernels were ground using a ball mill. The weight of the ground material was recorded and then extracted with 2 mL of hexane under reduced lighting. Total tocotrienols in single seed was expressed as μg/g kernel. An aliquot was reserved for methylation and subsequent fatty acid analysis.

Fatty Acid Composition

The fatty acid compositions for the major fatty acids of the crude corn oils were determined by GC by using a ZB-wax column (Phenomenex, Torrance, CA, USA) at 220 °C. About 50 μL of oil was diluted in 1 mL of hexane and methylated with trimethylsulfonium hydroxide [12, 13].

Free Fatty Acid Content

The free fatty acid (FFA) percentages in the crude oils were determined by using AOCS method Ca 5a-40 [11]. Percentage FFA was expressed as percentage of oleic acid.

Primary Lipid Oxidation Products Determined by Peroxide Value

Three samples of each extracted crude oil from the four bulk corn samples were oxidized at 60 °C for each of

9 days and analyzed by using the AOCS Cd 8-53 method adapted by Crowe and White [14].

Resistance to Oxidation as Induction Period (IP) Determined by Oil Stability Instrument (OSI)

Five samples of each extracted crude oil from the four bulk corn samples were analyzed by using an ADM OSI unit (Oxidative Stability Instruments, Omnion, Rockland, MA, USA). The 5 g samples were oxidized at 100 °C with airflow of 110 mL/min. Both the induction period and the actual plot of conductivity by time were recorded.

Statistical Analysis

All experiments were conducted with repeated treatments as noted. Data analyses were done by using Microsoft Office Excel 2003 (Microsoft Corporation, Redmond, WA, USA). One-way analysis of variance (ANOVA) was used and least significant difference was calculated at $P < 0.05$ ($LSD_{0.05}$).

Results and Discussion

Fatty Acid Composition

The major fatty acids in the crude extracted corn oil as molar percentages are given in Table 1. There were minimal differences in the profile of the four crude oils, and modification of the tocol pathway did not appreciably affect fatty acid composition beyond the minor differences that can normally be attributed to environmental conditions. Thus, fatty acid composition, one of the main determinants of oxidative stability, was not expected to influence the PV and OSI values obtained.

Free Fatty Acids

The FFA percentages in the crude oils expressed as oleic acid, were 10.69 for the 2005 control oil, 10.97 for the 2005 HGGT oil, 10.42 for the 2006 control oil and 12.30 for the 2006 HGGT oil. All values were much higher than expected for oils extracted from high quality, undamaged grains. A commodity corn grain sample extracted using the same equipment and process had a more reasonable FFA of 2.44%, indicating that the high FFA percentages observed did not result from the extraction process or laboratory storage conditions. No yield and other field performance data were provided with the corn, therefore, the cause is unknown. The initial hydroperoxide values for the crude oils were 1.45, 1.32, 1.10 and 1.04 meq/kg for the 2005 control, 2005 HGGT, 2006 control and 2006 HGGT, respectively, indicating no major damage or degradation of the seed during storage. The high FFA is a concern as FFA can be prooxidants [15–17]. Thus, the oxidative stability of the crude oils may not reflect the stability of the refined, bleached and deodorized final product after the FFA are removed. However, as both the control oils and the HGGT oils had similar FFA content, their influence on oxidative stability should also be similar. Ideally, the crude oil should have been fully refined and then evaluated for oxidative stability. However, due to the limited amount of oil obtained, refining was not possible.

Table 1 Fatty acid composition (molar %) of crude corn oils

	16:0	18:0	18:1	18:2	18:3
Control Corn Oil 2005	12.40	1.93	23.15	59.53	1.35
HGGT Corn Oil 2005	12.07	1.96	23.65	59.37	1.28
Control Corn Oil 2006	11.47	1.76	24.29	59.77	1.27
HGGT Corn Oil 2006	11.69	1.91	24.28	59.53	1.23

Tocol Content

Both samples from each year were grown at the same location. No significant differences were observed in the single kernel analysis of the control corn compared with HGGT corn for moisture, oil content, kernel weight and fatty acid composition. As expected, there were slight year-to-year differences. There were, however, significant differences in tocol amounts and tocol distribution between the control and the HGGT corn. The 2005 HGGT kernels segregated into two distinct populations, one with high T3 concentration ($\sim$75% total analyzed population) and one with low T3 concentration, similar to that of the control sample. This finding suggests that the dominant HGGT transgene was segregating in the 2005 HGGT sample. Thus, the genetics of this trait may not have been fully fixed in the parent seed until the following year. This phenomenon was not seen in the 2006 HGGT sample (Fig. 1).

