Analysis of Carotenoids in Corn Grain

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The unusually complex system of carotenoid polyenes in yellow corn grain has not permitted complete separation of each entity in a routine system of analysis. However, a procedure has been described for the elution of seven fractions from a magnesia chromatogram. Subsequent spectrophotometry provides values for three provitamins A and the eight predominant biologically-inactive carotenoids. In addition to *cis* isomers of the major polyenes, a number of minor components of the extract were recognized. Calculated provitamin A activity of the extracts from three corn samples agreed with bioassay results which were obtained with rats. The method shows that wide differences exist in carotenoid distribution in different inbred lines of corn.

Kuhn and Grundmann (*7*) were among the first to attempt to characterize the individual carotenoids of corn. They isolated zeaxanthin, cryptoxanthin, and β-carotene, but did not report the presence of any other pigments. Fraps and Kemmerer (*5*) identified α-carotene and a neo-cryptoxanthin in corn extracts, White, Zscheile, and Brunson (*10*) isolated lutein and a neo-zeaxanthin, and Callison *et al.* (*3*) reported neo-β-carotenes, but did not find α-carotene in yellow corn. Each of these authors attempted to devise a quantitative method of analysis for corn, but all were encumbered by the presence of unidentified fractions.

Recent work in this laboratory has eliminated some of these encumbrances. Isolation of the biologically-active β-zeacarotenes and of ζ-carotene from corn (*9*) has resolved the mixtures previously described as "*K*-carotene" and "unnamed carotene *I*" (*3, 5, 10*). A study of zeinoxanthin (*8*) has clarified the identity of the "monohydroxy-α-carotene-like substance" (*10*). These identifications, together with other studies described in this paper, have cleared the way for an improved scheme of analysis for the C_{40} polyenes of corn.

Table I. Chromatographic Fractions from the Corn Extract

Fraction	Major Component	Eluent, H:A:E[a]	Reading[b]	Absorptivity Value Used
1	Total hydrocarbons	95:5:0		
	Phytoene		286	85
	Phytofluene		331	98
	β-Carotene		478	228
	β-Zeacarotene		452	170
	ζ-Carotene		400	244
2	Zeinoxanthin	90:10:0	445	268
3	Cryptoxanthin	90:10:0	478	216
4	Esters (as zeaxanthin)	89:10:1	450	250
5	Lutein	89:10:1	447	256
6	Zeaxanthin	88:10:2	452	248
7	Polyoxy pigments	80:10:10	452	248

[a] Ratio of hexane to acetone to ethyl alcohol in per cent by volume.
[b] For the polyene fraction a reading was also made on the solution at 426 mμ, and concentrations of the three pigments were then determined by the ratio method as explained in the text.

Procedure

Solvents for extraction and chromatography were purified before use. Hexane (Skellysolve B) was percolated through a column of silica gel (28- to 200-mesh, Davison Chemical Co.). Both hexane and iso-octane were distilled over potassium hydroxide pellets before use. Acetone was purified by drying for 2 days, over anhydrous sodium sulfate and subsequently distilling it over metallic zinc. Ethyl alcohol (Commercial Solvents, 200 proof) was used without purification. Magnesia columns were packed with a mixture of equal weights of magnesia (Sea Sorb 43, Westvaco Chemical Corp.) and diatomaceous earth (Hyflo Supercel, Johns-Manville Co.).

For extraction, a 100-gram sample of freshly ground corn (20-mesh) was treated with 400 ml. of the extraction mixture consisting of 15% hexane, 75% acetone, and 10% water by volume. The resulting slurry was allowed to stand in the dark, at room temperature, for 48 hours and was then poured into a glass percolation column (35 × 5 cm.). An additional 500 ml. of the extraction mixture was allowed to percolate through the column into a 1-liter separatory funnel. After addition of 100 ml. of hexane and 100 ml. of water to the percolate, the hypophase was drawn off and re-extracted by swirling with 200 ml. of hexane. The combined epiphases were washed with five 100-ml. portions of water, then filtered through a layer of anhydrous sodium sulfate, and the solvent was removed under reduced pressure on a rotary evaporator, the bath temperature not exceeding 40° C. The resulting oil was made up to 100 ml. with hexane.

