flora, which were also observed by colleagues during previous experiments (see Acknowledgments). Microflora has been reported to cause intrinsic heating in grains to a range of 50-55 C (20) and was the probable source of heat in our experiments. Thus, aside from moisture content, the primary reason for our slightly lower temperature maxima was small sample size.

Oil from simulated storage-damaged beans differs from that of undamaged (control) soybeans in 3 significant respects: (a) higher FFA concentration; (b) much lower P concentration; (c) lower linolenic acid concentration. These trends concur with the literature (9,11,12) on the studies of actual bulk storage damage and field-damaged soybeans, and thus support the reliability of the adiabatic reactor.

Based on the data obtained from the apparatus described, a larger reactor is being constructed. This reactor will permit us to collect additional data, e.g., taste-panel evaluations, headspace analyses, enzyme effects, combustion temperatures, microflora composition and content, and to evaluate various oil extraction and refining methods, storage effects on unripe grains and aeration techniques.

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⁂High Performance Liquid Chromatography of the Tocols in Corn Grain[1]

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ABSTRACT

A sensitive and selective method was developed for analyzing the tocol isomers in corn grain by high performance liquid chromatography (HPLC) with fluorescence detection. The relative proportions and the total amounts of the tocol isomers (α-tocopherol, α-tocotrienol, γ-tocopherol and γ-tocotrienol) varied greatly among the 15 corn inbreds that were examined. Although γ-tocopherol has traditionally been considered to be the predominant vitamin E isomer in corn, inbreds with equal or higher levels of α-tocopherol have been discovered. No tocotrienols were found in corn germ oil, only α- and γ-tocopherols. Analysis of the tocopherols of the germ oils of inbreds and their reciprocal crosses indicated that the proportions of the α- and γ-isomers and the total amount of the tocopherols are heritable.

INTRODUCTION

The increased use of vegetable oils has modified the fatty acid composition of American diets. The amount of linoleic acid (18:2) in the diet increased from 20.2 g per capita per day in 1969 to 25.2 in 1979, a 25% gain (1). The daily requirement of vitamin E is markedly influenced by the polyunsaturated fatty acid content of the diet, and the adequacy of vitamin E in American diets is still being debated (2). Animal feed is being supplemented with α-tocopherol (α-T), because vitamin E deficiency can lead to muscle degeneration, central nervous disorders and lack of fertility in animals (3,4). The vitamin E biological activities of β-, γ-, and δ-tocopherols and the tocotrienols in animals are generally less than 15-25% of that of α-T (5). The functions of tocols (tocopherols and tocotrienols) in plants are little known, but one role may be as antioxidants to protect the unsaturated lipids. As an antioxidant, γ-tocopherol (γ-T) may be superior to α-T (6-8). This study was made to determine the variability for total tocol content and the proportions of the tocol isomers that might exist in various cultivars of corn.

Very few studies of the vitamin E isomers in corn grain have been done previously. Quackenbush et al. (9) determined the total tocols in inbreds well-known to corn breeders in 1963. These workers used the colorimetric Emmerie-Engel procedure (10). Karaiwanow et al. (11) also used a colorimetric method to determine the total tocol content of Russian and German hybrids with different endosperm colors. Grams et al. (12) analyzed the distribution of tocols within the germ, endosperm and pericarp fractions of 4 hybrids by thin layer chromatography (TLC). Yoshida and Kajimoto (13) used TLC to follow the tocopherol composition of corn grain during development. Slover (14) analyzed the tocols of one sample of dry, yellow corn by gas chromatography (GC). All of these procedures, colorimetric, TLC and GC, are time-consuming and, during the many required manipulations, the risk of losing the tocols by oxidation is

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great. We have developed a faster, milder method using high performance liquid chromatography (HPLC) to determine the tocols.

MATERIALS AND METHODS

Corn Samples

The corn inbreds and F_1 reciprocal crosses were grown on the Agronomy-Plant Pathology South Farm at the University of Illinois, Urbana, IL. Mature kernels were harvested 60-62 days after hand-pollination and were air-dried.

