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Stabilization of Carotene and Xanthophyll in Alfalfa Leaf Protein Concentrates

Cameron K. Lyon* and George O. Kohler

Carotene and xanthophyll are valuable constituents of alfalfa leaf protein concentrate (Pro-Xan), which is now prepared commercially. The effects of moisture content, pH, pelleting, inert atmosphere storage, and the addition of an antioxidant, fat, or alfalfa-soluble solids on the storage stability of these carotenoids were investigated. Earlier work on storage at low temperatures and in the dark is discussed. Greatest stability is obtained by addition of the antioxidant ethoxyquin (0.05%), storage in an inert atmosphere, and, if economically feasible, cold storage. Increases in stability, particularly of carotene, are also obtained by drying to a lower moisture content, addition of fat, or addition of the soluble solids remaining after separation of the alfalfa protein. It is essential that any added fat be stabilized with antioxidants and free of peroxides that promote the oxidation and destruction of carotenoids.

Alfalfa leaf protein concentrate (Pro-Xan), a feed product containing 50–60% protein and high levels of xanthophyll and β-carotene, is prepared by wet fractionation of fresh alfalfa. Early work on leaf protein concentrates was reviewed by Pirie (1971) and Kohler et al. (1978). Superior large-scale processes for its preparation have now been developed, and the yields and quality of Pro-Xan have been significantly improved (Edwards et al., 1978). Pro-Xan is now being produced commerically in this country by the Valley Dehydrating Co., Sterling, CO (Kohler and Edwards, 1980; Edwards et al., 1979).

Pro-Xan is a good source of protein and energy for poultry (Kuzmicky and Kohler, 1977) and because of its high xanthophyll content is particularly valuable as a pigment source for boilers and laying hens (Kuzmicky et al., 1977). To maintain this value, it is important that the carotenoid pigments be protected from oxidative degradation during storage, and means of accomplishing this have been investigated (Witt et al., 1971, 1972; Livingston et al., 1980). However, the earlier investigations were carried out before the current, preferred Pro-Xan process was developed so that the products had different exposures to heat and air during drying, different contents of water-soluble compounds, etc. Moreover, the effects of variables such as moisture content, added oil, or water-soluble compounds from alfalfa were not always consistent, so it seemed desirable to investigate the factors that could improve the stability of the carotenoids in Pro-Xan as currently prepared.

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EXPERIMENTAL SECTION

Leaf Protein Concentrates. In general, the concentrates were prepared in the pilot plant as described by Edwards et al. (1978) by pressing juice from ground alfalfa, adjusting its pH to 8.5 with ammonia, coagulating protein in the juice at 80 °C or higher by direct injection of steam, separating the protein curd in a continuous centrifuge, and drying the protein. Drying required air temperatures of about 220 °C for a few minutes in a fluidized bed dryer (No. 2052-1, Witte Co., Washington, NJ) or 70 °C for 2–3 h in 1.0–1.5-cm layers in a tunnel dryer. In one experiment, the concentrates were prepared on a small scale as described by Lyon et al. (1976) and dried in a force draft

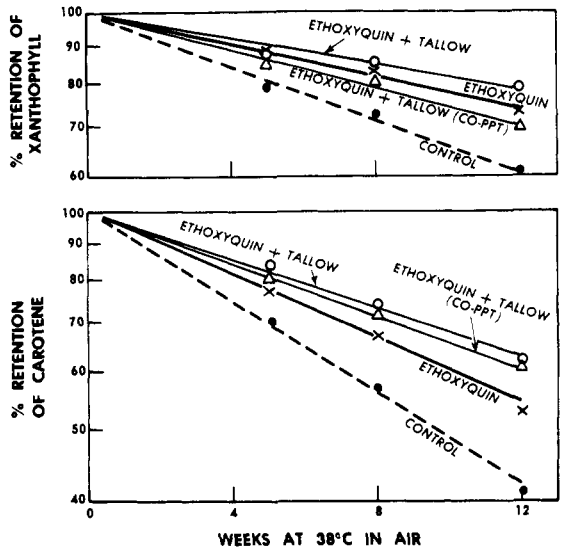

Figure 1. Effect of ethoxyquin (0.05%) and tallow (5%) on stability of carotenoids in Pro-Xan. Initial levels in control: 1175 mg of xanthophyll/kg; 1008 mg of carotene/kg. log percent retention vs. weeks.

oven at 55 °C for 20 h. Samples of granular and pelleted (6.5-mm diameter) Pro-Xan were obtained from the Valley Dehydrating Co.

Antioxidants or other potential stabilizers were added to certain samples. Ethoxyquin (6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline), 0.05 or 0.13%, was added to the Pro-Xan either before drying or after drying as done by Livingston et al. (1955). Melted tallow at about 50 °C was either added to the dry Pro-Xan or emulsified with the alfalfa juice at pH 8.5. When the mixture was heated to 80 °C or above, the tallow was coprecipitated with the protein (Lyon et al., 1976; Dinius et al., 1980). In most cases, ethoxyquin was dissolved in the tallow before addition. For one experiment, alfalfa-soluble solids (Edwards et al., 1978) were added to the Pro-Xan before drying to obtain levels of 8, 16, 24, and 32% (1, 2, 3, and 4 times the residual level in Pro-Xan). These soluble solids were added as a 50% solid concentrate of the supernatant remaining after centrifugal separation of the Pro-Xan curd.

Storage Tests. Most samples, 1–50 g, were stored in open containers in the dark at 38 °C and were analyzed after 4, 8, and 12 weeks. One set of samples was also stored in vacuum-sealed glass vials and tightly filled heat-sealed aluminum foil–polyethylene–paper composite bags.