For chromatographic separation of the carotenoids, a 25-ml. portion of the extract was placed on a magnesia column (12 × 1 cm.) on top of which was a 1-cm. layer of anhydrous sulfate. With vacuum applied, this solution was followed by 5 ml. of hexane and 100 ml. of 5% acetone in hexane, which sufficed to elute the polyene band (Fraction 1) from the column. Eluants for the subsequent six fractions are shown in Table I. Each solvent mixture was added to the column after complete elution of the previous fraction. After removal of the solvent under reduced pressure, Fraction 1 was diluted to 50 ml. with iso-octane. All other fractions were evaporated similarly and diluted to 50 ml. with hexane, except Fractions 5 and 6, which were diluted to

Table II. Absorbance Ratios for Different Pigment Mixtures

Pigment Ratio,[a] %	Ratio A, 426/478	Ratio B, 426/452
80–10–10	1.01	0.83
70–20–10	1.14	0.87
70–10–20	1.18	0.97
60–30–10	1.32	0.91
60–20–20	1.38	1.03
60–10–30	1.43	1.16
50–40–10	1.55	0.96
50–30–20	1.64	1.09
50–20–30	1.72	1.24
50–10–40	1.78	1.42
40–50–10	1.90	1.00
40–40–20	1.99	1.14
40–30–30	2.10	1.31
40–20–40	2.20	1.51
30–50–20	2.56	1.20
30–40–30	2.69	1.39
30–30–40	2.85	1.62
20–50–30	3.74	1.48
20–40–40	3.92	1.75

[a] Pigment ratio is the percentage ratio of β-carotene:β-zeacarotene: ζ-carotene in the solution.

Table III. Comparison of Extraction Methods for Carotenes

Method	Absorbance of Extract, 436 mμ	γ/G.
Acetone, hexane, water (75:15:10)[a]	0.129	2.6
Ethyl alcohol, 75%	0.121	2.4
Toluene, ethyl acetate, and ethyl alcohol (*4*)	0.105	2.1
Ethanolic potassium hydroxide (*2, 5*)	0.098	2.0
Acetone, water (*10*)	0.058	1.2

[a] Present work.

100 ml. with hexane. Absorbance values (Beckman DU spectrophotometer) were determined at appropriate wave lengths, and, using the corresponding absorptivity (Table I), the concentration in micrograms per gram of corn was calculated from the following formula:

$$C = \frac{AVD \cdot 1000}{aw}$$

where A is absorbance, V is original volume of extract, D is the dilution factor for reading on the spectrophotometer, a is the corresponding absorptivity of the pigment, and w is the sample weight.

To estimate the major components of the polyene fraction (Fraction 1), absorbance values were obtained at six different wave lengths (Table I). Calculations for phytoene and phytofluene were made directly from their corresponding absorptivity values. Calculations for β-carotene, β-zeacarotene, and ζ-carotene were made in three steps: Absorbance ratios were compared with values in Table II to approximate the percentage of each of the three pigments in solution. Amounts of other pigments present were considered to be negligible. The ratios in the table were based on the following absorptivities in iso-octane.

	Wave Length, Mμ 400	426	452	478
β-carotene	92	179	257	228
β-zeacarotene	127	185	170	17
ζ-carotene	244	245	...	...

The amount of the observed absorbance representative of each pigment was then calculated from the following equations:

$$A_{\beta\text{-carotene}} = A_{478} \frac{\%\ \beta \times 2.28}{(\%\ \beta \times 2.28) + (\%\ \text{zea} \times 0.17)}$$

$$A_{\beta\text{-zeacarotene}} = A_{452} \frac{\%\ \text{zea} \times 1.70}{(\%\ \text{zea} \times 1.70) + (\%\ \beta \times 2.57)}$$

$$A_{\zeta\text{-carotene}} = A_{400} \frac{\%\ \zeta \times 2.44}{(\%\ \zeta \times 2.44) + (\%\ \text{zea} \times 1.27) + (\%\ \beta \times 0.92)}$$

The amount of each pigment in micrograms per gram of corn was then calculated from this corrected absorbance value and the appropriate absorptivity from Table I.