The samples of corn germ were prepared by hand-dissection. First, the kernel was placed in boiling H_2O for 2 min to inactivate lipolytic enzymes and facilitate the removal of the pericarp. Next, the tip cap was snapped off below the black layer. Under a 1¾x magnifier, the pericarp was removed and the germ carefully dissected from the endosperm with a scalpel.

Oil Analysis

For determining the percentage of oil of each strain, ca. 25 g of corn was dried for 2 weeks in an oven at 60 C to 4.5% moisture or less and then scanned by wide-line nuclear magnetic resonance (NMR) as described by Alexander et al. (15).

Fatty Acid Analysis

For each sample, 3 kernels were crushed in a hand mill similar to that used by Paulis and Wall (16). The ground material was transferred to a 10 mL centrifuge tube. Petroleum ether (5 mL) (Skellysolve B, redistilled; b.p. 66-68 C) was added. The petroleum ether also contained 0.002% butylated hydroxytoluene (BHT) as an antioxidant to protect the polyunsaturated fatty acids. The atmosphere in the tube was replaced by N_2, and the tube was stoppered. The mixture was stirred on a vortex mixer, allowed to stand at least 30 min, stirred again and centrifuged. The extract was decanted into a screw-cap vial and taken to dryness under N_2. Methyl esters of the fatty acids of the triglycerides were prepared for GC by treating the lipids with boron trifluoride/methanol (17). The conditions for GC of the methyl esters have been described previously (18). The results reported are the means of 3 or more samples.

Tocol Analysis of Corn Kernels

The conditions for preparing the tocol samples for HPLC with fluorescence detection are very critical. All preparations of tocol extracts were done under a safe light to avoid inactivation of the vitamin E isomers. A safe light was prepared by placing a 1/8 in. thick sheet of clear Lexan (General Electric, Pittsfield, MA) plastic over a 25 W tungsten light bulb. Lexan is a polycarbonate plastic that cuts out ultraviolet (UV) light.

The absolute ethanol used in the extraction was redistilled in the presence of 0.02% by weight of potassium permanganate and KOH pellets. Water was deionized, boiled and cooled on ice. Hexane (J. T. Baker Chem. Co., Phillipsburg, NJ) and isopropanol (Burdick & Jackson Laboratories, Inc., Muskegon, MI) were HPLC grade.

Whole kernel samples must be saponified for complete extraction of the tocols. Ca. 4 g whole kernels of a given strain of corn was placed in a Spex ball mill (Spex Industries, Inc., Scotch Plains, NJ) container and cooled in ice. The sample was ground for 7 min. The container was cooled in ice again and grinding was continued for another 5 min. Three subsamples of 0.5 g were accurately weighed and each was placed in a 25 mL test tube. Ascorbic acid (100 mg) was added to maintain reducing conditions. The sample was suspended in 6 mL absolute ethanol. The tube was covered and heated for 4 min at 90 C to bring the ethanol to the boiling point. Then 0.12 mL 80% KOH was quickly added, and the sample was saponified for 10 min at 90 C in a tube oven. Each tube was agitated twice during the saponification. After saponification, the tube was placed in an ice bath, and 3 mL of H_2O and 3 mL of hexane added. The atmosphere in the tube was replaced with N_2. The tube was stoppered, and the contents were mixed with a vortex mixer. After separation, the hexane layer was transferred to a centrifuge tube. The extraction with 3 mL hexane was repeated. The combined hexane extracts were washed 4 times with ice-cold H_2O to remove residual KOH and dried with anhydrous Na_2SO_4. The hexane extract was centrifuged at 1600 rpm. Just before HPLC and fluorometric detection of the tocols, the volume of the hexane solution was reduced to 1.5-2 mL by a stream of nitrogen.

For UV detection, larger and more concentrated tocol samples were required. Ground corn samples of 0.7 g were used, and the hexane solutions were concentrated to 0.15-0.20 mL. The saponification procedure was the same except the hexane extracts were passed through 0.45 μm regenerated cellulose microfilters (Schleicher & Schuell, Keene, NH) by centrifugation. The microfilters could not be used with the fluorometric detector because some fluorescent material was extracted by the organic solvents.

