

J. M. White

(5)

Reprinted from Analytical Edition, INDUSTRIAL AND ENGINEERING CHEMISTRY, Vol. 14, Page 798, October 15, 1942  
Copyright 1942 by the American Chemical Society and reprinted by permission of the copyright owner

## Studies on the Carotenoids

### Spectrophotometric Determination of the Carotenoids of Yellow Corn Grain

JONATHAN W. WHITE, JR.<sup>1</sup>, ARTHUR M. BRUNSON, AND F. P. ZSCHEILE, Purdue University  
Agricultural Experiment Station, Lafayette, Ind.

IN 1937, Clark and Gring (3) presented a method for determining the carotenoids of corn [*Zea Mays L.*] but did not separate the carotene from cryptoxanthol. Consideration of cryptoxanthol in analytical methods for corn pigments is important because of its provitamin A activity. These workers reported the concentration of "xanthophyll" in several corn varieties, but the standard absorption coefficients which they used for this determination were much lower than those reported by others, leading to erroneous results for "xanthophyll" concentration. Later, Buxton (2) described a method for the determination of "carotene and/or cryptoxanthin" in yellow corn. He used adsorption on calcium carbonate for separation of carotene from cryptoxanthol in his final solution. Both workers used 90 per cent methanol for the separation of zeaxanthol from the provitamin pigments. Fraps and Kemmerer (5) recently reported an adsorption method for the determination of the pigments of corn. They separated the pigments epiphasic to 90 per cent methanol into five compounds: beta-carotene, alpha-carotene, K carotene, cryptoxanthol, and neocryptoxanthol. An abridged method for routine determination was developed in which the pigments were divided into two groups, caro-

tenes and cryptoxanthol plus neocryptoxanthol, by adsorption on magnesium carbonate. Calculation of the provitamin A potency of these two groups was based on their average composition as found in the preliminary study. This method is probably the best of the three, though losses from incomplete recovery of adsorbed pigments must not be overlooked. It is also necessary to test and standardize each new lot of adsorbent.

White, Zscheile, and Brunson (11) demonstrated the presence of luteol and gamma-carotene in corn, and noted the occurrence of unnamed carotene 1 which may be identical with the K carotene reported by Fraps and Kemmerer (5). In this paper it is shown that large amounts of neo isomers are present in corn extracts and must be considered in analysis. The solvents suggested in a previous paper (10) for the separation of the pigments into three groups—carotene, cryptoxanthol, and carotenol—have been applied to the separation of corn-grain pigments. Where possible, account is taken of the presence of pigments other than beta-carotene, cryptoxanthol, and zeaxanthol.

#### Experimental

For immature corn grain, the sample (20 to 40 grams, depending on water content) was extracted 5 minutes in a Waring

<sup>1</sup> Present address, Eastern Regional Research Laboratory, Wyndmoor, Penna.

Blendor with 150 to 200 ml. of acetone. Mature corn grain was ground in a hammer mill and 15 to 20 grams of the meal were tempered with about 6 ml. of water for 5 minutes and extracted in a Soxhlet extractor with acetone until all visible color was removed. One volume of ether was added to the acetone solution from either extraction and the pigment was transferred to ether by cautious addition of 3 volumes of water. The ether solution was washed cautiously five times with water and saponified by refluxing for 0.5 hour with 10 ml. of concentrated ethanolic potassium hydroxide. The alkali and soaps were washed from the ether solution and it was evaporated on a steam bath with a boiling stick in the beaker. The beaker was removed from the steam bath just before it became dry.

The residue was dissolved in a mixture of hexane and 78.5 per cent diacetone alcohol (100 parts of diacetone alcohol to 28 parts of water) and transferred to a separatory funnel. The volumes of the two phases were adjusted to approximately 25 ml. and after vigorous shaking the diacetone alcohol phase was removed. The hexane was extracted twice with fresh diacetone alcohol solution. The diacetone alcohol solutions were combined and extracted with about 15 ml. of hexane, which was in turn extracted with an equal volume of diacetone alcohol solution. An equal volume of ether was added to the combined diacetone alcohol extracts, the solution was shaken, and 2.5 to 3 volumes of water were added to force the pigments into the ether phase. The combined hexane extracts and the ether solution were each washed five times with water. The ether was evaporated on the steam bath with precautions noted above and the residue (carotenols except cryptoxanthol) dissolved in ethanol for spectrophotometric measurement.

