# Xanthophyll and Carotene Storage Stability in Commercially Dehydrated and Freeze-Dried Alfalfa

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Alfalfa commercially dehydrated to yield meals with different moisture contents and freeze-dried alfalfa were subjected to accelerated storage conditions and analyzed for xanthophylls and carotene at periods extending to 12 weeks. Individual xanthophylls were determined by thin-layer chromatography. Meals which had undergone the lowest losses during dehydration suffered the highest losses during storage. Freeze-dried samples were the most stable. Ethoxyquin treatment reduced oxidative losses but isomerization losses of neoxanthin and violaxanthin continued.

Research on stability of alfalfa carotenoids during storage has been largely concerned with carotene stability (Griffith and Thompson, 1949; Silker *et al.*, 1944; Taylor and Russell, 1938; Van Der Veen and Olcott, 1967). The importance of xanthophyll content has grown, however, as alfalfa is increasingly used in poultry rations to provide desired egg and skin pigmentation. Thompson and Maclay (1952) studied xanthophyll retention in commercially dehydrated alfalfa stored at ambient temperature and concluded that xanthophyll was retained better than carotene. Livingston *et al.* (1955) found that during storage of alfalfa-containing mixed feeds, xanthophyll losses were decreased by the addition of ethoxyquin or oils. Dua *et al.* (1965) stated that "xanthophylls generally are thought to be more stable than carotenes," but did not find differences in their relative losses in a storage study on yellow corn, although Quackenbush (1963) had found such differences in stored corn. Fritz and Wharton pointed out (1957) that "xanthophyll—i.e., lutein—is very unstable in dry products" and that "this and the assay problems make potency guarantees impractical and misleading." However, industry adoption of inert gas storage and antioxidant (ethoxyquin) treatment of alfalfa meal as earlier advocated (Graham, 1944; Hoffman *et al.*, 1945; Livingston *et al.*, 1955; Thompson, 1951), has improved storage stability, and development of improved analytical procedures (Kohler *et al.*, 1967; Nelson and Livingston, 1967) has made possible more precise and detailed evaluation of storage changes.

Pilot-scale dehydrator trials by this laboratory demonstrated that loss of xanthophyll during alfalfa dehydration is directly related to outlet temperature and inversely related to moisture content of the dehydrated material (Livingston *et al.*, 1966). In a further study these conclusions were confirmed using two commercial dehydrators (Livingston *et al.*, 1968). In that study carotene loss was lowest in the highest-moisture meals. Because the highest retention occurred under processing conditions outside the normal limits employed in industrial operations, the stability of the alfalfa meal carotenoids during storage was subsequently studied in more detail.

## EXPERIMENTAL

Meals produced in two types of commercial dryers were selected. In each dryer alfalfa was dehydrated to three moisture levels, approximately normal, above normal, and below normal for the industry. Outlet temperatures were varied in the operation of one dryer and held constant in the other. To demonstrate storage effects attributable to the dehydration process, samples of the fresh alfalfa were also freeze-dried (RePP sublimator Model 15 FFD, RePP Industries, Inc., Gardiner, N. Y.), ground, and stored with the dehydrated meals, thus serving as optimally dehydrated samples. In addition, 0.15% ethoxyquin by weight (Livingston *et al.*, 1955), dissolved in a solvent, was added to one freeze-dried meal and to one low-moisture, commercially dehydrated meal to assess the amount of oxidative storage loss taking place in untreated meals. Moistures were determined on all samples before storage and analyses were calculated on a dry solids basis.

Two-gram samples of the various alfalfa meals were stored in the dark in open vials at 90° F. After 3, 6, and 12 weeks of storage, duplicate samples of each were analyzed for total carotene and xanthophylls by the method of Kohler *et al.* (1967) and for individual xanthophylls (neoxanthin, violaxanthin, lutein, and three lutein isomers) (Table I) by the thin-layer chromatographic (TLC) method of Nelson and Livingston (1967). The amount of each xanthophyll present at the end of each storage period was calculated from the percentages of individual xanthophylls and the total xanthophyll found.