Tocol Analysis of Corn Germ

For each sample, germs were dissected from the number of corn kernels which weighed ca. 1 g. The 4-6 germs were accurately weighed and totalled ca. 100 mg. The germs were finely minced and allowed to stand under N_2 in 5 mL of hexane for 1 hr with occasional stirring on a vortex mixer. Two additional extractions with 5 mL portions of hexane were carried out for periods of at least 30 min. The combined hexane extracts were centrifuged and reduced under a stream of N_2 to 2 mL. Germ oil in hexane was injected directly into the HPLC and gave clean peaks without saponification. Three or more samples were prepared from each inbred and cross, and each sample was analyzed 3 times by HPLC.

HPLC

The tocol analyses were performed with a Beckman Model 322M HPLC (Beckman Instruments, Inc., Berkeley, CA). UV absorption was measured by a Model 155-409 Hitachi variable wavelength UV-VIS spectrophotometer (Beckman) set at 295 nm. Fluorescence was detected by a Kratos Model 970 variable wavelength Spectrofluoro Monitor (Kratos Analytical Instruments, Ramsey, NJ) with an excitation wavelength of 205 nm and a cut-off filter of 330 nm. The range was set at 0.5 μa, sensitivity at 4.5 and the time constant at 4 sec. The detectors were coupled to a Shimadzu Chromatopac C-R1A recording data processor (Shimadzu Scientific Instruments, Inc., Columbia, MD). All samples were injected via a fully loaded 20 μL loop. The tocol separations were achieved on a 25 cm × 4.6 mm i.d. stainless-steel column prepacked with 5 μm Ultrasphere-Si (Beckman). The proportions of the mobile phase were varied from 1.2% to 1.3% isopropanol in hexane, and the flow rate was varied from 1.25 mL to 1.45 mL/min to obtain optimum separations on a given day.

Tocol standards were included with each set of HPLC runs. α-T and γ-T were purchased from Eastman-Kodak Chemical Co. (Rochester, NY) and δ-tocopherol (δ-T) from Supelco Inc. (Bellefonte, PA). Some tocol standards were not available commercially and were isolated from natural sources and purified by TLC (19). β-Tocopherol

(β-T) was isolated from wheat germ oil, α-tocotrienol (α-T3) from the seed of the corn inbred B73 and γ-tocotrienol (γ-T3) from barley germ oil.

RESULTS AND DISCUSSION

Development of HPLC Method

A method to measure individual tocols was needed that was fast, accurate and required only small samples. The colorimetric procedure of Emmerie and Engel (10) has been widely used to determine tocopherols but a number of substances including vitamin A, carotenoids and fats interfere in the color development (20). TLC and GC have not given complete separation of all the tocol isomers (20). HPLC has several advantages over GC because less sample cleanup is usually required and the lower temperature and shorter time on the column reduce the loss of labile compounds, e.g., the tocols.

A silica gel column was chosen for analysis of the tocols. Although reversed-phase columns have been widely used in HPLC for simultaneous determinations of α-T and other fat-soluble vitamins in foods and feeds (21,22), absorption columns of silica gel have given the best separations of all the vitamin E isomers, the tocopherols and the tocotrienols (20,23-27).

Usually both the UV absorption and the fluorescence of tocols have been measured at 290-295 nm (21, 24-28). Hatam and Kayden (29) noted that the fluorescence spectrum of α-T showed 2 peaks with maxima at 212 nm and 292 nm and that the response was 20-fold greater at the shorter wavelength. These workers used an excitation wavelength of 205 nm to measure α-T in blood cells and plasma. Tangney et al. (30) used an excitation wavelength of 200 nm for fluorometric detection of α-T, β-T, γ-T and δ-T in some commercial vegetable oils and food. We used the short 205 nm wavelength to analyze the tocopherols and tocotrienols of corn grain and increased the sensitivity of our assay by 15-fold over UV detection. As little as 4 ng of a vitamin E isomer can be measured. The sample size could be reduced to one corn kernel, which weighs ca. 250 mg, or even a sample of ground corn weighing only 25 mg.

When we started to use fluorescence detection, we noticed a high level of background peaks between the α-T3 and γ-T peaks of the corn samples (Fig. 1A). This noise made the accurate measurement of α-T and α-T3 very difficult. The interfering peaks were found to be residual detergent (Sparkle Jet; Amsco, Westchester, IL) on the laboratory glassware (Fig. 1B). The peaks were eliminated by washing the glassware with a chromic-sulfuric acid solution (Chromerge; Fisher Scientific Co., Fairlawn, NJ) (Fig. 1C).

