from the technical grade monostearin absorb less moisture than do the products made from the purer or molecularly distilled monostearin.

Comparison of Permeability of Films of Acetostearin Products and Other Types of Coating Materials. There apparently are no data in the literature on the permeability of fats and fatlike products to moisture, but data are available for many other organic compounds. To provide a basis for comparing acetostearin products and other organic compounds, some of the literature data are reproduced in Table V. The permeability constants shown were recalculated to conform to the dimensions used in the present investigation.

It is evident from Tables III and V that the permeability constant for the acetostearin films is less than that of cellulose acetate and only slightly greater than that of nylon, ethylcellulose, polystyrene, and soft vulcanized rubber. Cellophane, polyethylene, and paraffin wax have permeability constants equal to 1/100 or less of those found for the acetostearin products.

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ALFALFA CAROTENOIDS

Xanthophylls in Fresh and Dehydrated Alfalfa

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The carotenoids of fresh and dehydrated alfalfa were separated chromatographically. Five xanthophylls (lutein, violaxanthin, cryptoxanthin, zeaxanthin, and neoxanthin) comprised 99% of the xanthophylls in fresh material. In addition, seven minor bands were present. The same five pigments comprised 87% of the xanthophylls of a dehydrated alfalfa meal. A total of more than 40 xanthophyll bands were shown to be present in dehydrated meal.

DEHYDRATED ALFALFA MEAL or another rich source of xanthophyll is required in chick feeds to give the finished poultry a desirable yellow color. However, until the kinds and amounts of the separate xanthophylls in the feed additives are known and their relative pigmenting value is assayed, it is difficult to make an accurate estimate of the amount of alfalfa or other supplement required in the diet.

The effect of dehydration on the formation of stereoisomers of β-carotene in alfalfa has been reported (*4*, *8*), but the literature reveals no comparable information on the xanthophylls present in fresh and dehydrated alfalfa. There is incomplete agreement in the literature in regard to the nature of the xanthophylls found in fresh leaf tissue. Strain (*7*) in an investigation of the leaves of some 50 different species of plants, observed the following xanthophylls in each: lutein, violaxanthin, neoxanthin, zeaxanthin, and cryptoxanthinlike pigments. Karrer *et al.* (*5*) were unable to confirm the presence of neoxanthin and zeaxanthin in fresh leaves and stated that leaves contain only two principal xanthophylls which they called xanthophyll (lutein) and xanthophyll - epoxide (violaxanthin). More recently, Moster, Quackenbush, and Porter (*6*), working with corn seedlings, confirmed the presence of neoxanthin and zeaxanthin in fresh leaf material. In addition, they found small amounts of other carotenoids hitherto not observed in green leaves. The present paper describes the separation, characterization, and quantitative determination of the xanthophylls present in fresh green alfalfa, and the changes that occur in the xanthophylls during the dehydration process.

Fresh Alfalfa

Extraction The alfalfa employed was a Chilean variety grown in a small plot near the laboratory. Alfalfa was harvested at the prebloom stage, taken immediately to the laboratory, and the pigments were extracted by a slight modification of a method published previously (*3*).

In this method, 30 grams of fresh plant material were blended with 100 ml. of acetone for 1 minute. A 50-ml. aliquot of the acetone solution was added to 50 ml. of hexane and 30 ml. of water in a separatory funnel. Gently swirling the funnel for a minute transferred most of the acetone into the lower water layer which was then withdrawn and dis-