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Table I. Loss of Carotene and Xanthophyll during Storage

| Alfalfa Preparation and % Moisture | Content before Storage, Mg/Kg. C[a] | N | V | L | Total xanth. | % Loss at 6 Weeks C | N | V | L | Total xanth. | % Loss at 12 Weeks C | N | V | L | Total xanth. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Arnold dehydrator | | | | | | | | | | | | | | | |
| Freeze-dried control, 3 trials | 362 | 91 | 130 | 494 | 788 | 33 | 14 | 66 | 26 | 31 | 47 | 22 | 84 | 28 | 38 |
| Dehydrated meal | | | | | | | | | | | | | | | |
| 9.2% | 342 | 55 | 15 | 346 | 508 | 64 | 56 | 100 | 54 | 53 | 78 | 56 | 100 | 65 | 64 |
| 7.8% | 269 | 40 | 29 | 222 | 368 | 55 | 50 | 100 | 36 | 43 | 68 | 44 | 100 | 47 | 52 |
| 2.3% | 291 | 11 | 29 | 159 | 286 | 51 | 20 | 100 | 42 | 37 | 66 | 29 | 100 | 53 | 52 |
| Stearns-Roger dehydrator | | | | | | | | | | | | | | | |
| Freeze-dried control, 3 trials | 364 | 97 | 174 | 493 | 825 | 25 | | 77 | | 28 | 40 | 21 | 82 | 25 | 37 |
| Ethoxyquin-treated | 282 | 66 | 135 | 353 | 598 | 39 | 44 | 77 | 14 | 32 | 43 | 57 | 82 | 22 | 39 |
| Dehydrated meal | | | | | | | | | | | | | | | |
| 12.2% | 353 | 82 | 46 | 350 | 574 | 72 | 65 | 100 | 53 | 60 | 84 | 70 | 100 | 69 | 71 |
| 7.1% | 342 | 64 | 33 | 306 | 484 | 71 | 62 | 100 | 53 | 57 | 84 | 65 | 100 | 67 | 68 |
| 2.5% | 293 | 35 | 20 | 207 | 346 | 59 | 44 | 100 | 29 | 38 | 76 | 44 | 100 | 48 | 52 |
| 2.5%, ethoxyquin-treated | 309 | 40 | 22 | 232 | 388 | 25 | 39 | 100 | 7 | 19 | 37 | 61 | 100 | 17 | 31 |

[a] C = total carotene; N = neoxanthin; V = violaxanthin; L = lutein.

[Relative humidity of the storage room was 25 to 35%. Bailey *et al.* (1949) found that for this relative humidity, equilibrium moisture content of alfalfa meal is 4 to 5%; they also showed that 5 to 8% moisture content is optimum for carotene retention during storage of open alfalfa meal samples. In the present study, moisture equilibration of the stored alfalfa samples undoubtedly occurred, tending to improve the storage stability of both low- and high-moisture meals. Nevertheless, the storage differences found should be closely related to the composition of the meals as a result of dehydration treatment.]

## RESULTS AND DISCUSSION

**Carotene Stability.** Retention of carotene during storage was highest in freeze-dried alfalfa, by a large margin. Ethoxyquin treatment after freeze-drying did not improve this excellent storage stability. In the commercially dehydrated alfalfa, carotene storage stability was 50% higher in the low-moisture meals than in the highest-moisture meals. Ethoxyquin treatment of one low-moisture dehydrated meal resulted in greatly improved carotene stability, comparable to that of freeze-dried alfalfa. Carotene was less stable than xanthophyll in every case, for the 12-week period, although the differences were more pronounced in dehydrated meals than in freeze-dried alfalfa.

**Xanthophyll Stability in Freeze-Dried Alfalfa Meals.** Freeze-dried alfalfa meal showed higher xanthophyll retention than the most stable of the commercially dehydrated meals (Figure 1). Total xanthophyll losses during 12 weeks' storage of freeze-dried alfalfa averaged 38%, compared with losses of 52 to 71% in the dehydrated meals. Although total xanthophyll losses in the former were high (milligrams per kilogram), the xanthophyll content at the end of storage was still nearly equal to that of the best dehydrated meals before storage exposure. Two thirds of the loss in freeze-dried alfalfa occurred during the first 3-week period, while in the dehydrated meals a smaller proportion (26 to 60%) of the over-all loss occurred during that period. An explanation for this difference in initial rate of loss was sought. Furthermore, as there are significant differences in the pigmenting potency of various xanthophylls (Fritz and Wharton, 1957; Kuzmicky *et al.*, 1968; Marusich *et al.*, 1960; Quackenbush *et al.*, 1965), it is important to know how much each of the major xanthophylls is involved in the total loss.

