Technical

Carotenoids and Tocols of Corn Grain Determined by HPLC[1]

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A high performance liquid chromatographic (HPLC) procedure has been developed that permits determinationof carotenoids and tocols in the same sample preparation of corn grain. For 15 inbreds, the total carotenoids ranged from 16 to 77 μg/g dry wt and the total tocols from 30 to 128 μg/g dry wt. For four inbreds, total carotenoids were concentrated in the horny endosperm (83 ± 2%) and total tocols in the germ (77 ± 6%). After six months storage at room temperature, the mean loss of total carotenoids for four inbreds was 42 ± 4%, while the tocols had a mean loss of 5%.

Both humans and animals are unable to biosynthesize carotenoids and must assimilate them through their diets. Yellow corn is the only animal feedstuff available in large quantities that contains an appreciable amount of carotenoids without a high fiber level (1,2). If the original level of carotenoids in corn is low or losses occur during storage, the feed may be deficient in provitamin A and pigments.

Two general classes of carotenoids, carotenes and xanthophylls, are primarily responsible for the yellow color of corn grain. The carotenes are precursors of vitamin A, and xanthophylls impart a desirable yellow color to egg yolks and the skin of poultry. The various forms of the carotenoids have different vitamin, antioxidant and pigmenting activities. For example, the yellow-orange zeaxanthin is approximately twice as efficient as the light-yellow lutein as a pigmenting agent for egg yolks (1). To determine the carotenoid feeding value of corn grain, the various forms must be separated from each other, identified, and measured quantitatively.

Consumers associate the yellow skin color of broilers with healthy chickens and the yellow color of egg yolks with "country fresh" eggs. In addition, the demand has increased for deep orange-pigmented egg yolks for commercial use in egg noodles, yellow cake mixes and many other products. Premium prices are paid for eggs with these dark-colored yolks. Corn and corn gluten meal supply most of the xanthophylls in poultry feed, but other pigmenting sources must be added, particularly for the dark-yolk rations. Dehydrated alfalfa meal is a good source of xanthophylls but it is so high in fiber and low in energy that only levels of 2.5-5.0% can be used in poultry feeds. Marigold petal meal is a concentrated source of pigments, but it is expensive and has no nutritional value other than the xanthophylls. Poultry specialists have estimated that a feed formulated for production of dark yolks by supplementation with corn gluten, alfalfa or marigold meals will cost consumers at least three to four cents more per dozen eggs than a regular poultry feed (3). Corn with known high levels of carotenoids would have increased value as a feedstuff.

Little work has been done on carotenoids in corn grain since 1963 (4,5). Corn inbreds widely used by breeders at that time were analyzed, but current inbreds have not been examined. The analytical methods

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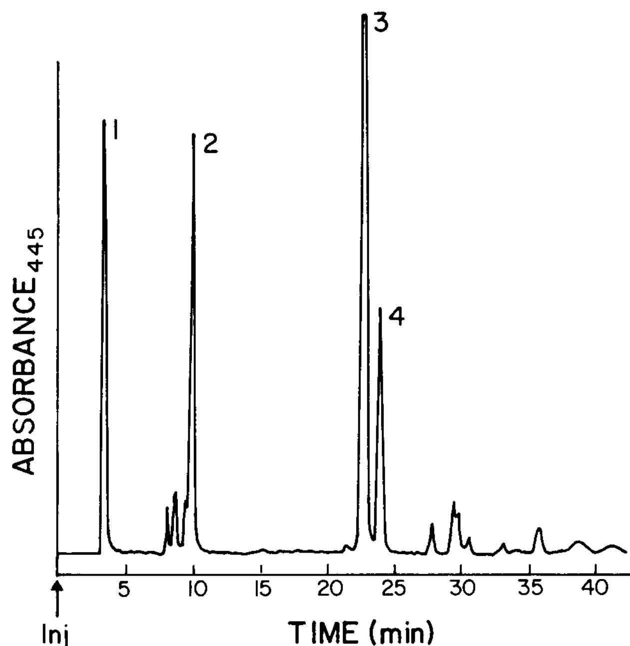

Fig. 1. HPLC analysis of the carotenoids of corn inbred B73. Normal phase system on Ultrasphere-Si. Chromatographic conditions are described in text. Peak number: 1, carotenes; 2, β-cryptoxanthin; 3, lutein; 4, zeaxanthin.

used in the 1960s (6,7) included long extraction times (3-48 hr) and slow column chromatography. We have developed a procedure with a shorter extraction time and quantitation by high performance liquid chromatography (HPLC). This procedure is faster and safer for the oxygen- and light-sensitive carotenoids.