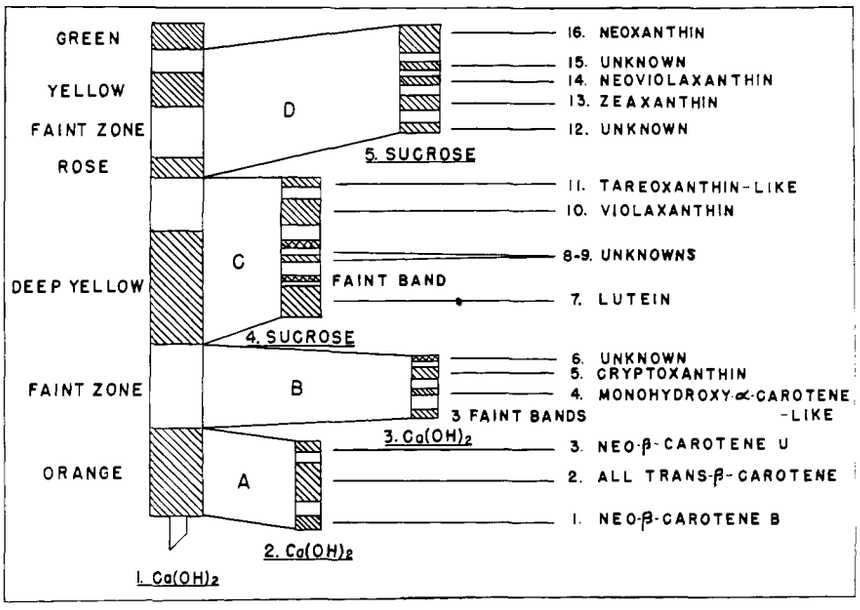


Figure 1. Chromatographic separation of the carotenoids of fresh alfalfa

1 and 3. Developed with hexane-acetone (9 to 1)
2. With hexane–p-cresyl methyl ether (98.5 to 1.5)
4 and 5. With hexane-alcohol (99.5 to 0.5)

carded. The hexane layer was washed several times with water to remove the acetone, after which the extract was ready for chromatography. The interval from harvest to chromatography was less than 15 minutes. To minimize the possibility of isomer formation during the analysis, the extraction and chromatography were performed under dim red light.

Chromatographic Separation

Procedure 1. Two alternative procedures were employed to separate the xanthophylls. Initially, the method of Moster *et al.* (*6*) was followed. This method utilizes a column of calcium hydroxide, as a first step in separation of the pigments, to remove the chlorophylls, separate zeaxanthin from lutein, and remove the xanthophylls from the remaining carotenoids. However, it did not separate the two main pigments, lutein and violaxanthin, from each other.

In this method as modified in this laboratory, 20 ml. of the alfalfa extract in hexane were poured on a column (34 mm. diameter × 500 mm. height) containing a one to one mixture of calcium hydroxide and diatomaceous earth (*7*). The column was developed with a mixture of hexane-acetone (9 to 1) to produce a chromatogram (column 1, Figure 1). The fractions were collected separately by continued development until each zone passed completely through the column. (In the procedure for this chromatogram and those mentioned later, after elution from columns the pigment solutions were washed with water to remove acetone or alcohol in preparation for further chromatographic study.) The carotene fraction, A, was chromatographed on a second lime column (11 × 120 mm.), and developed with 1.5% *p*-cresyl methyl ether. Fraction B was readsorbed on a second lime column using hexane-acetone (9 to 1) as the developer. Fractions C and D were adsorbed on a column (34 mm. diameter × 500 mm. height) containing a one to one mixture of sucrose and diatomaceous earth (*6*). The sucrose was dried under reduced pressure for 24 hours at 90° C. prior to use. The chromatograms were developed with hexane-alcohol (99.5 to 0.5). Each of the zones was removed from the developed column and eluted separately. The pigments were eluted from the sucrose with hexane-alcohol (9 to 1), and rechromatographed on a second sucrose column to assure homogeneity.

Procedure 2. In this procedure the recommendation of Strain (*7*) was followed using a column of sucrose as the first step in the separation of the pigments. It had an advantage over the first procedure in that violaxanthin and neoxanthin were separated without the necessity of rechromatography on a second column. Twenty milliliters of the alfalfa extract in hexane were poured on a column of sucrose (34 × 550 mm.). The column was developed with hexane-alcohol (99.5 to 0.5) which produced the chromatogram shown in Figure 2. Fractions A and B were eluted and collected separately by the flowing chromatographic technique. Fraction A was rechromatographed on lime as in Procedure 1. Fraction B, which included zeaxanthin and cryptoxanthin with the lutein, was readsorbed on a column, 24 mm. in diameter and packed to a height of 24 cm., with a mixture of hydrated lime and diatomaceous earth. The column was developed with hexane-acetone (9 to 1) which separated the minor bands readily. After the elution of fractions A and B, the adsorbent was extruded, and the yellow bands of fraction C carved out. Because the pigments appeared as homogeneous bands on the original sucrose column, it was not necessary to rechromatograph them for further purification. The entire chromatographic procedure could be completed in 1 hour by this technique.

