CAROTENOIDS OF CORN AND SORGHUM
I. Analytical Procedure[1]

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ABSTRACT

An improved procedure for determining total carotenes and total xanthophylls was developed to serve as a basis for studying the variation of carotenoid pigments in corn and sorghum. The method, based on chromatography and spectrophotometry, is also applicable to grain fractions from wet or dry milling. The effectiveness of alcohols as extracting solvents is attributed to their wetting action and to the breaking of carotenoid-protein complexes. The method produces a sharp separation of carotenes from free xanthophylls. The carotenes fraction includes cryptoxanthin esters, which comprise 13 to 33% of the carotenes and 1–5% of the total carotenoids in corn and sorghum. Saponification is unnecessary in studies on gross variation in xanthophyll level in cereal grains. Relative amounts of noncarotenoid pigments are also estimated by the improved procedure. Other factors influencing the determinations are discussed.

Carotenoid pigments in cereal grains are grouped in two general classes – the carotenes and the xanthophylls. The carotenes are important in feed grains as vitamin A precursors and as a source of yellow color in milk and body fat of cattle. Xanthophylls impart desirable yellow color to egg yolks and to the skin of broilers and fryers (12). Variability in the carotenoid content of corn and sorghum, and their products, has caused concern to the feed processor who desires a dependable low-fiber source of these pigments.

Although numerous procedures are available for measuring carotenes in a variety of materials (4), detailed methods for determining xanthophylls in cereal grains are lacking. A previously published procedure (3) was modified to adapt it to a more routine measure of these pigments in corn and sorghum. The method also provides an estimate of the relative amounts of noncarotenoid pigments which may be highly colored but lacking in comparable biological value. Interference from such pigments undoubtedly has been responsible for high results in phase separation procedures (1).

Analytical Procedure

Preparation of Sample. Grind a clean sample of whole grain in a Wiley Mill, Intermediate Model,[2] using a 40-mesh screen. Grinding

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[2]Mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

must be done with as little temperature elevation as possible because carotenoids are sensitive to heat. Store all samples in the refrigerator. Avoid exposure of materials and extracts to direct sunlight during the entire analytical procedure.

Extraction. Weigh sample (limit total pigments to 100 γ) into a 25- × 80-mm. glass extraction thimble with either a glass-wool plug or glass frit. Cover sample with a layer of glass wool. Place thimble in a Butt extraction tube and attach to a 50-ml. round-bottomed flask containing boiling chips. Add 25 ml. absolute ethanol to top of sample and allow solvent to drain. Extract for 2 hours using heating mantles as the source of heat.

Removal of Interfering Materials. Allow solvent to cool to approximately 35°C. and add approximately 0.5 g. Celite Analytical Filter-Aid followed by 25 ml. reagent-grade diethyl ether (peroxide-free). Swirl to promote precipitation. Place the sample in the refrigerator (approximately 5°C.) for 30–60 minutes to improve filtration characteristics of the precipitate. Precipitation prior to refrigeration prevents the formation of an insoluble gel which retains pigments.

Prepare a filter (under vacuum) by plugging a column, 25 × 110 mm., with cotton and adding a 10-mm. layer of dry Celite Analytical Filter-Aid followed by a 20-mm. layer of Celite Hyflo-Super Cel. Wash filter with 25 ml. 50 absolute ethanol-50 diethyl ether (by volume) to remove fine material. Filter the pigment mixture into a 100-ml. volumetric flask with a Fisher Filtrator or comparable apparatus. Wash flask and filter with sufficient 50 absolute ethanol-50 diethyl ether (by volume) to make the final volume 100 ml. Read absorbance at 445 mμ for total pigment determination.

Place the mixture in a 250-ml. round-bottomed flask, add 0.5 g. Celite Analytical Filter-Aid, and remove the solvents under vacuum on a rotary evaporator at 30°–40°C. Add 10 ml. 90 n-hexane-10 acetone (by volume) to the residue and take to dryness. (The complete removal of ethanol is essential to prevent premature elution of xanthophylls into the carotenes fraction during chromatography.) Avoid prolonged drying.