The tocols (tocopherols + tocotrienols) are forms of vitamin E. We have reported previously on HPLC of the tocols of corn grain (8). In the present study, we have devised an extraction procedure by which aliquots of the same sample can be used for both carotenoid and tocol analyses. This procedure has been used to determine the distribution of carotenoids and tocols among various corn kernel fractions (horny endosperm, floury endosperm and germ). The stability of carotenoids and tocols in corn grain during storage was also investigated.

MATERIALS AND METHODS

Corn samples. The corn inbreds were grown on the Agronomy-Plant Pathology South Farm at the University of Illinois, Urbana, IL. Mature kernels were harvested 60-64 days after hand pollination. All of the corn samples were protected from light. Half of the grain from each ear was stored in a freezer at −20 C, and the other half was stored at room temperature in a cloth bag in a closed plastic container.

To separate the corn kernels into horny endosperm, floury endosperm and germ plus tip cap fractions, the kernels were placed in boiling water for 2 min to inactivate lipolytic enzymes and to facilitate removal of the pericarp. Then the tip cap was snapped off below the

TABLE 1

Carotenoids of Yellow Corn Inbreds

		Carotenoids				
	Kernel dry wt	Carotenes	β-Cryptoxanthin	Lutein	Zeaxanthin	Total
Inbred	(mg)			(%)		(μg/g)
A619	230	50.5	8.3	24.7	16.4	69.3
A632	234	10.7	9.4	8.0	71.9	15.8
B37	253	25.5	7.0	52.7	14.7	59.0
B73	222	11.6	14.7	60.3	13.4	28.5
B84	312	10.6	6.6	64.6	18.2	27.4
C103D	247	6.5	7.0	54.5	31.9	32.3
H51	214	18.4	12.5	51.9	17.2	48.2
Mo17	370	9.0	7.0	44.4	39.5	34.4
NY16	158	17.5	8.3	60.1	14.1	31.2
Oh43	236	28.8	7.8	55.2	8.1	49.2
Oh45	288	32.8	5.0	54.9	7.3	76.9
R802A	219	50.0	10.5	34.7	4.8	73.6
R806	305	28.1	12.0	43.4	16.5	49.9
T220	184	39.4	8.0	38.7	13.9	51.2
W64A	177	12.2	13.2	46.0	28.6	36.1

black layer, and the pericarp was removed under a 1 3/4× magnifier; the germ was dissected from the endosperm, and the floury endosperm was carefully scraped from the horny endosperm with a scalpel. All dissection work was done under a safe light (8) because of the lability of the carotenoids and tocols to natural and fluorescent lighting.

Carotenoid and tocol standards. α-Carotene, β-carotene and lycopene were purchased from Sigma Chemical Co., St. Louis, Missouri. The other carotenoids were not available commercially and were isolated from corn by thin layer chromatography (TLC). The carotenoids were separated on silica gel G:MgO (1:2, w/w) 0.3 mm thick. The solvent system was acetone:hexane (10:90, v/v). The carotenoids were extracted from the TLC adsorbents with 5% ethanol in acetone. Lutein, zeaxanthin and β-cryptoxanthin were isolated. Their UV-visible absorption spectra maxima determined on a Beckman scanning spectrophotometer (Model UV5230) were identical to published data (9).

α-Tocopherol and γ-tocopherol were purchased from Eastman-Kodak Chemical Co. (Rochester, New York). The tocotrienols were prepared from natural sources and purified by TLC (10). α-Tocotrienol was isolated from the seed of corn inbred B73 and γ-tocotrienol from barley germ oil.

Extraction of the carotenoids and tocols. All sample preparations were done under a safe light and kept in a nitrogen atmosphere wherever possible, because the carotenoids and tocols are very sensitive to oxygen, light, heat and pH, and especially to combinations of these factors, e.g., light and oxygen (11,12). Butylated hydroxytoluene (BHT) was added to the samples as an antioxidant. The water used for extraction was deionized, freshly boiled and cooled on ice.