Rechromatography on magnesia, hydrated lime, and powdered sucrose indicated that all isolated fractions con-

Figure 2. Chromatographic separation of the carotenoids of fresh alfalfa

1. Developed with hexane-alcohol (99.5 to 0.5)
2. With hexane–p-cresyl methyl ether (98.5 to 1.5)
3. With hexane-acetone (9 to 1)

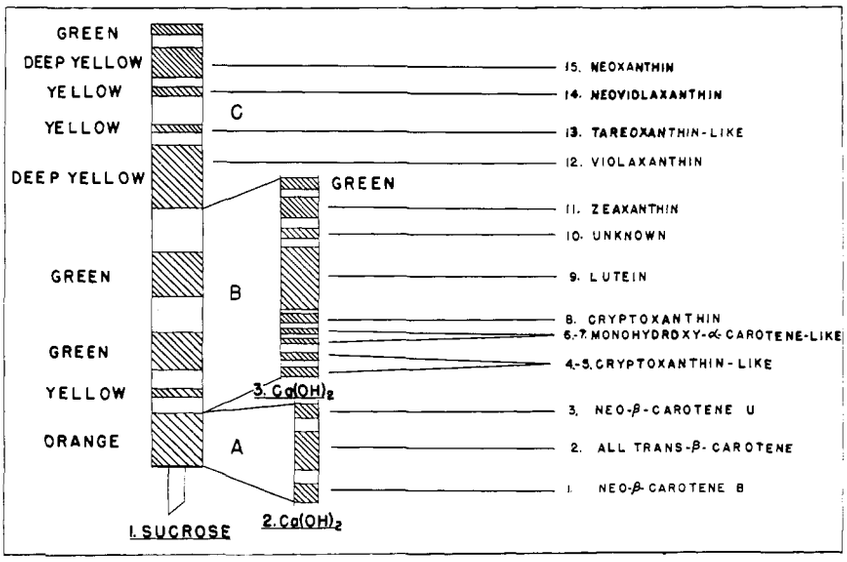


Table I. Various Bands Formed from Neoxanthin During Storage in the Dark

(For 48 hours at room temperature)

Pigment	Maximum		Minimum		Maximum		Minimum		Maximum	
	mμ[a]	I_0/I[b]	mμ	I_0/I	mμ	I_0/I	mμ	I_0/I	mμ	I_0/I
Neoxanthin	412	0.665	422	0.600	436	1.000	453	0.540	466	0.935
1	411	0.665	421	0.585	436	1.000	452	0.505	465	0.940
2	406	0.775	416	0.725	430	1.000	446	0.665	458	0.850
3	420	0.740	424	0.735	443	1.000	461	0.730	472	0.860
4	...	...	...	...	433	1.000	453	0.725	463	0.825
5	420	0.785	425	0.780	443	1.000	461	0.730	471	0.830
6	415	0.730	424	0.705	439	1.000	457	0.655	470	0.930

[a] Actual wave length absorption, mμ.
[b] Corresponding log (I_0/I) values.

sisted of only one band. Lutein, violaxanthin, neoxanthin, cryptoxanthin, and zeaxanthin were identified by position on the column as well as by comparison of their spectrophotometric properties with constants obtained from the literature. Authentic preparations of cryptoxanthin and zeaxanthin aided in the identification of these pigments. Crystalline lutein was prepared from fresh alfalfa at this laboratory. Neoxanthin and violaxanthin were also isolated from fresh alfalfa and purified by chromatography on hydrated lime and sucrose. Their purity was further ascertained by comparison with spectrophotometric data from the literature (*6*).