The hexane solution was added to 25 ml. of 91.8 per cent (100 to 9) 2-methyl-2,4-pentanediol in a dry separatory funnel and extracted three times with that solvent. About 15 ml. of hexane were added to the combined extracts and the cryptoxanthol was transferred to hexane by the addition of 300 ml. of water. Occasionally a second extraction was necessary. The original hexane solution and the cryptoxanthol solution were each washed five times with water and made to volume for spectrophotometric measurement. It was not necessary to dry the solutions for clarification.

Absorption spectra of these three solutions showed the type of "degradation" from the curves of the pure pigments that is caused by the presence of neo-type pigments (1). [Absorption spectra were determined on an improved photoelectric spectrophotometer, previously employed in pigment studies (1, 12, 13), of the type described by Hogness *et al.* (6).] Chromatography on magnesium oxide-Supercel did not separate the neo pigments from their isomers. Absorption spectra of the pigments from single zones indicated the presence of neo isomers.

**CAROTENE FRACTION.** In addition to the presence of neo-beta-carotene, the spectra of the carotene fractions showed the presence of unnamed carotene 1, as noted previously (11). Attempts were made to analyze the carotene fraction as a beta-carotene-neo-beta-carotene system as outlined by Beadle and Zscheile (1). Agreement between wave lengths 4780 and 4850 Å. was poor, owing to the presence of other colored compounds (compare values of  $R_2$  in Table I with those of Table III).

TABLE I. RATIO VALUES FOR CERTAIN CAROTENOIDS

Pigment	$R_1 = \frac{\log \text{ at } 4350 \text{ \AA.}}{\log \text{ at } 4250 \text{ \AA.}}$	$R_2 = \frac{\log \text{ at } 4950 \text{ \AA.}}{\log \text{ at } 5000 \text{ \AA.}}$
$\beta$ -Carotene	1.08	1.73
Neo- $\beta$ -carotene	1.16	1.77
Unnamed carotene 1	0.35	
$\gamma$ -Carotene	0.98	0.83
33% neo-67% $\beta$ -carotene.	1.10	1.74

TABLE II. ANALYTICAL CONSTANTS FOR CAROTENOLS

Wave Length Å.	Specific Absorption Coefficients in Ethanol Solution		
	Zeaxanthol	Luteol	Neozeaxanthol II
	Liters per gram cm.		
4275	ca. 172	ca. 172	ca. 172
4500	247	247	174
4783	219	219	103
4850	203	152	59.5
4900	166	98.3	35.5
4950	119	53.7	23.0

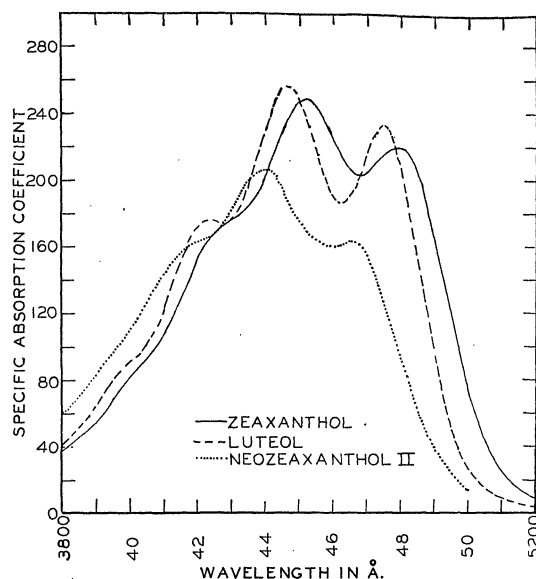


FIGURE 1. ABSORPTION SPECTRA OF CAROTENOLS IN ETHANOL

Accurate analyses for unnamed carotene 1 and for gamma-carotene are not possible because quantitative absorption spectra for these components are not yet available. As an indication of the presence of these compounds in corn-grain extracts, the ratios of  $\log \frac{I_0}{I}$  values at 4350 Å. to those at 4250 Å. were determined for the carotene fractions from corn, as well as the ratios of  $\log \frac{I_0}{I}$  values at 4950 Å. to those at 5000 Å.

Table I shows these ratios for certain pure pigments for comparison with the values for corn extracts reported in Table III.

**CRYPTOXANTHOL FRACTION.** The cryptoxanthol fraction was treated as a mixture of cryptoxanthol and neocryptoxanthol for analysis, using the absorption spectra previously presented (11, 13).