Individual xanthophyll losses in freeze-dried meals were, therefore, determined by TLC analysis. Loss of violaxanthin was most rapid, 83% of that originally present being lost, largely during the first 3 weeks. Since violaxanthin comprised about 20% of the original total, its instability contributed significantly to the early loss of total xanthophylls in stored freeze-dried alfalfa. By contrast, neoxanthin proved the most stable during storage, averaging only 21% loss for the 12-week period. Lutein storage losses were significantly lower than the over-all average, 27% being lost in the 12-week period. This

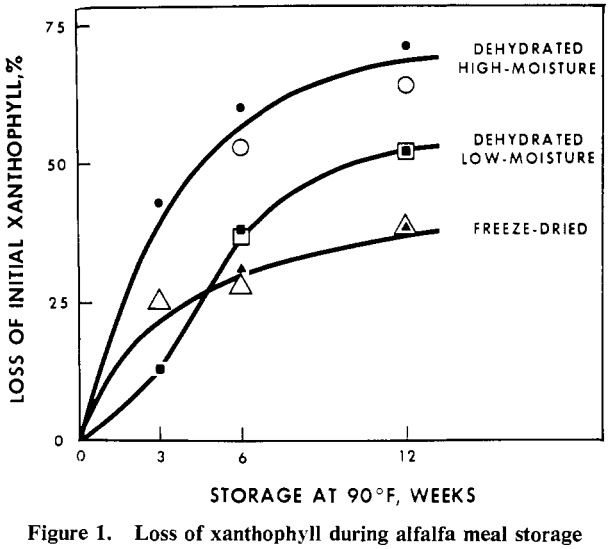


**Figure 1. Loss of xanthophyll during alfalfa meal storage**

Stearns-Roger dehydrator trials. ●, ▲, ■
Arnold dehydrator trials. ○, △, □

Table II. Visible Spectra of Xanthophylls of Stored Alfalfa

| Treatment | Xanthophyll[a] | $\lambda_{max}$, Mμ, in Hexane-Acetone, 7 to 3 Initial | After 3 weeks | After 6 weeks | After 12 weeks |
|---|---|---|---|---|---|
| Freeze-dried | N | 437, 465 | 436–7, 465 | 437, 465 | 437, 466 |
| | V | 441, 471 | 445.5, 470 | 445, 470.5 | 446, 470.5 |
| | L | 446, 474.5 | 446, 474.5 | [b], 475 | 447, 475 |
| Freeze-dried + ethoxyquin | N | [b] | 436, 464 | 445, 465 | 447, 465 |
| | V | [b] | 445, 466 | 439, 467 | 440, 468 |
| | L | [b] | 446, 474 | 446.5, 475 | 446, 474.5 |
| Meal | N | 445, 465 | 440, 463 | 435–45, 465 | 445, 465 |
| | V | 435–40, 467 | None | None | None |
| | L | 445, 474 | 446, 474.5 | 446, 474.5 | 445.5, 474.5 |
| Meal + ethoxyquin | N | [b] | 445, 465 | 435–45, 464 | 422, 449 |
| | V | [b] | None | None | None |
| | L | [b] | 446, 474 | 446, 474 | 447, 475 |

[a] See Table I for letter abbreviations. [b] Not recorded.

is important, since lutein comprised 61% of the xanthophyll present at the start of storage and is the most potent pigmenter of the alfalfa xanthophylls (Kuzmicky *et al.*, 1968).

**Xanthophyll Stability in Commercially Dehydrated Meals.** Although xanthophyll storage losses in the commercially dehydrated alfalfa meals were more difficult to interpret, because of the larger amount of isomers present, certain definite trends appeared. Total xanthophyll losses in dehydrated meals were 30 to 100% greater than in the corresponding freeze-dried samples. These losses were consistently high in the highest-moisture meals and low in the lowest-moisture meals. The normal, intermediate-moisture meals from the two dehydrators showed storage losses 38 and 90% greater than losses in freeze-dried meals. The lower dehydrator outlet temperature (250° C.) used in the latter case may help to explain the larger loss but the fate of individual xanthophylls in these meals is also informative.