Quantitative Estimation

The quantitative estimation of the xanthophylls was performed by measuring the absorbance of each of the purified solutions in a suitably standardized colorimeter (*2*). Chromatographically homogeneous solutions of known concentration of each of the five main xanthophylls found in alfalfa were employed to prepare the colorimetric calibration curves. Measurements were made with a filter-type colorimeter at 440mμ. Specific absorption coefficients, taken from the literature (*6*), were used for obtaining the concentration of each solution spectrophotometrically. The calibration curves obtained with lutein, cryptoxanthin, and zeaxanthin were very similar to that of β-carotene. Therefore, it was possible to employ the carotene calibration curve for these xanthophylls. The quantity of the minor pigments, for which no specific absorption coefficients are available, were estimated by utilizing the carotene calibration curve without correction. The relative absorbances for violaxanthin and neoxanthin differ from β-carotene to the extent that assays based on a carotene calibration curve for these two pigments must be multiplied by 120 and 125%, respectively, to obtain the corrected xanthophyll concentration.

Dehydrated Alfalfa

A sample of commercial dehydrated alfalfa meal was used for this work. The preparation of an extract for study from dehydrated alfalfa was similar to a previously published assay method for carotene (*3*). A 10-gram sample of the meal was rehydrated on filter paper with hot water. The sample and filter paper were blended at high speed in an electric blender with 100 ml. of acetone. A 50-ml. aliquot was then taken and transferred to hexane as described under fresh plant tissue. Thirty-five milliliters of the extract was chromatographed on a magnesium oxide-Celite column (34 × 430 mm.). The magnesia employed was special chromatographic No. 2642 obtained from Westvaco Chemical Division, Food Machinery & Chemical Corp., New York, N. Y. The column was developed with hexane-acetone-alcohol (82.5 to 15 to 2.5), which produced the chromatogram shown in Figure 3.

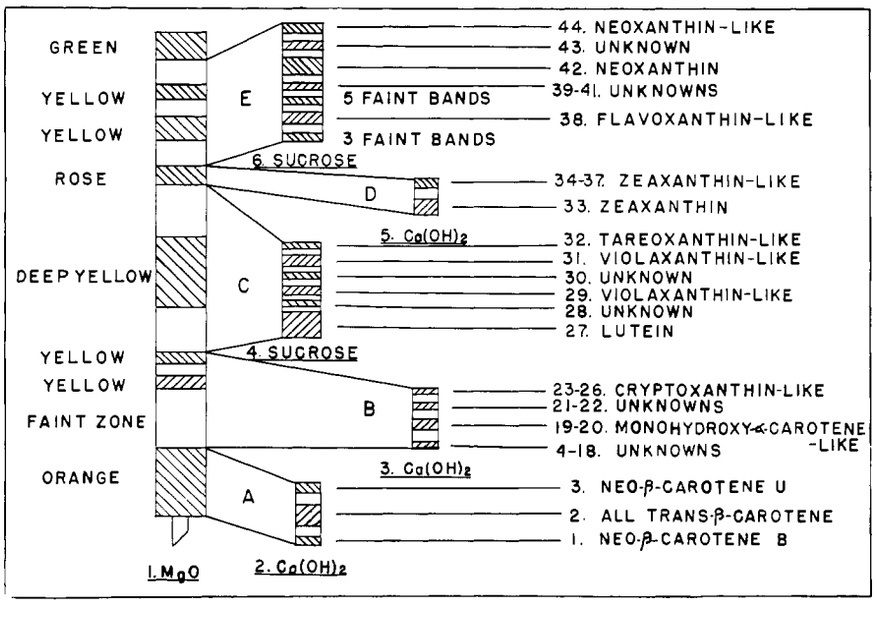
Fractions A, B, C, and D were eluted by continued development of the column. Then the column was extruded, fraction E was carved out and eluted with 100% alcohol. Fraction A was adsorbed on lime–diatomaceous earth as in Procedure 1 for fresh alfalfa. Fractions B and D were readsorbed on columns (24 × 200 mm.) of lime and diatomaceous earth, and developed with hexane-acetone (9 to 1), and fractions C and E, on sucrose–diatomaceous earth columns (34 × 400 mm.), developed with hexane-alcohol (99.5 to 0.5). Each band was removed from the developed column, eluted separately and rechromatographed to assure homogeneity. On completion of all the fractionation steps indicated in Figure 3, known volumes of the individual pigments were prepared and the quantity of each fraction was determined as described for fresh alfalfa. The spectrophotometric curve of each band was obtained with a Cary automatic recording spectrophotometer.