The composition of these extracts in terms of percentage neocryptoxanthol varied from 28 to 90 per cent. The average values are considerably higher than those present in equilibrium mixtures of pure cryptoxanthol and neocryptoxanthol. Since agreement between wave lengths 4500 to 4850 Å. and 4500 to 4900 Å. was only fair, other compounds probably interfered with this analysis (11).

**CAROTENOL FRACTION.** The carotenol fraction from a corn-grain extract was chromatographed on magnesium oxide-Supercel. The zeaxanthol zone was eluted and the pigment adsorbed on calcium hydroxide-Supercel, resulting in 3 zones, similar in appearance to those resulting from the isomerization of zeaxanthol (11). Analysis of the carotenol fraction as a mixture of zeaxanthol, luteol, and neozeaxanthols I and II (11), ignoring the presence of neoluteols, is impractical because the curves for luteol and neozeaxanthol I are so close together.

Therefore, the carotenol fraction was analyzed as a mixture of zeaxanthol, luteol, and neozeaxanthol II by the procedure given below. Figure 1 shows the spectra (11, 13) used as standards for the analysis and Table II presents the analytical constants at selected wave lengths.

*Step 1.* At wave lengths 4500 and 4783 Å. the curves for zeaxanthol and luteol intersect and are considerably higher than the curve for neozeaxanthol II. The method of simultaneous

TABLE III. CAROTENOIDS OF CORN GRAIN

Age Days after pollination	Water Content %	Total <sup>a</sup> Carotenes	Total <sup>b</sup> Crypto- xanthols	Zea- xanthol	Neozea- xanthol	Luteol	Ratio	
							R <sub>1</sub>	R <sub>2</sub>
Micrograms per gram dry weight								
Inbred K-156								
32	55.2	2.48	2.25	0.53	8.40	12.8	1.00	1.73
Mature	9.9	2.30	1.67	1.03	7.83	9.30	0.97	1.66
Mature	..	2.01	1.74	0.83	6.71	11.1	0.98	1.38
Inbred 38-11								
40	35	5.17	6.54	15.6	5.68	5.78	0.98	1.28
44	38	5.70	5.58	17.4	6.76	6.79	0.99	1.35
44	..	..	6.30	17.4	5.48	7.45	..	..
Mature	9.0	5.06	2.98	20.7	7.78	4.45	0.93	1.37
Mature	9.6	3.42	3.62	18.9	8.48	4.36	..	..
Mature	..	4.40	3.43	16.8	6.17	3.69	..	..
Inbred M4B								
24	71.5	5.87	10.3	9.71	9.41	18.0	1.06	1.47
28	65.2	6.71	7.67	7.72	8.76	16.4	1.04	1.58
36	48.8	6.96	7.95	16.9	5.62	13.7	0.92	1.18
40	48.0	7.35	12.6	16.2	3.92	18.3	0.96	1.09
Mature	11.6	7.41	10.4	14.0	7.32	12.8	0.87	1.30
Mature	..	8.95	10.7	14.5	7.98	11.9	0.90	1.31
Inbred Kys								
32	54.4	8.51	14.7	33.1	8.87	10.7	0.90	1.57
36	47.5	8.75	15.7	17.7	7.65	11.3	0.86	1.71
40	..	9.45	12.1	21.8	3.88	6.53	0.86	1.62
Mature	9.1	5.64	6.78	21.3	11.8	3.33	0.73	1.54
Mature	..	5.53	7.36	20.7	10.3	4.54	0.73	1.60

<sup>a</sup> Sum of beta-carotene and neo-beta-carotene.  
<sup>b</sup> Sum of cryptoxanthol and neocryptoxanthol.

equations which has been previously applied in such cases (4) was used here to obtain the concentrations of luteol plus zeaxanthol and of neozeaxanthol II.

Step 2. At wave lengths 4850, 4900, and 4950 Å. in the region of greatest spread between the spectra of luteol and zeaxanthol,

the absorptions (absorption =  $\frac{\log_{10} \frac{I_0}{I}}{l}$ ) due to neozeaxanthol were

calculated from the concentration of neozeaxanthol obtained from step 1 and from its absorption coefficients. These absorptions were then subtracted from the observed absorptions of the mixture at these wave lengths to give the absorption due to the luteol and zeaxanthol.

Step 3. Then with the value for concentration of luteol plus zeaxanthol from step 1 and the absorption of luteol plus zeaxanthol from step 2, the percentage of luteol was calculated by the method used by Beadle and Zscheile (1) for beta- and neo-beta-carotenes.