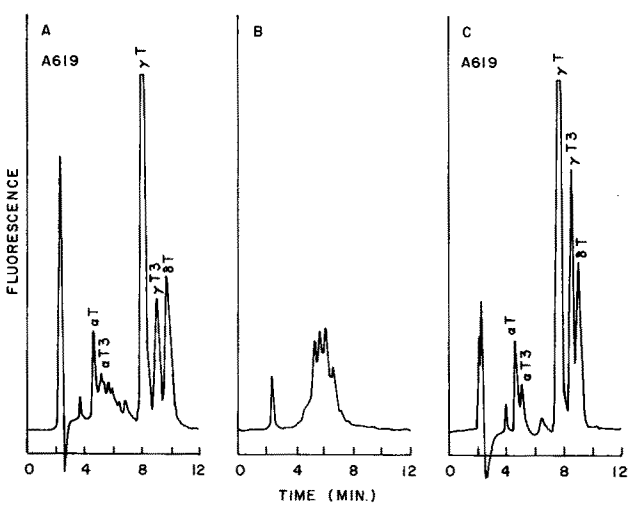

FIG. 1. Fluorescence spectra of (A) tocols of corn inbred A619 + background fluorescing material; (B) detergent for washing laboratory glassware; (C) tocols of corn inbred A619 prepared in glassware washed with acid.

Because no δ-T was found in the corn grain or oil samples, it was chosen as an internal standard. The selection of δ-T was based on its commercial availability, response and retention time. Since δ-T elutes immediately after γ-T3, the analysis time is short, ca. 10 min.

Oil, Linoleic Acid and Tocol Content of Corn Inbreds

Wide ranges were observed for kernel weight, oil content, linoleic acid (18:2) and tocol isomers among the 15 corn inbreds that were analyzed (Table I). The oil content, on a percentage basis, varied from 2.3% to 7.4% with 10 inbreds in the normal range of 3.5-4.5%. The percentage range for 18:2 was 43 to 70% with 8 inbreds in the 61 to 68% range.

The predominant tocol isomer in corn oil has traditionally been considered to be γ-T (20,31), and γ-T was dominant in most of the inbreds that we examined. The inbreds are arranged in Table I in order of increasing weights of γ-T/g kernel weight. A619 had the largest amount of γ-T of all the inbreds, 69.6 μg/g kernel weight. W64A had the highest proportion of γ-T, 82.6%. Some unusual patterns were also observed. Inbreds K6 and B37 had nearly equal amounts of α-T and γ-T. In A632, the level of α-T was higher than γ-T (42.2% α-T compared to 25.8% γ-T).

In general, the level of the tocotrienols paralleled that of their respective tocopherols. These mutual relationships might be expected since the tocopherols are thought to be formed by hydrogenation of the tocotrienol unsaturated side chains, but alternate pathways for synthesis of the tocopherols may also exist (32). In B84, for example, although γ-T made up 67.4% of the tocols, the percentage of γ-T3 was very low, only 1.5%. In most of the inbreds, the levels of the tocotrienols were lower than their corresponding tocopherols, but in W64A, the percentage of α-T3 was higher than that of α-T (6.5% α-T3 compared with 2.5% α-T).

The total tocol weights ranged from 28.2 μg/g kernel weight in C103D to 101.6 μg/g kernel weight in NY16. These 2 inbreds had nearly identical oil weights but NY16 had 1.5 times larger weight of 18:2 than C103D.

The weight data from Table I were used to determine correlation coefficients for oil, 18:2, and the tocols (Table II). Very few strong correlations were found. Linoleic acid