The concentrations of tocopherols and tocotrienols in the oils studied are presented in Table 2. Modification of the tocol biosynthetic pathway in HGGT corn resulted in dramatic increases of all tocotrienols, especially γ-tocotrienol. As there was no modification of the endogenous tocol methyltransferase, excess tocols would be expected to accumulate as the gamma homologue. There was a slight decrease in α- and γ-tocopherols and a slight increase in β- and δ-tocopherols in the modified HGGT corn oil compared with the unmodified control oil. Major tocotrienols were less abundant in the 2005 HGGT oil than the 2006 HGGT oil. The reduction may have been a result of the segregating population in 2005.

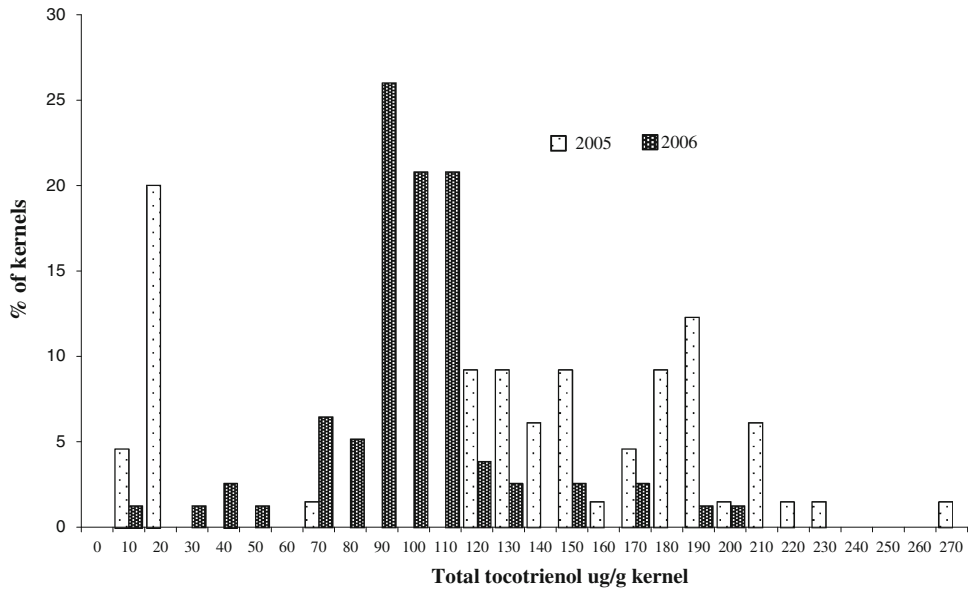

Fig. 1 Histogram of total tocotrienols in corn kernels modified with a homogentisate geranylgeranyl transferase (HGGT) gene

Table 2 Tocol composition of extracted crude corn oil in ppm and relative %

	α-	β-	γ-	δ-	Total	α-	β-	γ-	δ-	Total
	Tocotrienols (ppm)					Tocopherols (ppm)				
Control 2005	192	ND	55	1	248	501	20	730	28	1,279
HGGT 2005	643	18	3,081	442	4,184	301	26	641	56	1,024
Control 2006	187	1	108	7	303	373	29	647	23	1,072
HGGT 2006	690	20	4,051	618	5,379	185	31	565	60	841
	Tocotrienols (%)					Tocopherols (%)				
Control 2005	12.6	ND	3.6	0.1	16.2	32.8	1.3	47.8	1.8	83.8
HGGT 2005	12.3	0.3	59.2	8.5	80.3	5.8	0.5	12.3	1.1	19.7
Control 2006	13.6	0.1	7.9	0.5	22.0	27.1	2.1	47.1	1.7	78.0
HGGT 2006	11.1	0.3	65.1	9.9	86.5	3.0	0.5	9.1	1.0	13.5