Two to three corn kernels at a time were crushed in a hand mill similar to that used by Paulis and Wall (13). Approximately 2 g (8-10 kernels) of the coarsely ground corn was placed in a Spex ball mill (Spex Industries, Inc., Scotch Plains, New Jersey) container that had been cooled in ice. The sample was ground for 10 min. An accurately weighed sample (ca. 300 mg) was placed in a test tube (18 mm O.D. × 150 mm). Triplicate samples were extracted. Ethanol (5 ml) and BHT (0.5 mg) were added to each tube. The test tube was covered with a glass marble and heated for 5 min in a tube oven at 90 C (TOTCO, Glendale, California) to bring the ethanol to the boiling point. Then 0.08 ml of 80% KOH was quickly added and, after stirring with a vortex mixer, the sample was saponified for 10 min at 90 C. To prevent loss of solvent, the top of the tube was cooled with a stream of cool air. The sample was agitated once during saponification. After saponification, the tube was placed in an ice bath and 2.5 ml of ice-cold water and 2.7 ml of hexane:toluene (10:8, v/v) were added. The contents were mixed with a vortex mixer for 20 sec and then centrifuged at 2100 rpm for 4 min. The upper layer was transferred to a separatory funnel containing 15 ml cold water. Extraction of the sample with 2.7 ml hexane-toluene was repeated three times. The combined upper layers were washed five times with 10 ml cold water to remove residual KOH. The combined hexane layers were taken to dryness on a rotary evaporator, and the sample was diluted to a known volume with 1.2% 2-propanol in hexane for HPLC. All samples were kept under nitrogen at −20 C until analysis.

HPLC. The carotenoid and tocol analyses were performed with a Beckman Model 322M HPLC (Beckman Instruments, Inc., Berkeley, California) connected to a Shimadzu Chromatopac C-R1A recording data processor (Shimadzu Scientific Instruments, Inc., Columbia, Maryland). The 25 cm × 4.6 mm i.d. stainless steel column was prepacked with 5 μm Ultrasphere-Si (Beckman) and protected with an Adsorbosphere silica cartridge guard column (Alltech Associates, Inc., Deer-

field, Illinois). All samples were injected via a fully loaded 20-μl loop. The solvents, hexane and 2-propanol, were HPLC grade (J.T. Baker Chem. Co., Phillipsburg, New Jersey).

Absorption of the carotenoids was measured at 445 nm by a Model 155-409 Hitachi UV-visible, variable wavelength spectrophotometer. Detector sensitivity was set at 0.05 absorbance unit full scale. A step gradient program was used to shorten the time of analysis of the carotenoids. The initial values were 3.1% 2-propanol in hexane for the mobile phase and 0.86 ml/min for the flow rate. After β-cryptoxanthin was eluted from the column (at 10 min) (Fig. 1), the flow rate was increased to 1.5 ml/min and the percentage of 2-propanol to 4%. These changes decreased the retention times of lutein and zeaxanthin. After the xanthophylls were eluted (at 24 min), the flow rate was increased to 2 ml/min for 10 min to clear the column; then the initial values of flow rate and mobile phase were reinstated.

The tocol analysis was carried out on an aliquot from the same corn sample extract that was used for carotenoid analysis. The same Ultrasphere-Si HPLC column was used, but the ratio of 2-propanol to hexane was reduced to 1.2% 2-propanol in hexane and the tocols were determined by fluorescence detection (8).

RESULTS AND DISCUSSION

Analytical method. Saponification of the corn samples was necessary to obtain complete extraction of the carotenoids and tocols. This alkaline hydrolysis eliminated contaminating lipids and released the carotenoids and tocols that were present as esters. To avoid detrimental effects on the carotenoids and tocols, saponification time was shortened and the KOH concentration was lowered compared to previously published procedures (9,12).

Both normal-phase and reversed-phase systems have been used for provitamin A analysis of fruits and vegetables, but quantitative HPLC methods dealing with the carotenoids of feedstuffs have not been reported previously (14). A normal-phase HPLC column was chosen because standard xanthophylls, the major carotenoids in corn, are well separated on a silica adsorption column (15). HPLC on a normal phase silica column did not separate the carotenes of corn. The carotenes were eluted as an early, single peak (Peak 1, Fig. 1). With a scanning detector, the concentrations of the individual carotenes could be calculated from their absorbance values (6).