TLC analyses for individual xanthophylls in dehydrated meals revealed that all the initial violaxanthin present was lost within the first 3 weeks of storage. Neoxanthin was less stable in dehydrated meal than in freeze-dried alfalfa. It showed greater loss at 6 weeks than lutein or the total xanthophylls. However, little neoxanthin loss occurred thereafter, and at the end of 12 weeks, it ranked slightly above the other xanthophylls in storage stability. The final amount of neoxanthin was small in all samples. Lutein percentage losses were generally lower than total xanthophyll losses after both 6- and 12-week storage periods. Both neoxanthin and lutein showed the tendency to higher storage losses in the high-moisture meals; percentage losses were higher in the 250° C. than in the 310° C. outlet-temperature meals.

**Xanthophyll Stability in Ethoxyquin-Treated Meals.** In freeze-dried alfalfa addition of ethoxyquin did not enhance storage stability of violaxanthin, lutein, or total xanthophyll, and higher neoxanthin losses were measured after 12 weeks' storage. Similar antioxidant treatment of low moisture commercially dehydrated meal failed to prevent loss of violaxanthin, but reduced loss of lutein, 65% and total xanthophylls, 40% during 12 weeks. It again permitted a higher neoxanthin percentage loss at 12 weeks, although the loss in milligrams was low. The antioxidant preservative effect on lutein is far more significant than the increased rate of loss of neoxanthin.

**Storage Changes Suggested by Visible Spectra of Xanthophylls.** The visible spectra of the individual xanthophyll fractions isolated by TLC (Table II) were examined qualitatively, and an attempt was made to relate the observed changes to storage losses. For comparison, the visible spectra of purified preparations of the xanthophylls discussed are presented in Figures 2 and 3. These preparations were obtained from alfalfa extracts by column or thin-layer chromatography and by HCl-isomerization of neoxanthin and luteoxanthin, and spectra were taken in hexane-acetone 7 to 3.

Freeze-dried alfalfa showed no apparent spectral change in the neoxanthin or lutein fractions following storage, but the increased spectral absorbance of the violaxanthin fraction in the 445-mμ region may indicate considerable isomerization of violaxanthin (5,6:5′,6′-diepoxy-5,5′,6,6′-tetrahydro-β-carotene-3,3′-diol) to luteoxanthin(5,6:5′,8′-diepoxy-5,5′,6,8′-tetrahydro-β-carotene-3,3′-diol) (Chichester and Nakayama, 1965; Curl and Bailey, 1954; Glover and Redfearn, 1953; Strain, 1954) during the first 3-week period (Figure 4). Loss of neoxanthin and lutein, and of violaxanthin after the first storage period, appears therefore to have been due largely to oxidation rather than to isomerization. The fairly slow rate of loss was probably due to the natural antioxidants present (Griffith and Thompson, 1949; Polesello and Vistarini, 1966).

Addition of ethoxyquin in solvent to freeze-dried alfalfa may have permitted some isomerization of the partially dissolved xanthophylls before the solvent evaporated (Weedon, 1965). The violaxanthin fraction was mixed, even at 3 weeks' storage (apparently with luteoxanthin); surprisingly, the spectra became more characteristic of pure violaxanthin as storage proceeded. This could be