Chromatographic Separation. Prepare a chromatographic column by packing dry under vacuum a 25 × 30 mm. layer of Celite Hyflo-Super Cel followed by a 25 × 30 mm. layer of Seasorb 43-Celite Hyflo-Super Cel (1:1, by weight). Add 10 mm. layer of anhydrous sodium sulfate.

Transfer the residue to the chromatographic column by washing the flask with five 5-ml. portions of 90 n-hexane-10 acetone (by

volume). Elute carotenes with additional 90 n-hexane-10 acetone to make the total volume 50 ml. Wash flask which contained sample with two 5-ml. portions of 75 n-hexane-25 absolute ethanol (to ensure complete removal of xanthophylls) and place this mixture on the column. Elute xanthophylls with additional 75 n-hexane-25 absolute ethanol (by volume) to make the total volume 50 ml.

Spectrophotometric Determination and Calculation. Determine absorbance of xanthophylls fraction at 445 mμ and carotenes fraction at 445 and 451 mμ. For convenience, normal hexane may be used as a blank for all absorbance measurements without introducing significant error. Calculate concentration of pigments in the total extract, xanthophylls fraction, and carotenes fraction as follows:

$$\text{Pigment, p.p.m.} = \frac{A \times V \times 1{,}000}{a \times L \times W}$$

A = absorbance
V = volume
L = cell path in cm.
W = sample weight in grams
a = absorptivity of carotenoid

Calculations are based on the following wave lengths and absorptivities (2):

	Wave Length mμ	*Absorptivity* *l/g-cm*
Total pigments (as equivalent xanthophylls)	445	231
Xanthophylls	445	231
Carotenes	451	251
Carotenes (as equivalent xanthophylls)	445	231

Carotenes are recalculated as equivalent xanthophylls to adjust all values to the same wave length and reference standard.

The "other pigments" value, expressed as equivalent xanthophylls, is then calculated as follows:

Other pigments, p.p.m. = p.p.m. total pigments − [p.p.m. xanthophylls + p.p.m. carotenes, as equivalent xanthophylls]

Discussion

Grinding the sample to 40-mesh in a Wiley Mill, Intermediate Model, gives sufficient fineness, since grinding to 60-mesh did not improve extraction characteristics. Extraction studies on several corn and sorghum varieties indicate that a 2-hour extraction time is adequate and that this period minimizes loss of carotenoids due to heat and isomerization. A Butt-type extractor is used to eliminate hold-up volume of the solvent. Glass thimbles with either a glass-wool

plug or glass frit prevent "creep" and retention of the pigments which occurs with filter paper or paper thimbles.

The possible existence of carotenoid-protein complexes in plants and animals has been reported by several investigators (5,6,8). Mahadevan *et al.* (6) reported that nonpolar solvents, such as petroleum ether, extracted only small amounts of carotenoids from chicken liver, but that the addition of absolute ethanol gave complete extraction. The effectiveness of ethanol was attributed to protein denaturation, which permitted the extraction of carotenoids by ether or petroleum ether.

Numerous solvents were investigated in the present work to determine their effectiveness in extracting all pigments from ground corn and sorghum. Solvent systems included hydrocarbons, ketones, esters, ethers, alcohols, and their mixtures. Alcohols gave the most complete extraction, based on both visual observations and spectrophotometric determinations. Methanol and ethanol were equally effective, but isopropanol did not give complete extraction for high-xanthophyll corn in 2 hours (Table I). However, ethanol is preferred, since

TABLE I

COMPARISON OF EXTRACTION SOLVENTS IN REMOVING TOTAL PIGMENTS FROM HIGH-XANTHOPHYLL CORN[a]

SOLVENT	CAROTENES	XANTHOPHYLLS	OTHER PIGMENTS[b]
	ppm	*ppm*	*ppm*
Methanol	3.0	35.4	4.5
Ethanol	3.0	35.9	3.7
Isopropanol	2.3	32.0	3.2

[a] A 2-hour extraction.
[b] Pigments retained on the column following chromatography of the alcohol extract.

methanol extracts additional amounts of extraneous material (zein, carbohydrates, etc.) which later interferes with the chromatographic procedure by retaining carotenoids and other yellow pigments. The extraneous material extracted by ethanol is, however, adequately removed by precipitation with equal volumes of diethyl ether. Filtration through Celite produces a filtrate suitable for absorbance measurements and chromatography. These results support the theory that carotenoid-protein complexes exist in corn and sorghum.