Table IV. Typical Analyses of Corn Samples from Different Parentage

Fraction	Chief Component	Corn 1 (Os420)	Corn 2 (H60)	Corn 3 D.C.[a]	Corn 4 Oh45	Corn 5 Hy
		Micrograms per Gram				
1	Phytoene	21.1	15.5	19.3	25.6	10.7
	Phytofluene	4.8	3.8	3.7	9.3	1.3
	β-Carotene	2.7	1.8	2.0	4.4	0.6
	β-Zeacarotene	0.4	0.5	1.2	4.0	0.1
	ζ-Carotene	0.9	0.3	1.0	4.2	0.2
2	Zeinoxanthin	2.3	1.1	2.5	2.2	0.6
3	Cryptoxanthin	1.4	3.1	2.1	3.2	1.0
4	Esters	0.8	1.1	1.1	4.0	0.5
5	Lutein	11.7	11.9	14.1	29.2	10.5
6	Zeaxanthin	6.0	11.3	6.8	5.4	3.9
7	Polyoxy pigments	0.8	1.1	1.8	1.3	0.8
	Total polyenes	52.9	51.5	55.6	92.8	30.2

[a] Seed from the double cross (A25 × W8a) × (Kys × 38-11) grown under open pollination conditions.

Table V. Comparison of Results of Single Analyses of Corn 3 Performed on 10 Different Days by Each of Two Different Analysts

Fraction	Av., γ/G.	One Analyst Std. dev.	One Analyst Coeff.,[a] %	Two Analysts Std. dev.	Two Analysts Coeff.,[a] %
Total pigment	33.0	±1.5	5	+1.5	5
Phytoene	19.3	±4.4	22	±4.4	22
Phytofluene	3.7	±0.5	11	±0.5	14
1	4.2	±0.2	4	±0.2	4
2	2.5	±0.2	10	±0.3	11
3	2.1	±0.2	12	±0.2	12
4	1.1	±0.5	52	±0.6	54
5	14.1	±0.7	5	±0.7	5
6	6.8	±0.4	7	±0.4	7
7	2.6	±0.7	26	±0.8	29

[a] Coefficient of variation.

As a check on the total recovery of all pigments from the chromatogram, it was found helpful to dilute a 5-ml. portion of the original hexane extract to 50 ml., read the absorbance at 450 mμ, and calculate total pigment as lutein (a = 250). The sum of all pigment values was usually within a few per cent of this figure.

Results and Discussion

Extraction of Carotenoids. The method of extraction by percolation of the rehydrated ground corn was adopted only after a comparison with other techniques which had been used by previous workers (Table III). Total absorbance of carotenes in the extract, observed in hexane at 436 mμ and calculated as β-carotene, showed the highest yield of pigment was obtained with this method. Typical results obtained with a ground corn sample are shown in Table III. The procedure requires little apparatus and avoids heating of the sample or of its extract. Good reproducibility was shown in the results of 10 replicate extractions of portions from a large sample of Corn 3, a double cross (Table IV). Total pigment values (450 mμ) on the 10 extracts (50 ml. volume) averaged 33.0 γ per gram, with a standard deviation of 1.5 (Table V).

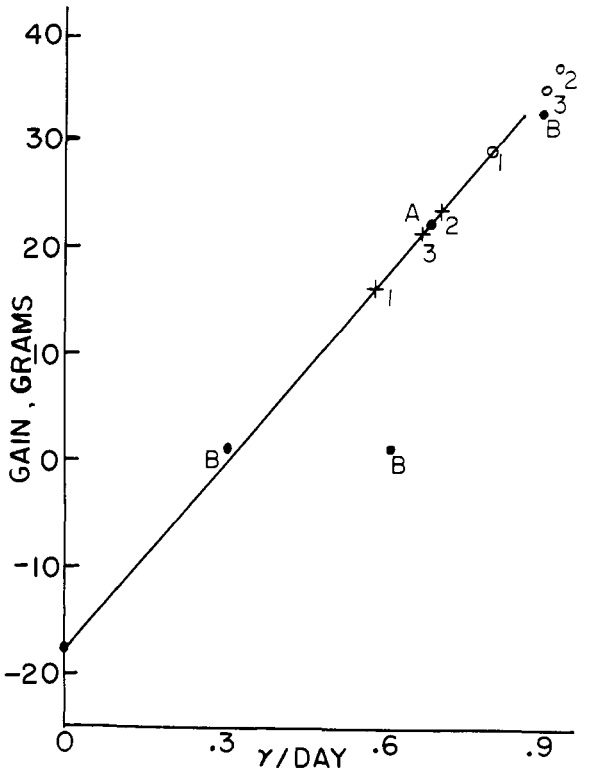

Figure 1. Average body weight responses of rats to vitamin A standards and corn supplements