Step 4. Since all three absorption spectra are close together at 4275 Å. an approximate value for the total concentration of all three components can be obtained by use of an approximate absorption coefficient at this wave length. This value should check the sum of the two concentrations obtained in step 1.

Results of the application of the above methods to several inbred corn lines are shown in Table III. The inbreds were self-pollinated and each sample is a composite from at least 3 ears.

CHROMATOGRAPHIC CONFIRMATION OF SPECTROPHOTOMETRIC RESULTS. The approximate amounts of cryptoxanthol in the carotene fraction and vice versa were determined on the solutions from the mature samples with the aid of chromatographic technique. This separation was approximately 80 per cent efficient (10)

The carotenol fractions from the same samples were transferred to ether and chromatographed to obtain values representing the relative amounts of zeaxanthol and luteol present. Pigments were eluted from zones and made to volume and the concentration was determined spectrophotometrically. Results are shown in Table IV.

These data indicate that the determination of all the neo-type pigments in the solution as neozeaxanthol II did not introduce serious error as far as the relative amounts of luteol and zeaxanthol were concerned.

The carotenol fraction of a corn sample was analyzed spectroscopically as described above and also as a mixture of luteol, zeaxanthol, and neozeaxanthol I. Results are given in Table V.

The agreement between determinations of total pigment content of the carotenol solution by step 1 and step 4 is shown in Table VI.

## Discussion

It is evident that accurate spectroscopic determination of the carotenoids is complicated by the presence of neo-type isomers as well as by the occurrence of pigments not heretofore recognized in corn extracts. In some applications it may be desirable to employ a single wave length to determine pigment groups. In analysis for total pigment content of the carotene, cryptoxanthol, and carotenol fractions, a careful choice of wave lengths is necessary to minimize errors due to varying composition. This is accomplished by selection of wave lengths where the absorption spectra of the component pigments are coincident or nearly so.

For the carotene fraction a specific absorption coefficient of 185 at 4325 Å. is obtained by assuming a specific absorption coefficient for unnamed carotene 1 of 250 at 4250 Å., and using the spectrum for gamma-carotene reported by Kuhn and Brockmann (7). The absorption coefficients at 4325 Å. are then: beta-carotene, 187; neo-beta-carotene, 182; gamma-carotene, 170; and unnamed carotene 1, 150. Since most of the pigment present is beta- or neo-beta-carotene, 185 is taken as a weighted mean.

In a solution in which the only significant components are cryptoxanthol and its neo isomer, the choice of the average coefficient 186 of the two components at 4375 Å. as a standard absorption coefficient should give results with small error, since the curves are only 12 units apart.

As previously pointed out, determination of total carotenols at 4275 Å. gives good agreement with spectrophotometric analysis for the components. The specific absorption coefficient used was 172.

TABLE IV. COMPARISON OF CHROMATOGRAPHIC AND SPECTROPHOTOMETRIC DETERMINATIONS OF LUTEOL

Inbred	Luteol Content of Carotenol Fraction	
	By adsorption %	By spectroscopic analysis %
Kys	27	18
M4B	46	45
38-11	22	18
K-156	83	90

TABLE V. ANALYSIS OF CAROTENOL FRACTION BY TWO SYSTEMS

System	Zea- xanthol Mg./l.	Luteol Mg./l.	Neozea- xanthol Mg./l.	Total at 4275 Å. (Step 4)	Total by Addition (Step 1)	Luteol %
				Mg./l.	Mg./l.	
Neozeaxanthol II system	1.21	1.26	0.75	3.21	3.22	49
Neozeaxanthol I system	1.39	1.51	0.33	3.25	3.23	48

TABLE VI. RELIABILITY OF CAROTENOL DETERMINATION

Inbred	No. of Determinations	Average Difference between Results by Steps 1 and 4		Maximum Difference %
		%		
Kys	17	3.3		7.7
M4B	11	1.9		4.0
38-11	19	2.0		11.4
K-156	4	2.4		3.0

TABLE VII. COMPARISON OF RESULTS

	Cryptoxanthol		Total Carotenols Micrograms/g
	Carotene Micrograms/g.	Carotene plus Cryptoxanthol Micrograms/g.	
Buxton (2)	0.34-0.85	5.9-13.5	4.2-9.3
Clark and Gring (3)	.....	.....	0.10-1.11
Kuhn and Grundmann (3)	0.5-0.7	6.5-14.0	5.1-7.5
Peterson, Hughes, and Payne (9)	.....	.....	0.8-9.4
Frap and Kemmerer (5)	0.4-3.7	0.73-2.14	0.9-7.7
This paper (ma- ture samples)	2.0-8.9	0.7-1.2	3.7-19.6
			18.2-35.9

A comparison of the results of several investigators is shown in Table VII.