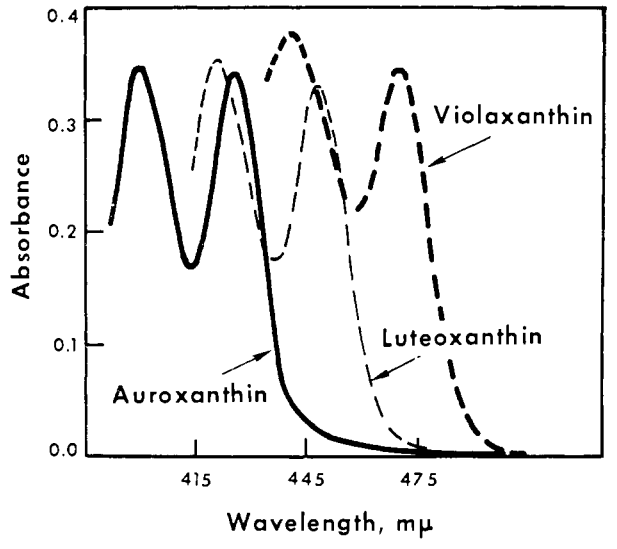


Figure 2. Visible spectra of violaxanthin, luteoxanthin, and auroxanthin

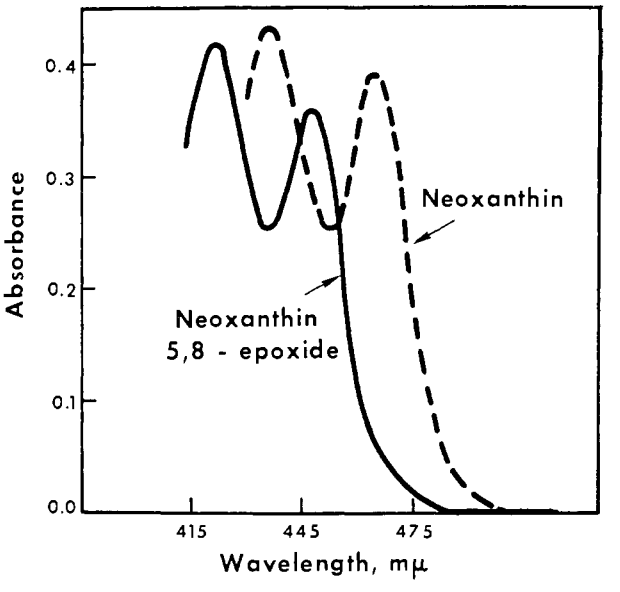


Figure 3. Visible spectra of neoxanthin and neoxanthin 5,8-epoxide

explained by isomerization of luteoxanthin to auroxanthin (5,8:5′,8′ - diepoxy - 5,5′,8,8′ - tetrahydro - β - carotene-3,3′-diol) (Curl and Bailey, 1954; Goodwin, 1958; Karrer and Jucker, 1945), which would not alter the absorbance at 475 mμ but would shift the spectrum in the 445-mμ region as observed. The neoxanthin fraction appeared to be reasonably pure at 3 weeks' time but showed spectral changes—viz., increased absorbance at 447 mμ—with further storage, suggesting partial isomerization to the 5,8-monoepoxide (Figure 5). This conversion decreases the absorbance measured at 465 mμ and the calculated per cent of neoxanthin present. The failure to demonstrate an over-all protective effect on xanthophylls by ethoxyquin treatment is probably due, therefore, to natural antioxidant protection in the freeze-dried alfalfa, and to isomerization losses which are not prevented by ethoxyquin.

In the untreated commercially dehydrated meal, violaxanthin, as previously noted, was absent after 3 weeks' storage. While oxidative changes were probably also involved, the TLC analyses indicated formation of isomers with altered visible spectra and chromatographic $R_f$'s. The neoxanthin fractions apparently contained the 5,8-epoxide isomer, as evidenced by an increase in the absorbance at 440 to 445 mμ. This mixed fraction was apparently stable and persisted throughout the storage period. Little further loss of neoxanthin occurred after the first 6 weeks. Visible spectra of the lutein fractions were characteristic of pure lutein throughout the storage periods.

In the ethoxyquin-treated dehydrated meal, violaxanthin was absent following storage, only its isomers being found. Neoxanthin in this meal was mixed, probably with its 5,8-epoxide isomer, from 3 weeks onward, and the 12-week "neoxanthin" fraction spectrum was almost entirely characteristic of the 5,8-isomer. Little or no oxidative loss need be inferred in either case, particularly in view of the excellent protection of lutein against oxidative loss in this sample. (The all-*trans* lutein showed no evidence of isomerization during storage: The fractions isolated showed very good spectral homogeneity regardless of source or treatment, and the three minor isomer fractions determined by TLC could not account for any significant loss of lutein. Therefore, those losses which occurred in untreated lutein were oxidative, but ethoxyquin provided good protection against such loss.) Thus in commercially dehydrated meal, ethoxyquin apparently protected neoxanthin and violaxanthin against oxidative