Suitable HPLC internal standards have not been identified. Therefore, calibration curves of β-carotene and γ-tocopherol were used as external standards. The lower limits of adequate detection under the HPLC settings described were 1 ng for carotenoids and 0.5 ng for tocols; thus, one corn kernel could be analyzed for genetic studies.

Recovery studies were carried out by spiking corn samples with known amounts of β-carotene or γ-tocopherol before extraction. The recovery mean for β-carotene was 93.5% with a standard error (SE) of 6.2 in triplicate analyses. The recovery of γ-tocopherol was 98.2 ± 0.2%.

Carotenoid analysis of corn inbreds. The carotenoid analyses of the corn inbreds in Table 1 were carried out within a month after harvest on samples that had been

TABLE 2

Distribution of Carotenoids in Hand-Dissected Corn Kernel Fractions

			Carotenoids					
	Kernel	Dry weight	Carotenes	β-Crypto-xanthin	Lutein	Zeaxanthin	Total carotenoids	
Inbred	fraction	(%)		(%)			(μg/g)	(%)
B37	Whole kernel	100.0	28.2	10.7	50.8	10.3	59.0	100.0
B84		100.0	15.3	7.5	64.0	13.2	27.4	100.0
Oh45		100.0	37.9	8.5	48.2	5.4	76.9	100.0
Canadian flint		100.0	23.1	14.2	53.7	9.0	19.8	100.0
B37	Horny endosperm	69.5	25.0	6.8	45.3	10.2	47.7	87.3
B84		66.3	10.1	4.0	53.6	11.9	19.1	79.4
Oh45		69.3	31.3	5.4	37.6	3.8	54.0	78.1
Canadian flint		65.7	21.2	12.0	43.5	9.1	15.2	85.8
B37	Floury endosperm	14.9	3.2	2.2	4.6	1.0	6.0	11.0
B84		18.6	2.6	1.9	9.9	2.5	4.1	16.9
Oh45		17.4	7.4	3.3	8.5	0.9	13.9	20.1
Canadian flint		19.8	3.6	3.1	5.3	0.9	2.3	12.9
B37	Germ + tipcap	15.6	0.4	0.2	0.8	0.3	0.9	1.7
B84		15.0	0.6	0.3	2.0	0.7	0.9	3.6
Oh45		13.3	0.5	0.1	1.0	0.2	1.2	1.8
Canadian flint		14.5	0.2	0.1	0.8	0.2	0.2	1.3

TABLE 3

Distribution of Tocols in Hand-Dissected Corn Kernel Fractions

			Tocols					
Inbred	Kernel fraction	Dry weight (%)	α-Tocopherol	α-Tocotrienol (%)	γ-Tocopherol	γ-Tocotrienol	Total tocols (μg/g)	Total tocols (%)
B37	Whole kernel	100.0	39.3	15.0	35.9	9.8	44.8	100.0
B84		100.0	19.9	12.5	56.3	11.3	36.9	100.0
Oh45		100.0	6.5	3.4	57.3	32.8	62.3	100.0
Canadian flint		100.0	8.2	7.1	59.9	24.8	38.7	100.0
B37	Horny endosperm	69.5	0.1	9.3	-[a]	5.7	6.3	15.1
B84		66.3	-	5.7	0.2	0.5	1.9	6.4
Oh45		69.3	-	2.8	0.2	22.8	14.5	25.8
Canadian flint		65.7	-	4.8	0.5	14.4	8.3	19.7
B37	Floury endosperm	14.9	0.2	0.9	0.1	1.6	1.2	2.8
B84		18.6	0.3	1.0	-	1.6	0.9	2.9
Oh45		17.4	0.5	1.7	0.6	8.6	6.4	11.4
Canadian flint		19.8	-	1.6	0.4	6.8	3.7	8.8
B37	Germ + tip cap	15.6	48.7	-	32.5	0.8	34.4	82.0
B84		15.0	25.0	-	65.7	-	27.7	90.7
Oh45		13.3	9.6	-	50.5	2.7	35.2	62.8
Canadian flint		14.5	8.2	0.7	59.0	3.6	30.0	71.5

[a]Not detected.

stored at −20 C immediately after harvest. For 15 yellow corn inbreds, the total carotenoids ranged from 15.8 μg/g for A632 to 76.9 μg/g for Oh45 (Table 1).