Wide variation is found among corn varieties with respect to pigment content. All varieties studied here were inbreds; this was not true of the other work reported in Table VII. The grain of inbred lines usually has a higher proportion of pigmented hard starch than the grain of hybrids, which may account for the relatively high values obtained in this study. Clark and Gring (3) found an inverse relationship between grain size and carotenoid content. The outstanding differences between the results presented here and those of other workers are the higher values for carotene and the consequently lower cryptoxanthol-carotene ratio.

The method of separation of carotene and cryptoxanthol used here tends to give slightly high results for carotene because of the inclusion of about 10 per cent of the cryptoxanthol in the carotene fraction. Since a corresponding percentage of the carotene is found in the cryptoxanthol fraction, error is introduced by incomplete separation only when the cryptoxanthol-carotene ratio deviates appreciably from unity. The results of Fraps and Kemmerer show ratios of the same order of magnitude as those reported here.

Approximation of the amount of unnamed carotene 1 in the inbreds from  $R_1$  values in Table III gives results varying from 5 to 35 per cent of the total carotene fraction. Fraps and Kemmerer (5) reported K carotene in 22 corn varieties to average 15 per cent of the total carotene fraction.

### Summary

A method is presented for the determination of the carotenoids of corn grain which involves a separation of the pigments into three fractions by partition between immiscible solvents and spectrophotometric estimation of the pigment concentration.

The analysis of the carotene and cryptoxanthol fractions of corn-grain carotenoids in terms of normal and neo-type pigments was not highly successful, but it is probable that analyses of the fractions for total pigment content are reliable at 4325 Å. for the carotene fraction and at 4375 Å. for the cryptoxanthol fraction.

Analysis of the carotenol fraction for luteol, zeaxanthol, and total neocarotenols was made by spectrophotometric methods. Analysis for total pigment in this fraction was made at 4275 Å.

Four inbred corn lines were studied. Variations in content of mature grain were as follows: total carotenols twofold, total carotenes fourfold, total cryptoxanthols sixfold, luteol fourfold, and zeaxanthol twenty-five fold.

### Literature Cited

- (1) Beadle, B. W., and Zscheile, F. P., *J. Biol. Chem.*, **144**, 21 (1942).
- (2) Buxton, L. O., *IND. ENG. CHEM., ANAL. ED.*, **11**, 128 (1939).
- (3) Clark, G. L., and Gring, J. L., *IND. ENG. CHEM., ANAL. ED.*, **9**, 271 (1937).
- (4) Comar, C. L., and Zscheile, F. P., *Plant Physiol.*, **17**, 198 (1942).
- (5) Fraps, G. S., and Kemmerer, A. R., *IND. ENG. CHEM., ANAL. ED.*, **13**, 806 (1941).
- (6) Hogness, T. R., Zscheile, F. P., Jr., and Sidwell, A. E., Jr., *J. Phys. Chem.*, **41**, 379 (1937).
- (7) Kuhn, R., and Brockmann, H., *Ber.*, **66**, 407 (1933).
- (8) Kuhn, R., and Grundmann, C., *Ibid.*, **67**, 593 (1934).
- (9) Peterson, W. J., Hughes, J. S., and Payne, L. F., *Kans. Agr. Expt. Sta., Tech. Bull.* **46** (1939).
- (10) White, J. W., Jr., and Zscheile, F. P., *J. Am. Chem. Soc.*, **64**, 1440 (1942).
- (11) White, J. W., Jr., Zscheile, F. P., and Brunson, A. M., *Ibid.* in press.
- (12) Zscheile, F. P., and Comar, C. L., *Bot. Gaz.*, **102**, 463 (1941).
- (13) Zscheile, F. P., White, J. W., Jr., Beadle, B. W., and Roach, J. R., *Plant Physiol.*, **17**, 331 (1942).

JOINT contribution from the Departments of Agricultural Chemistry and Botany of the Purdue University Agricultural Experiment Station and the Division of Cereal Crops and Diseases, Bureau of Plant Industry, U. S. Department of Agriculture. Journal Paper No. 35 of the Purdue University Agricultural Experiment Station. Studies on the Carotenoids No. 5.