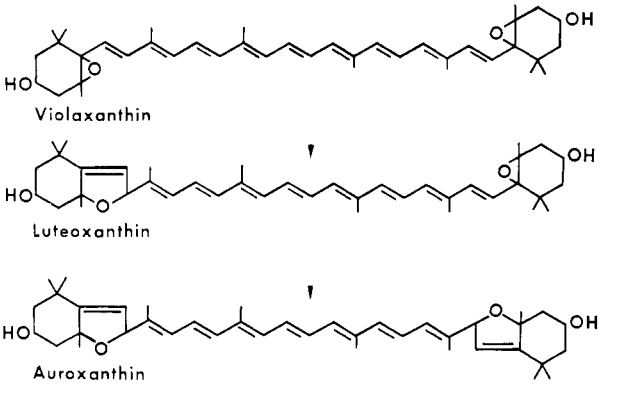


Figure 4. Isomerization of violaxanthin and luteoxanthin

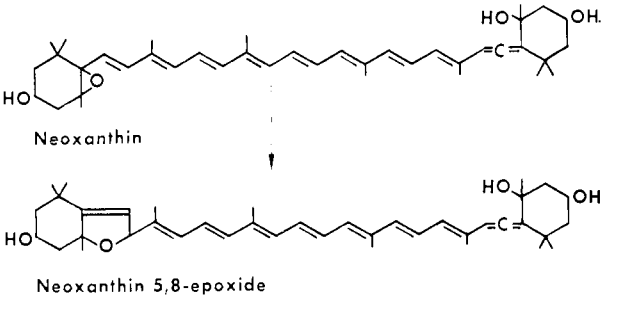


Figure 5. Isomerization of neoxanthin

Table III. Loss of Carotene and Xanthophyll during Dehydration Plus Storage

| Moisture in Meal, % | Outlet Temp., °F. | Carotenoids in Fresh Alfalfa, Mg./Kg. | | | Carotenoids Following Dehydration | | | | | | Carotenoids Following Dehydration Plus 12 Weeks' Storage | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Remaining, Mg./Kg. | | | Loss, % in Fresh | | | Remaining, Mg./Kg. | | | Loss, % in Fresh | | |
| | | Carotene | Total xanth. | Lutein | Carotene | Total xanth. | Lutein | Carotene | Total xanth. | Lutein | Carotene | Total xanth. | Lutein | Carotene | Total xanth. | Lutein |
| Arnold Dehydrator | | | | | | | | | | | | | | | | |
| 9.2 | 300 | 377 | 834 | 550 | 342 | 508 | 346 | 9 | 39 | 37 | 75 | 185 | 121 | 80 | 78 | 78 |
| 7.8 | 310 | 355 | 735 | 425 | 269 | 368 | 222 | 24 | 50 | 48 | 86 | 176 | 119 | 76 | 76 | 72 |
| 2.3 | 330 | 353 | 796 | 508 | 291 | 286 | 159 | 18 | 64 | 69 | 97 | 136 | 75 | 73 | 83 | 85 |
| Stearns-Roger Dehydrator | | | | | | | | | | | | | | | | |
| 12.2 | 250 | 342 | 800 | 480 | 353 | 574 | 350 | +3 | 28 | 27 | 53 | 167 | 108 | 85 | 79 | 78 |
| 7.1 | 250 | 412 | 862 | 517 | 342 | 484 | 306 | 17 | 44 | 41 | 57 | 156 | 101 | 86 | 82 | 80 |
| 2.5 | 250 | 337 | 814 | 482 | 293 | 346 | 207 | 13 | 58 | 57 | 68 | 165 | 108 | 80 | 80 | 78 |

deterioration during storage, but isomerization of these epoxide-containing xanthophylls occurred and was responsible for most of their apparent losses.

**Total Carotene and Xanthophyll Losses during Dehydration and Storage.** During unprotected storage of commercially dehydrated alfalfa meal, carotene and xanthophyll losses rank in the same order as moisture contents of the dehydrated meals, whereas the reverse order had been found, generally, with respect to losses during dehydration. For practical purposes it is important to know the total loss of alfalfa carotenoids during both dehydration and storage (Table III). Carotene over-all losses were highest finally in the high-moisture meals; likewise, the favorable effects on total xanthophyll content produced by dehydrating alfalfa to higher meal moisture contents were almost completely compensated for by opposite storage effects. The same was true of the most valuable single poultry pigmenter in alfalfa, lutein, during unprotected storage.