Each inbred had its own characteristic pattern of carotenoids. Lutein was the predominant carotenoid in 11 of the 15 inbreds (Table 1), but the proportions of lutein varied greatly (from 8.0% in A632 to 64.6% in B84). Inbreds could be selected for high percentages of carotenes (A619, 50.5%, and R802A, 50.0%) or of zeaxanthin (A632, 71.9%). The differences by weight varied among the inbreds by approximately 32-fold for lutein (A632, 1.3 μg/g to OH45, 42.2 μg/g) and by approximately 4-fold for zeaxanthin (R802A-3.5 μg/g to Mo17-13.6 μg/g). The only strong positive correlation coefficients ($P<0.001$) observed were for carotenes and lutein versus total carotenoids.

Our HPLC procedure has also been used to determine the concentration of carotenes and xanthophylls in high-oil corn varieties that were being evaluated for poultry feed (16). The levels of the plasma carotenoids and the shank pigmentation scores of the chicks corresponded to the levels of the carotenoids in the corn varieties that the chicks were fed.

Distribution of carotenoids and tocols in the corn kernel. Blessin and coworkers have examined the distribution of carotenoids (17) and tocols(18) in the corn kernel, but not of both carotenoids and tocols in the same inbred corn sample as was done in this study.

TABLE 4

Recovery of Carotenoids in Four Corn Inbreds After Storage at Room Temperature for Six Months

			Recovery of initial value of individual carotenoids			
Inbred	Initial wt of total carotenoids μg/g)	Recovery of total carotenoids (%)	Carotenes	β-Cryptoxanthin (%)	Lutein	Zeaxanthin
B84	27.4	66.7	64.6	76.1	64.7	68.7
H51	48.2	53.7	56.5	63.5	51.0	52.0
Oh45	76.9	62.7	74.2	82.0	54.0	53.8
R802A	73.6	49.8	48.2	61.1	49.7	58.6
Mean		58.2	60.9	70.7	54.8	58.3

The weight percentages of the hand-dissected kernel parts exhibited little variation among four corn inbreds (Table 2). The horny endosperm accounted for the largest proportion of the kernel (66-69%), while the floury endosperm accounted for 15-20% of the kernel weight and the germ plus tip cap 13-16%.

The carotenoids were localized mainly in the horny endosperm with 78-87% of the total carotenoids in this kernel fraction (Table 2). Floury endosperm contained 11-20% and the germ only a negligible percentage (1-4%). These results are similar to those of Blessin et al. (17), who analyzed the carotenoid content of hand-dissected fractions of two corn hybrids.

The distribution of individual carotenoids in the kernel is also shown in Table 2. Lutein was the predominant carotenoid in all of the kernel fractions, as it was in the whole kernels of the four inbreds. If the ratios of the carotenoids are calculated, only in floury endosperm was there a marked trend in all four corn inbreds away from the original carotenoid composition of the whole kernel. The proportion of β-cryptoxanthin was increased and the proportion of lutein was decreased in floury endosperm compared to the carotenoids of the whole kernel. In germ + tip cap, the only consistent change was an increase in the proportion of zeaxanthin. These results differed from those of Blessin et al. (17). These workers separated the carotenoids into only two fractions, the carotenes and the xanthophylls, and found that the ratio of carotenes to xanthophylls was higher in germ than in the whole kernel. In the data presented here, three of the four inbreds exhibited a decreased proportion of carotenes in the germ as compared to whole kernel.

The percentages of the tocols varied more widely among the kernel parts than did the carotenoids. The major portion of the tocols was concentrated in the germ (63-91%) (Table 3), while the horny endosperm contained 6-26% of the tocols and floury endosperm 3-11%. The wide variation among the inbreds in the percentages of the tocols in the kernel fractions was due to the unique distribution of the tocopherols and the tocotrienols in the corn kernel. The tocopherols were concentrated almost exclusively in the germ and the tocotrienols in the endosperm. The small amounts of these tocols that appeared in the other kernel fractions might have been due to cross contamination of the kernel fractions. Completely clean separation, even by hand-dissection, is very difficult.

Grams et al. (18) found that the endosperm fraction contained all of the measurable tocotrienols that occurred in the whole grain of the four hybrids that they examined. They did not separate the endosperm into floury and horny fractions. The data presented here show that the level of tocotrienols in horny endosperm was usually at least twice that in the floury endosperm. The compartmentation of tocopherols in germ and tocotrienols in endosperm is interesting because it has been proposed that the tocopherols are formed by biohydrogenation of the tocotrienols (19).