- ● Controls (A, vitamin A acetate, 0.67 γ/day; B, β-carotene, 0.3, 0.6, and 0.9γ/day)
- \+ Extracts of Corns 1, 2, and 3 fed at 0.67γ vitamin A equivalent/day, calculated from analyses
- ○ Whole ground corn samples fed at levels equivalent to the extracts

Main Components of Each Fraction. Under the conditions described above, it was possible to separate from the magnesia chromatogram seven well defined zones of pigmentation. Each of these zones contained one or more predominant pigments together with smaller amounts of isomers or other pigments. A summary of the components given below for a single cross, Wf9 × M14, and the double cross, Corn 3, is typical of results obtained with corn samples of various parentage.

Fraction 1 contained all the hydrocarbons, including phytoene, phytofluene, α- and β-carotenes, α- and β-zeacarotenes, ε-carotene, and ζ-carotene. Small amounts of neo-isomers of some of these polyenes were also isolable, probably as a result of interconversion in solution after extraction.

Fraction 2 consisted almost wholly of zeinoxanthin and its neo-isomer.

Fraction 3 was predominantly cryptoxanthin; however, the upper portion of the band contained light-colored components from which two distinct pigments were isolable on lime columns. Both were monohydroxy compounds in their phasic behavior; in spectral characteristics, one corresponded to β-zeacarotene, the other to α-carotene.

Fraction 4 consisted of xanthophyll esters, predominantly helenien, with some physalien. This band was eliminated when the pigments were subjected to saponification before chromatography.

Fraction 5 consisted of lutein and a small amount of neo-lutein.

Fraction 6 consisted of zeaxanthin with a small amount of neo-zeaxanthin.

Fraction 7 was a mixture of strongly hypophasic pigments which partitioned almost equally between hexane and 80% aqueous methanol. Chromatography on lime showed the predominant pigment to give a spectral curve similar to that of zeaxanthin. Also isolable from this mixture were substances with spectral curves similar to β-zeacarotene and ζ-carotene, apparently xanthophyll analogs of these pigments. Fluorescence of some eluates from this fraction as well as from Fraction 3 further suggested the presence of hydroxy derivatives of phytoene and/or phytofluene in the corn extracts.

Reproducibility of the Method. Ten different portions of a uniform sample of hybrid corn (Corn 3, Table IV) were extracted and chromatographed on different days by two different analysts over a period of several weeks. The data (Table V) show fairly good reproducibility for most fractions. Variations in phytoene are probably due mainly to residual solvent impurities (especially the hexane). Variations in Fraction 4 reflect difficulties in separating this band from adjacent bands, especially that representing Fraction 3. This separation is particularly difficult with chromatograms which flow unevenly. Variations in Fraction 7 reflect a difficulty in complete elution from the column and, perhaps, also some losses into the hypophase, immediately after extraction of the pigments. Differences between analysts were found to be negligible.

Provitamin A Activity. The basal diet and the procedure for the animal experiments has been described previously (*1*). When growth ceased, the animals were divided into groups of eight with no sibs of the same sex in any group. Supplements were then given, three times a week for 4 weeks, to the different groups as follows: β-carotene at three levels, 0.30, 0.60, and 0.90 mg. per day; vitamin A acetate, 0.67 mg. per day; extracts from three different samples of ground corn (Corns 1, 2, and 3) in amounts calculated to supply 0.67 mg. of β-carotene equivalent per day. In these calculations the cryptoxanthin fraction was assumed to have half the activity of β-carotene, and the β-zeacarotene was assumed to have one third the activity of β-carotene (*9*). The extracts were evaporated under reduced pressure, and the residues were diluted with Wesson oil. Each rat received a 1 mg. supplement of *dl*-α-tocopherol per day. Finally, three groups of rats also received an equivalent amount of each of three ground corn samples: 0.44 gram of Corn 1; 0.45 gram of Corn 2; and 0.45 gram of Corn 3. On this diet, responses to β-carotene are essentially the same as those to an equal weight of vitamin A (*1*).