For practical purposes, the study shows that carotene and xanthophyll preserved by careful dehydration, maintaining adequate minimum moisture in the meal—e.g. 7 to 9%—can readily be lost during unprotected storage. The value of antioxidant protection, as by addition of ethoxyquin, or of inert gas storage, is emphasized. However, a means for preventing isomerization losses has yet to be found.

## LITERATURE CITED


Bailey, G. F., Atkins, M. E., Bickoff, E. M., *Ind. Eng. Chem.* **41,** 2033 (1949).

Chichester, C. O., Nakayama, T. O. M., "Chemistry and Biochemistry of Plant Pigments," p. 451, T. W. Goodwin, Ed., Academic Press, New York, 1965.

Curl, A. L., Bailey, G. F., J. AGR. FOOD CHEM. **2,** 685 (1954).

Dua, P. N., Day, E. J., Grogan, C. O., *Agron. J.* **57,** 501 (1965).

Fritz, J. C. Wharton, F. W., Jr., *Poultry Sci.* **36,** 1118, Abstract, (1957).

Glover, J., Redfearn, E. R., *Biochem. J.* **54,** *Proc. Biochem. Soc.*, p. viii (1953).

Goodwin, T. W., *Biochem. J.* **68,** 503 (1958).

Graham, W. R., Jr. (to U. S. Dept. of Agr.), U. S. Patent **2,351,853** (1944).

Griffith, R. B., Thompson, C. R., *Botan. Gaz.* **111** (2), 165 (1949).

Hoffman, E. J., Lum, F. G., Pitman, A. L., *J. Agr. Res.* **71,** 361 (1945).

Karrer, P., Jucker, E., *Helv. Chim. Acta* **28,** 427 (1945).

Kohler, G. O., Knowles, R. E., Livingston, A. L., *J. Assoc. Offic. Anal. Chemists* **50** (3), 707 (1967).

Kuzmicky, D. D., Kohler, G. O., Livingston, A. L., Knowles, R. E., Nelson, J. W., *J. Poultry Sci.*, in press, 1968.

Livingston, A. L., Bickoff, E. M., Thompson, C. R., J. AGR. FOOD CHEM. **3,** 439 (1955).

Livingston, A. L., Knowles, R. E., Israelsen, M., Nelson, J. W., Mottola, A. C., Kohler, G. O., J. AGR. FOOD CHEM. **14,** 643 (1966).

Livingston, A. L., Knowles, R. E., Nelson, J. W., Kohler, G. O., J. AGR. FOOD CHEM. **16,** 84 (1968).

Marusich, W., DeRitter, E., Bauernfeind, J. C., *Poultry Sci.* **39,** 1338 (1960).

Nelson, J. W., Livingston, A. L., *J. Chromatog.* **28,** 465 (1967).

Polesello, A., Vistarini, S., Proc. VI Intern. Symp. Agr. Chem., p. 104, Varenna, Italy, September 1966.

Quackenbush, F. W., *Cereal Chem.* **40,** 266 (1963).

Quackenbush, F. W., Kvakovszky, S., Hoover, T., Kogler, J. C., *J. Assoc. Offic. Anal. Chemists* **48** (6), 1241 (1965).

Silker, R. E., Schrenk, W. G., King, H. H., *Ind. Eng. Chem.* **36,** 831 (1944).

Strain, H. H., *Arch. Biochem. Biophys.* **48,** 458 (1954).

Taylor, M. W., Russell, W. C., *J. Nutr.* **16** (1), 1 (1938).

Thompson, C. R. (to U.S. Dept. of Agr.), U.S. Patent **2,562,970** (1951).

Thompson, C. R., Maclay, W. D., *Feed Age* **2** (10), 22 (October 1952).

Van Der Veen, J., Olcott, H. S., J. AGR. FOOD CHEM. **15,** 682 (1967).

Weedon, B. C. L., "Chemistry and Biochemistry of Plant Pigments," p. 113, T. W. Goodwin, Ed., Academic Press, New York, 1965.



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