This chromatographic procedure separates the total carotenoids into two major fractions — the carotenes and the xanthophylls. Individual carotenoids in each fraction were determined by detailed column chromatography and spectrophotometry. Carotenoids in the carotenes fraction in corn and sorghum are alpha-carotene, beta-carotene, band II (3), and that portion of cryptoxanthin which existed

as an ester in the original extract. Other xanthophyll esters were not detected in this fraction. Band II appears to be a mixture of zeta-carotene and zeacarotenes (10). Carotenoids in the xanthophylls fraction were band I (3), zeaxanthin, lutein, zeinoxanthin (9), and free cryptoxanthin. Band I may contain neoxanthin (13) in addition to trollixanthin. Large amounts of esters give a high carotenes value at the expense of a lowered xanthophylls value. To determine the relative importance of esters, samples were selected to cover a wide range (2.1 – 218.5 p.p.m.) of total carotenoid content (Table II).

TABLE II
XANTHOPHYLL ESTERS IN CORN AND SORGHUM

Sample	Nonsaponified		Saponified			Percentage Carotene Fraction	Percentage of Total Carotenoids
	Carotenes	Xanthophylls	Carotenes	Xanthophylls	Xanthophyll Esters		
	ppm	*ppm*	*ppm*	*ppm*	*ppm*	%	%
High-xanthophyll corn	3.0	35.9	2.6	36.3	0.4	13	1
Hybrid yellow corn	2.0	20.1	1.6	20.5	0.4	20	2
Corn gluten (60% protein)	16.9	201.6	13.2	205.3	3.7	22	2
Red sorghum	0.3	1.8	0.2	1.9	0.1	33	5
Sorghum gluten (60% protein)	0.8	6.5	0.6	6.7	0.2	25	3

Xanthophyll esters comprise 13–33% of the carotenes fraction but only 1–5% of the total carotenoid content. Therefore saponification is unnecessary in studies on gross variation in level of xanthophylls. However, any research directed toward a precise determination of carotenes or xanthophylls or both should involve saponification.

The total pigment determination measures the absorption of carotenes, xanthophylls, flavonoids, and chlorophyll-like compounds at 445 mμ. The relative amount of other pigments, such as flavonoids or chlorophylls, is determined at this wave length, since the xanthophylls normally comprise the major portion of the carotenoid pigments in corn and sorghum.

Average absorptivity constants must be selected for the calculations, since each fraction contains several carotenoids with different absorption maxima and absorptivities (3). The values proposed by Bickoff *et al.* (2) for comparable fractions from alfalfa were adopted for the calculations in this procedure. The suitability of these constants was established by calculating the average absorptivity from the

yields of individual carotenoids separated from each fraction by chromatography. Absorptivities taken from the literature (4,7) were recalculated to adjust all values for individual carotenes and xanthophylls to 451 and 445 mμ respectively. Average absorptivity values were essentially the same as the constants proposed by Bickoff.

Absorbance values obtained with instruments equivalent to a Beckman (Model B or DU) or Cary spectrophotometer may be used without further correction. Instruments similar to the Model 14 Coleman Universal or Spectronic "20" should be calibrated with the spectrophotometers mentioned for optimum results. If this is not possible, a calibration curve with beta-carotene of established purity should be prepared.

Although the absorptivities of the carotenoids differ 2–4% in the three solvent mixtures used in this procedure (4,7,11), the variations are within the experimental limits of the method.

A standard deviation of 0.4 p.p.m. for carotenes and 1.3 p.p.m. for xanthophylls indicates the improved procedure is satisfactory for routine determinations of carotenoids in corn and sorghum.

Acknowledgments

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