Results

In preliminary studies, the method employed for the preparation of the xanthophyll extracts, prior to the chromatography step, was based on a procedure that had been developed for studying the xanthophylls present in eggs (*9*). This technique involved the treatment of fresh tissue with alcoholic potassium hydroxide in the dark for 16 hours. During the isolation of the xanthophylls from the alfalfa extracts, a very large number of bands were found, many in extremely small quantity. Also, there was a tendency for interconversion of some during the preparative procedure which was caused partly by the ease with which xanthophyll bands form stereo-

Figure 3. Chromatographic separation of the carotenoids of dehydrated alfalfa

1. Developed with hexane-acetone-alcohol (82.5 to 15 to 2.5)
2. With hexane–p-cresyl methyl ether (98.5 to 1.5)
3 and 5. With hexane-acetone (9 to 1)
4 and 6. With hexane-alcohol (99.5 to 0.5)



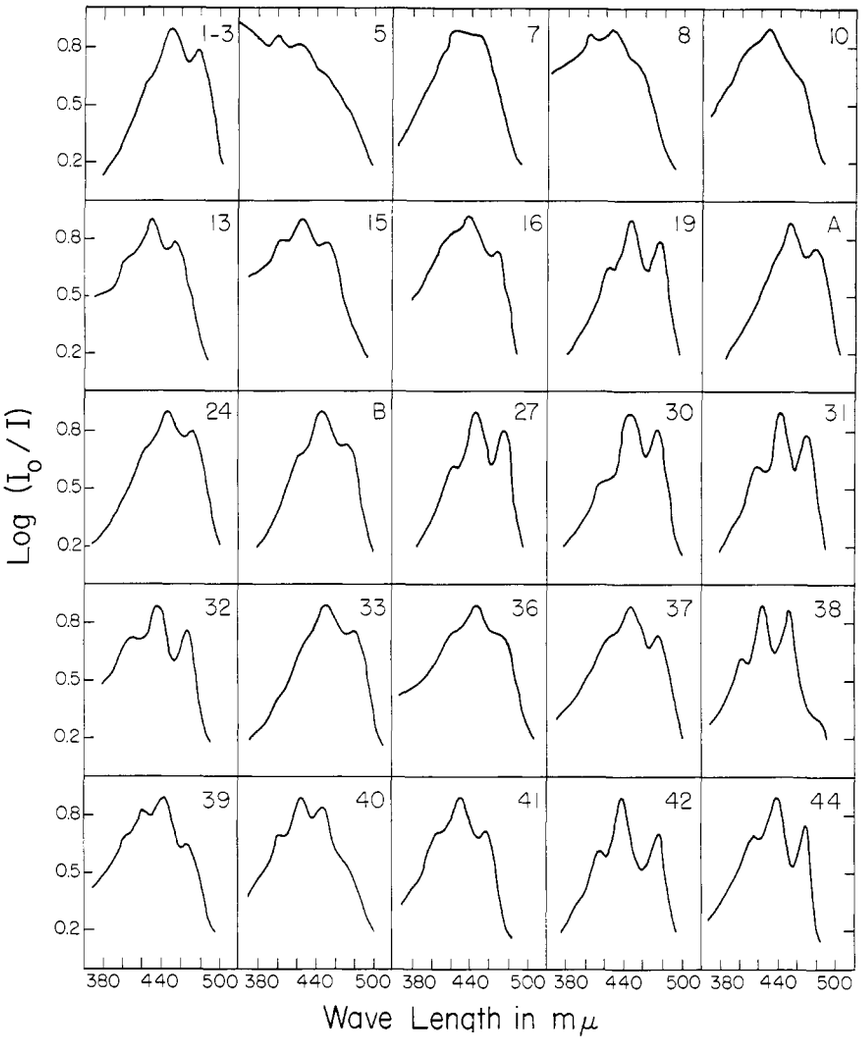


Figure 4. Spectral absorption curves of some carotenoid bands isolated from dehydrated alfalfa meal