ND not detectable

Accumulation of Primary Oxidation Products

The rates of hydroperoxide development in the crude corn oils are given in Table 3. Due to the linearity of PV development during the sampling period as shown in Fig. 2, expressing the rate of PV change as Δ-PV/day was reasonable and thus used. The R^2 for the linear relationship ranged from a low value of 0.9826 for the 2006 control to a high value of 0.9976 for the 2005 control (Fig. 2). The rate of PV development of the HGGT crude corn oil was significantly lower ($P < 0.05$) than that in the control crude oils of the same year. Thus, even at unusually high concentrations, the tocols appear to have an antioxidant effect in the crude oil. Results from our model system [18] and research by others suggest, however, that the optimum levels of tocotrienols for antioxidation in bulk oils should be lower [10, 19, 20]. The higher concentrations used in these other experiments promoted the formation of the primary lipid oxidation products in the purified fats and oils but this was not observed with the crude extracted corn oils. Other native antioxidants in the crude oils, such as carotenoids, may provide synergies not found in highly purified oils [17, 21]. This would allow the tocotrienols and tocopherols to retain their effectiveness at very high levels. These endogenous compounds provide an alternate path for sequestering free radicals resulting from tocol oxidation [21, 22].

Table 3 Change in average daily peroxide value ± one standard deviation over 9 days in crude corn oil at 60 °C in the dark

Δ-PV/day	2005	2006
Control	11.96 ± 1.06	9.19 ± 0.34
HGGT	9.83 ± 0.32	8.48 ± 0.27

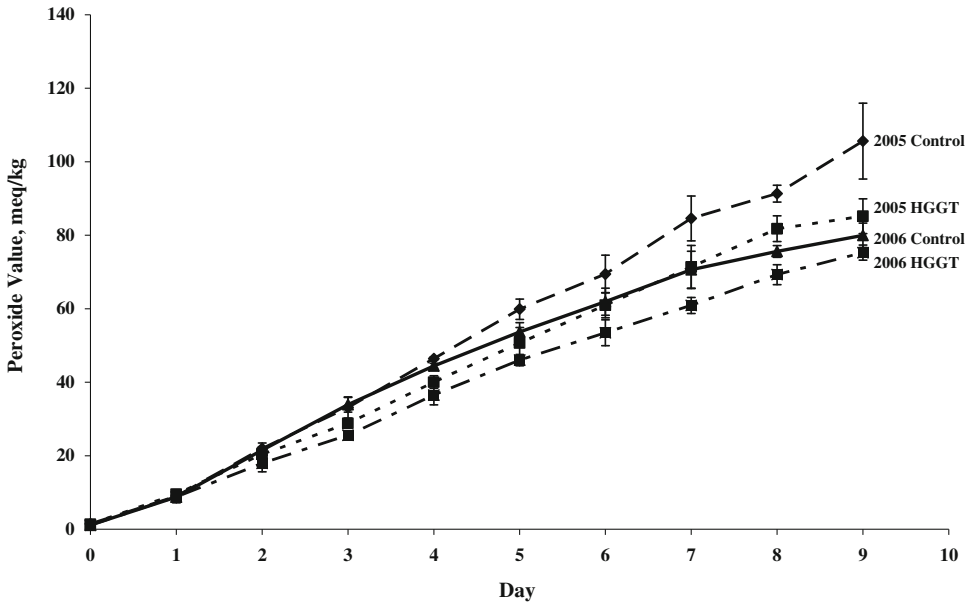

Fig. 2 Peroxide values of the extracted crude corn oil from control kernels and kernels modified with homogentisate geranylgeranyl transferase (HGGT) from 2005 to 2006 at 60 °C and in the dark

Oxidative Stability of Crude Oils as Measured by OSI

The IP and conductivity values at the IP time are given in Table 4. Although IP time is typically used to differentiate oil stabilities, a better understanding of the stability index and accumulation of polar degradation products is deduced from the graph of the actual conductance of the five samples as seen in Fig. 3. Both the control and HGGT crude oils from 2005 had the same IP value but exhibited different curve shapes and different conductivities at the IP time. The control oil had a lower, much sharper and well-defined inflection point than the HGGT crude oil containing increased tocotrienol levels. The 2006 samples also exhibited the same pattern but this similarity is less obvious as the IPs were significantly different from each other (Fig. 3). Similar findings of a smoothing of the curve and less defined inflection point of high tocol oils have also been reported when hexanal formation at lower temperatures was used to track secondary oxidation [22]. Because of the high temperature and artificial conditions used to generate the IP time, the value of this type of testing has caused considerable controversy, but it continues to be widely used.