Average weight gains of the rats on different supplements (Figure 1) showed that the calculated activity of the extract agreed well with the amount observed by bioassay. Average responses to Corn 1, both as the extract and the whole ground corn, were slightly less than to the other two corn samples. In all cases, responses to the whole ground corn supplements were about one third greater than responses to the corresponding extract. The reason for this is not known. Prolonged extraction of corn samples failed to yield more than traces of additional pigments. It is possible that corn contains a growth factor in addition to extractible carotenoids. The nature of such a factor is not surmised; however, this observation is reminiscent of one made earlier with alfalfa meal and its extracts by Frey and Wilgus (*6*).

In selecting corn samples for bioassay, it was attempted to include samples which had about the same total provitamin A and polyene content but

which differed substantially in the relative amounts of their provitamin A components (Table IV). Two additional inbreds (Corns 4 and 5) included in the table show that wide differences in carotenoid distribution also occur in yellow corn grain samples of different genetic origin.

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ALFALFA ESTROGENS

Quantitative Determination of Coumestrol in Fresh and Dried Alfalfa

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A simplified procedure has been developed for the paper chromatographic fluorometric determination of the plant estrogen, coumestrol, in fresh and dried alfalfa. The method is sensitive to about 2 p.p.m. of coumestrol, with a maximum error of about ±5%. The method makes feasible the assay of large numbers of samples required in studies correlating estrogenic activity and stage of maturity, heredity, environment, and other factors.

THE PRESENCE (*3*) and subsequent qualitative determination (*8*) of the plant estrogen coumestrol in alfalfa and other forages were given in previous reports. Estrogenic substances present in forages have been implicated as the cause of reproductive disturbances in sheep (*4*) and have also been suggested as having beneficial effects such as increased rate of growth and milk production (*5*). The bioassay procedures (*2*) used for determining the estrogenic activity of these plants are laborious and not adapted to assaying large numbers of samples required in studies correlating estrogenic activity with stage of maturity, heredity, environment, and other factors. However, since coumestrol appears to be the predominant plant estrogen in alfalfa, its quantitative determination should correlate closely with the total estrogenic activity of alfalfa.

A recent report from this laboratory (*7*) presented factors which are involved in the paper chromatographic, fluorometric measurement of coumestrol. Based on these observations a rapid quantitative paper chromatographic procedure has been developed for fresh and dried alfalfa. In addition to the proposed procedure two alternative methods have been devised to confirm the results obtained by the fluorometric procedure. In the first paper chromatography is used to purify the extract, and the developed chromatogram is read directly in an ultraviolet spectrophotometer. The second utilizes silicic acid chromatostrips (*6*) to purify the extract, followed by ultraviolet spectrophotometry to measure the solution of coumestrol eluted from the chromatostrips.

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Experimental Procedure

Extraction. FRESH ALFALFA. The fresh plant material is cut into 1-inch lengths and uniformly mixed, and a 10-gram sample is taken for moisture determination. For analysis, 400 grams of the material is blended with 1 liter of 95% alcohol at high speed for 1 minute in an electric blender. The mixture is filtered on a Büchner funnel through Whatman No. 1 paper, under very light suction to minimize evaporation losses. The filtrate thus obtained is an aliquot of the whole extract.

In cases where it is not convenient to perform the assay immediately, the fresh plant material may be stored conveniently in a sealed container with alcohol, in the proportions described above, for periods up to at least 3 months with no measurable loss of coumestrol.

DRIED ALFALFA. A 20-gram sample of the dried forage, ground to a fine meal, is rehydrated with 80 ml. of water in a 500-ml. Erlenmeyer flask for 1 hour at room temperature. Three hundred and twenty milliliters of 95% alcohol is then added and, after gently swirling to assure mixing, the flask is stored in the dark at room temperature for 24 hours. At the end of this time the mixture is filtered by the procedure used for the extract of the fresh sample, and an aliquot obtained as before for purification and analysis.

Purification. SOLVENT SEPARATION. A 100-ml. aliquot of the alcohol extract from either the fresh or dried alfalfa is shaken with four successive 35-ml. portions of petroleum ether, in a 250-ml. separatory funnel. The petroleum ether removes most of the waxes and fatty materials which interfere with later paper chromatography. The alcohol-water phase is then concentrated in a rotary evaporator at reduced pressure to a volume of about 20 to 25 ml. The remaining concentrated aqueous mixture is transferred to a 125-ml. separatory funnel and extracted successively with one 15-ml. and three 8-ml. portions of ethyl ether. The water phase is discarded and the combined ether extracts are concentrated in a 50-ml. round-