isomers. Precautions, such as avoidance of excessive light or heat on the samples during the preparation, working rapidly, and keeping manipulations to a minimum, tended to lessen this isomerization. Because many of the bands found by this technique may have been artifacts or stereoisomers not originally present in fresh plant tissue, less drastic methods of extraction were sought. Moster *et al.* (*6*) described the preparation of carotenoids from corn seedlings in which the pigments were extracted with organic solvents, and only a portion of the extract was treated with alkali. However, they permitted their solution to stand for 18 hours prior to chromatography. By their chromatographic technique, they were able to distinguish 31 separate yellow bands in addition to β-carotene and its isomers. Many of these were present in extremely small amounts and were not further characterized. By using their suggested chromatographic adsorption analysis technique, which involved preliminary separation on hydrated lime and followed by adsorption on sugar, 18 to 25 different bands were obtained in this laboratory at various times. Some of these bands were present in such extremely small amounts that there was insufficient material to obtain their spectrophotometric properties.

When chromatographically homogeneous bands of the main pigments were stored in the dark, additional bands formed in all cases. The results of one storage experiment are presented in Table I. All the additional bands shown were developed from the neoxanthin during storage in the dark. Strain showed that most of the xanthophylls present in fresh leaves could be separated by chromatography on sugar (*7*). When the extract, obtained from fresh alfalfa tissue by the rapid method, was chromatographed on a sugar–diatomaceous earth column according to Procedure 2, the column developed very rapidly (less than an hour). Further reduction in the formation of stereoisomers was accomplished by wrapping the entire column in dark paper during chromatography. Under these conditions, 12 xanthophyll bands were found to be present in fresh alfalfa (Table II). Five pigments make up about 99% of the total xanthophyll. The seven additional bands, present in very small

Table II. Xanthophylls Found in Fresh Alfalfa

Name	% of Total Xanthophylls	Pigment Number	Maximum		Minimum		Maximum		Minimum		Maximum	
			mμ[a]	I_0/I[a]	mμ	I_0[b]/I	mμ	I_0/I	mμ	I_0/I	mμ	I_0/I
Cryptoxanthinlike	0.2	4					447	1.000	465	0.810	474	0.840
Cryptoxanthinlike	0.2	5					443	1.000	464	0.890	469	0.895
Monohydroxy-α-carotene-like	0.2	6	422	0.780	427	0.780	445	1.000	462	0.770	473	0.855
Monohydroxy-α-carotene-like	0.2	7	421	0.705	426	0.700	444	1.000	461	0.695	473	0.870
Cryptoxanthin	4	8					447	1.000	465	0.795	473	0.820
Lutein	40	9	422	0.680	427	0.680	447	1.000	463	0.715	476	0.895
Unknown	0.2	10	419	0.720	426	0.705	443	1.000	460	0.685	472	0.880
Violaxanthin	34	11	417	0.690	425	0.635	441	1.000	459	0.580	471	0.950
Zeaxanthin	2	12					449	1.000	466	0.790	477	0.865
Tareoxanthinlike	0.2	13	412	0.725	421	0.685	436	1.000	453	0.605	465	0.860
Neovioleoxanthin	0.2	14	414	0.755	419	0.750	436	1.000	452	0.670	465	0.850
Neoxanthin	19	15	412	0.665	422	0.600	436	1.000	453	0.540	466	0.935

[a] Wave length absorption, mμ.
[b] Corresponding log I_0/I values.