The actual slopes or curve shapes generated by OSI suggest that the mechanism and antioxidative properties may have shifted as the concentrations of tocols increase in bulk oils. The initial rate of the evolution of degradation products may have increased slightly more rapidly in high tocol oils than in those oils containing less tocols. Yet the high tocol oils continued to inhibit polar or acidic compounds generation for a longer time. The classical model of a long induction period of little polar compounds formation followed by a very rapid increase in these compounds as measured by OSI is less useful for oils having very high tocol concentrations. Using IP time alone to compare crude oils with high tocol levels is thus insufficient to determine the actual oxidative effects.

Table 4 OSI induction period (IP, h) and conductivity (at IP) ± one standard deviation of the extracted crude corn oil

	OSI Induction period (h)	OSI conductivity at induction time
Control corn oil 2005	11.57 ± 0.22^{b}	$6{,}650 \pm 403^{c}$
HGGT corn oil 2005	11.61 ± 0.51^{b}	$10{,}548 \pm 756^{b}$
Control corn oil 2006	9.86 ± 0.57^{c}	$6{,}869 \pm 1{,}550^{c}$
HGGT corn oil 2006	13.82 ± 1.04^{a}	$13{,}015 \pm 1{,}804^{a}$
$LSD_{0.05}$	0.87	1,673

Values not sharing a common superscript are significantly different
OSI temperature is 100 °C

Conclusion

Crude oil from corn expressing tocotrienols at 4,200–5,400 ppm exhibited no prooxidant effects. High tocotrienol concentrations displayed slight antioxidative properties in the reduction of the formation of lipid hydroperoxides in crude oils. The induction period was the same or extended for these oils but the curve of the oxidative stability index shifted away from a sharp inflection point indicating a possible shift in the oxidation kinetics. Crude corn oil with increased tocol levels remains as oxidatively viable as crude oil from non-enhanced corn.

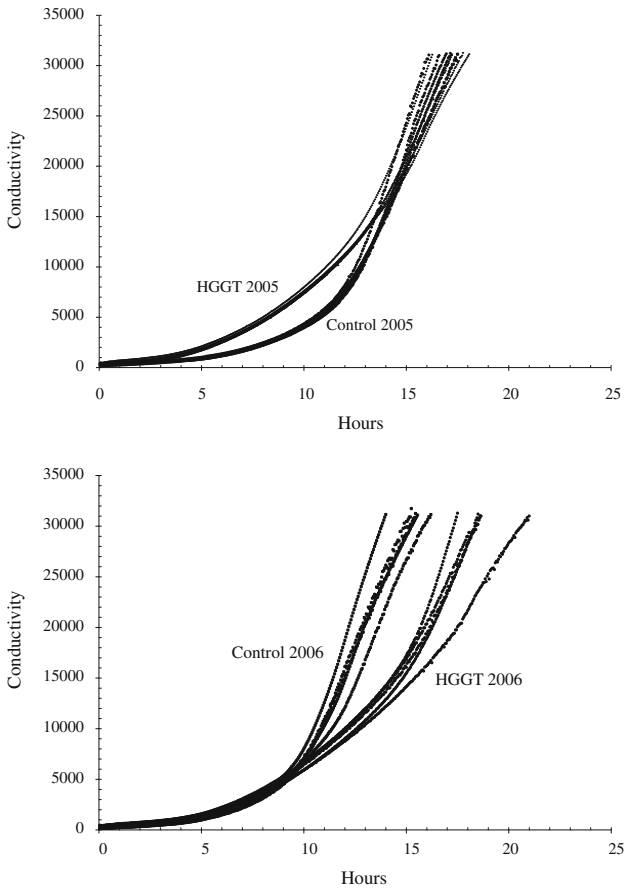

Fig. 3 Oxidative stability index at 100 °C of five subsamples each of extracted crude corn oil from control kernels and kernels modified with homogentisate geranylgeranyl transferase

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