Stability of carotenoids and tocols during storage. Four inbreds (B84, H51, Oh45, R802A) were sampled for the storage studies. The recovery of total carotenoids after six mo of storage at room temperature ranged from 49.8-66.7% of the harvest values (Table 4). B84, which had the lowest initial weight of total carotenoids, showed the lowest loss (33.3%) of total carotenoids. Dua et al. (20) observed that a commercial hybrid with one-half the carotenoid content of two experimental strains showed a lower percentage loss than the two high-carotenoid strains after storage for one year. Similar results are presented in this study.

The same trend was observed among the individual carotenoids. Recovery was greatest for β-cryptoxanthin which was present in low concentrations initially (Table

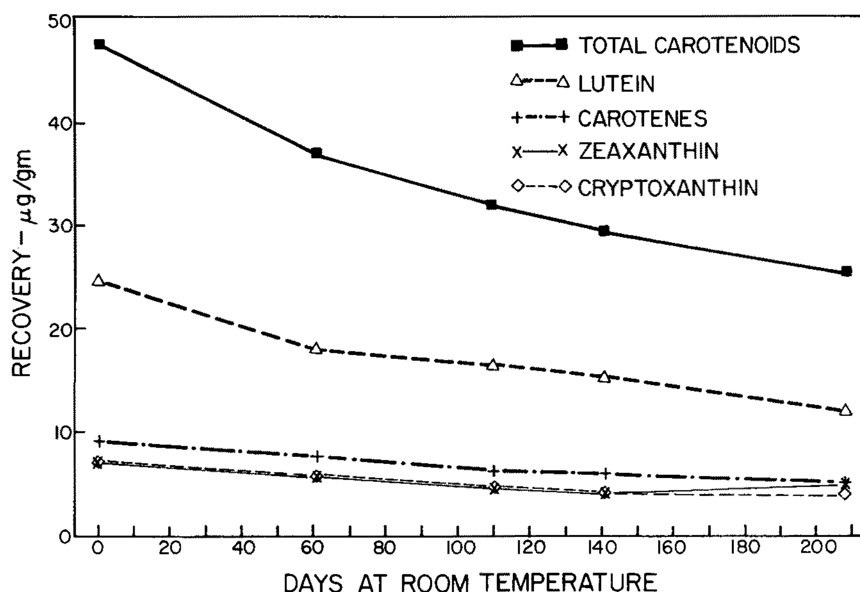

Fig. 2. Loss of carotenoids from whole grain of corn inbred H51 during storage at room temperature. Each symbol represents the mean of three replicates. The standard error of the means was < ± 1 μg/g.

TABLE 5

Recovery of Tocols in Four Corn Inbreds After Storage at Room Temperature for Six Months

Inbred	Initial wt of total tocols (μg/g)	Recovery of total tocols (%)
B84	36.9	97.1
H51	80.5	96.2
Oh45	62.3	96.4
R802A	91.5	90.4
Mean		95.0

1). The general pattern for the other carotenoids was that the individual carotenoid with the highest initial concentration suffered the greatest loss as shown for lutein in H51 (Fig. 2). Quackenbush (21) suggested that the carotene fraction was the least stable during storage, but he analyzed only one hybrid.

The corn samples stored at room temperature were also analyzed for tocols. Over the six-mo period, the average loss of tocols for the four inbreds was 5% (Table 5). The tocols showed much greater stability than did the carotenoids. The severe loss of carotenoids over a short period of storage at room temperature is a serious problem, especially if one considers the long time periods that corn may remain in government storage.

Recent investigations have shown that many livestock rations are low in vitamin A (2) and in vitamin E. Combs and Combs (22) analyzed the α- and γ-tocopherol contents of 42 corn varieties that were obtained from a major corn-breeding company. When the vitamin E activity of these corn varieties was calculated, the average fell below the vitamin E content of corn that is listed in feed formulation tables. The use of the average vitamin E value listed in current feed tables may at times significantly overestimate the vitamin E contribution of corn to livestock feed.

The levels of carotenoids and tocols in corn can be increased either by selection and breeding for higher levels or by learning how to decrease the loss of carotenoids during storage. Our HPLC procedure is faster and less degradative for carotenoid and tocol determinations than previous analytical methods and will be useful for screening corn varieties and other feedstuffs.

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