Table III. Quantitative Estimation of Carotenoids Found in Dehydrated Alfalfa

Name	% of Total Carotenoids	Pigment Number	Maximum		Minimum		Maximum		Minimum		Maximum	
			mμ[a]	I_0/I[b]	mμ	I_0/I	mμ	I_0/I	mμ	I_0/I	mμ	I_0/I
β-Carotene	22.6											
Unknowns 4–18[c]	0.5											
Monohydroxy-α-carotene-like	0.1	19	421	0.715	427	0.710	446	1.000	463	0.705	475	0.885
Monohydroxy-α-carotene-like	0.1	20	421	0.770	427	0.765	445	1.000	463	0.725	474	0.845
Unknown	0.1	21					441	1.000	458	0.840	468	0.955
Unknown	0.1	22	423	0.725	426	0.720	444	1.000	461	0.745	472	0.875
Cryptoxanthinlike	1.2	23					444	1.000	463	0.765	471	0.790
Cryptoxanthinlike	0.9	24					442	1.000	463	0.840	468	0.845
Cryptoxanthinlike	1.5	25					446	1.000	463	0.855	472	0.880
Cryptoxanthinlike	1.4	26					445	1.000	463	0.860	471	0.870
Lutein	35.3	27	420	0.685	426	0.675	445	1.000	461	0.685	474	0.890
Unknown	0.7	28	422	0.760	426	0.760	443	1.000	459	0.770	472	0.890
Violaxanthinlike	3.6	29	416	0.705	424	0.700	440	1.000	458	0.655	470	0.920
Unknown	1.0	30	423	0.730	427	0.730	443	1.000	461	0.745	473	0.895
Violaxanthinlike	8.5	31	416	0.685	423	0.670	440	1.000	456	0.665	468	0.870
Tareoxanthinlike	1.9	32	410	0.840	419	0.830	436	1.000	454	0.665	466	0.835
Zeaxanthin	1.5	33					451	1.000	469	0.825	478	0.845
Zeaxanthinlike	1.2	34					450	1.000				
Zeaxanthinlike	0.2	35					446	1.000	464	0.830	472	0.840
Zeaxanthinlike	0.8	36					445	1.000				
Zeaxanthinlike	0.6	37					448	1.000	467	0.770	474	0.800
Flavoxanthinlike	1.7	38	400	0.690	407	0.665	423	1.000	437	0.725	449	0.980
Unknown	1.8	39	420	0.920	428	0.845	442	1.000	460	0.710	466	0.735
Unknown	0.5	40	403	0.785	408	0.780	424	1.000	437	0.875	447	0.935
Unknown	1.0	41	409	0.790	413	0.785	429	1.000	446	0.755	457	0.800
Neoxanthin	10.5	42	414	0.700	422	0.665	437	1.000	454	0.585	465	0.825
Unknown	0.2	43	420	0.855	425	0.850	439	1.000	458	0.675	468	0.780
Neoxanthinlike	0.5	44	416	0.765	422	0.745	437	1.000	455	0.620	466	0.820

[a] Wave length absorption, mμ.
[b] Corresponding log (I_0/I) values.
[c] Probably oxidation products of carotene.

quantities, may be isomers formed during the extraction and chromatographic procedure. Relative amounts of major xanthophylls found in fresh alfalfa under these conditions are shown in Table II.

Chromatographic separation of the xanthophylls of dehydrated alfalfa was considerably more difficult than the separation of those of the fresh alfalfa. Sucrose proved unsuitable for the initial chromatography of the extract because the number of chlorophyll degradation products obscured most of the yellow xanthophylls. Therefore, it was necessary to make a preliminary separation on magnesia (Figure 3), followed by readsorption on lime or sucrose of the separated fractions. By this procedure, 41 bands, in addition to the carotene, were separated and spectrophotometric absorption curves for each were determined (Table III).

Eight additional bands were observed but were present in too small quantities for separation and study. The five main xanthophylls, shown in Table III, comprised 87% of the total xanthophylls of dehydrated alfalfa. Each of the totals of violaxanthin, zeaxanthin, neoxanthin, and cryptoxanthin include its probable isomers. The close relationship of these isomers to the parent pigment can be verified by Figure 4, which includes absorption curves of some of the pigments present in very small quantities as well as those of the major xanthophylls. Among these, an isomer (B, Figure 4), prepared from authentic cryptoxanthin (A, Figure 4), is comparable to the isolated cryptoxanthinlike isomers. No xanthophyll comparable to a flavoxanthinlike pigment and isolated from corn seedlings by Moster (*6*) was found in fresh alfalfa, but this pigment was isolated from the dehydrated meal (band 38). In the dehydrated alfalfa, 15 faint bands, comprising less than 0.3% of the total yellow color, were found between the neo-β-carotene U and the monohydroxy - α - carotenelike carotenoids. The spectral absorption curves of a number of these bands are given in Figure 4.

Two violaxanthinlike xanthophylls (bands 29 and 31) were isolated from the dehydrated meal. Below each was a related pigment band which appeared similar to what was termed neolutein by Moster (*6*).

No bands were found on the column above neoxanthin in the fresh plant extract. However, two bands were isolated above neoxanthin from dehydrated alfalfa, one of which (band 44) may be an isomer of neoxanthin.

Acknowledgment

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