TABLE I

Tocols of Corn Inbreds

Inbred	Kernel wt[a] mg	Oil wt mg/g[b]	18:2 mg/g[b]	α-T	α-T3	γ-T	γ-T3	Total T
				μg/g kernel wt				
A632	248	37.5	24.9	14.9	7.9	9.1	3.3	35.3
C103D	225	44.9	19.7	7.2	4.2	11.2	5.6	28.2
T220	267	41.0	18.1	4.6	4.9	14.7	9.6	33.9
Mo17	337	37.4	25.4	12.2	5.3	16.2	4.1	37.8
K6	225	24.9	14.4	15.5	8.8	16.3	2.2	42.8
B37	248	46.3	28.2	23.0	7.4	22.8	5.1	58.3
B73	269	40.5	23.6	9.3	8.1	29.1	15.4	62.0
B84	285	36.8	24.4	10.1	4.3	31.2	0.7	46.3
H51	195	50.3	24.1	5.3	2.2	39.4	10.3	57.2
Oh43	217	35.8	23.4	13.5	6.4	46.8	16.4	83.0
W64A	151	36.8	24.2	1.4	3.7	47.1	4.8	57.0
NY16	182	42.0	29.3	15.0	7.6	51.5	27.5	101.6
R802A	216	75.3	46.1	17.4	3.9	56.6	13.6	91.4
C105-H	115	22.6	9.9	5.7	5.2	57.0	4.8	72.7
A619	267	46.0	29.8	9.3	4.6	69.6	16.9	100.4

[a] Dry weight basis

[b] mg per g dry wt of kernel

TABLE II

Correlation Coefficients (r) among Kernel Weight, Oil Weight, Linoleic Acid Weight, and Tocol Weights for 15 Corn Inbreds

	Oil wt	18:2 wt	α-T wt	α-T3 wt	γ-T wt	γ-T3 wt	TT wt
Kernel wt	0.14	0.22	0.28	0.15	-0.48	-0.14	-0.35
Oil wt		0.89**	0.26	-0.40	0.24	0.35	0.32
18:2 wt			0.45	-0.19	0.34	0.41	0.49
α-T wt				0.59*	-0.13	0.11	0.21
α-T3 wt					-0.33	0.12	-0.01
γ-T wt						0.55*	0.90**
γ-T3 wt							0.78**

*,**Significant at the 0.05 and 0.01 levels, respectively.

was strongly correlated with oil weight. Among the tocols, γ-T and γ-T3 were strongly correlated with the total tocol weight. Not as strong a correlation was found between the tocols and oil weight or 18:2 weight as might be expected if the tocols are acting as the sole antioxidants for the polyunsaturated linoleic acid.

Tocol Content of Corn Germ from Inbreds and Their Reciprocal Crosses

Because so much variability exists in tocols among corn inbreds, perhaps the level of the tocols can be increased by breeding. To study genetic control of the tocol levels, we hand-dissected germs from corn inbreds and their F_1 reciprocal crosses. Inheritance is more easily determined in the diploid germ, which has equal inheritance from both parents, than in the triploid endosperm. No tocotrienols were found in germ oil, only α-T and γ-T; this finding also made the study of the tocols of the germ less complicated. Grams et al. (12) found that the endosperm fraction contained all of the measurable tocotrienols that occurred in the whole grain of the 4 corn hybrids that they examined.

Analysis of the tocopherols of the germ oils of the inbreds and the reciprocal crosses of B37 × C103D, Oh43 × W64A and A632 × W64 indicated that the proportions and amounts of the tocopherols are heritable (Table III). The α-T and γ-T levels of the crosses were intermediate to the parents. The maternal parent may have had some influence on the γ-T levels of the B37 by C103D and the Oh43 by W64A crosses. With the large variability in tocols that was observed among the corn inbreds, breeding of hybrids with selected proportions of vitamin E isomers may be possible.

TABLE III

Tocopherol Content of Germ Oil from Corn Inbreds and Their Reciprocal Crosses

	α-Tocopherol	γ-Tocopherol
	μg/g germ	
B37	109 ± 3[a]	154 ± 15
B37 × C103D	45 ± 6	133 ± 9
C103D × B37	58 ± 4	115 ± 10
C103 D	30 ± 5	54 ± 12
Oh43	80 ± 5	343 ± 11
Oh43 × W64A	37 ± 3	221 ± 17
W64A × Oh43	41 ± 3	193 ± 4
W64A	6 ± 2	143 ± 5
A632	53 ± 3	48 ± 5
A632 × W64A	34 ± 4	109 ± 13
W64A × A632	31 ± 2	104 ± 5
W64A	6 ± 2	143 ± 5

[a]Standard deviation of mean.

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