Carotenoid Analyses. Carotene and nonepoxide (pigmenting) xanthophyll were determined in duplicate on 1-g samples by the procedure of Knuckles et al. (1971).

Headspace Gas Analysis. The gas in sealed bags filled with Pro-Xan was sampled with a gas syringe and analyzed by mass spectroscopy. Each bag was sampled once and then opened for analysis of the Pro-Xan.

Determination of Phenols. Pro-Xan was extracted for 20 h at room temperature with 80% methanol; then the extract (1 mL from 20 mg of Pro-Xan) was analyzed for phenols by using the Folin–Ciocalteu reagent without added cupric ion (Lowry et al., 1951). The extract contains a mixture of phenols but results were expressed as percent ferulic acid.

Peroxide Analysis. Peroxide content of tallow was determined by Official Method CD 8-53 (AOCS, 1980).

RESULTS AND DISCUSSION

Ethoxyquin Addition. Ethoxyquin has been shown to be very effective in increasing the storage stability of the carotenoids in Pro-Xan (Witt et al., 1971, 1972) and is now added at the 0.05% level to Pro-Xan produced by the Valley Dehydrating Co. (Edwards et al., 1979). The stability conferred by adding 0.05% ethoxyquin may be seen in Figure 1. This level had been found (Livingston et al., 1980) to be more effective than 0.0125% and almost as effective as 0.125%.

Table I. Statistical Analysis of Effects of Ethoxyquin and Tallow on Carotenoid Stability[a] in Pro-Xan Stored at 38 °C for 12 Weeks

ethoxyquin, %	tallow, %	linear regression coeff (slope), log % retention/weeks: xanthophyll	carotene
0	0	$0.0179^{A,b}$	0.0316^{A}
0.05	0	0.0110^{B}	0.0225^{B}
0.05	5	0.0090^{C}	0.0168^{C}
0.05	5 coppt.	0.0126^{B}	0.0179^{C}

[a] Calculated from data plotted in Figure 1. log percent retention = regression coefficient × weeks + 2. [b] Coefficients in the same column without a common superscript are different ($P < 0.05$).

Table II. Effect of Ethoxyquin and Tallow on Carotenoid Stability in Pro-Xan[a] Stored in Air at 38 °C for 12 Weeks

ethoxyquin, %	tallow,[b] %	initial levels, mg/kg: carotene	xanthophyll	retention of inital value, %: carotene	xanthophyll
0	0	746	1005	12	31
0.13	0	737	975	36	58
0	24	651	669	43	43
0.13	24	785	924	64	56

[a] Small laboratory samples dried for 20 h at 55 °C. [b] Tallow coprecipitated with protein during processing.

Tallow Addition. The storage stability of carotene and xanthophyll in Pro-Xan containing tallow (5%) and ethoxyquin or ethoxyquin alone is indicated in Figure 1, and a linear regression analysis of these data is listed in Table I. Tallow increased the stability of carotene more than that of xanthophyll, but the increase was significant ($P < 0.05$) in both cases compared to the stability with ethoxyquin alone.

The stabilizing effects of tallow (24%) and ethoxyquin, added separately, are shown in Table II. Tallow alone stabilized carotene better than did ethoxyquin but was a less effective stabilizer for xanthophyll than was ethoxyquin. Witt et al. (1971, 1972) had found, similarly, that maximum stability of carotene was obtained with the addition of ethoxyquin and vegetable oil. However, they reported that in the presence of ethoxyquin, vegetable oil did not increase the stability of xanthophyll and, in some cases, actually decreased it. Their samples contained more water-soluble compounds, including natural antioxidants (Ben Aziz et al., 1968), but had probably suffered more oxidation during drying. Also, the added vegetable oil was less saturated than tallow and may have contained traces of peroxides. The effects of peroxides may be seen in Figure 2. In this experiment, the 5% tallow added contained 2.5 mequiv of peroxide/100 g. Instead of the increased stability found on addition of peroxide-free tallow (Figure 1), stability was decreased on addition of the peroxidized tallow—very markedly in the case of carotene. Budowski and bondi (1960) demonstrated the prooxidant effect of oxidized fat on carotene and suggested that the stabilizing effect of fat on the carotenoids in alfalfa meal was probably due to improved contact between carotenoids and antioxidants and to dilution of the carotenoids. The content of polyunsaturated fatty acids should be mini-

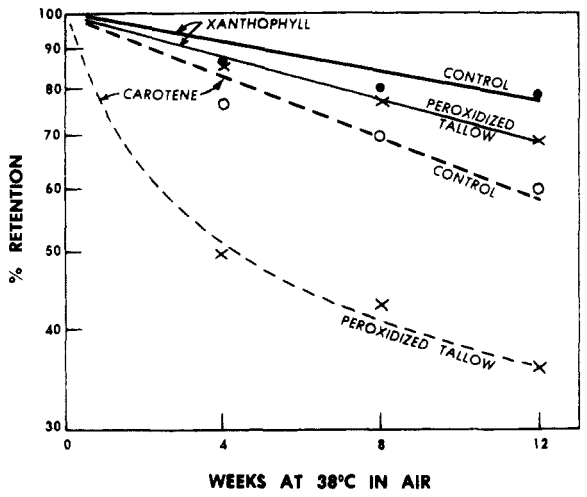

Figure 2. Effect of peroxidized tallow (5%) on stability of carotenoids in Pro-Xan (containing 0.05% ethoxyquin). Initial levels: 1032 and 1142 (control) mg of xanthophyll/kg; 858 and 944 (control) mg of carotene/kg. log percent retention vs. weeks.

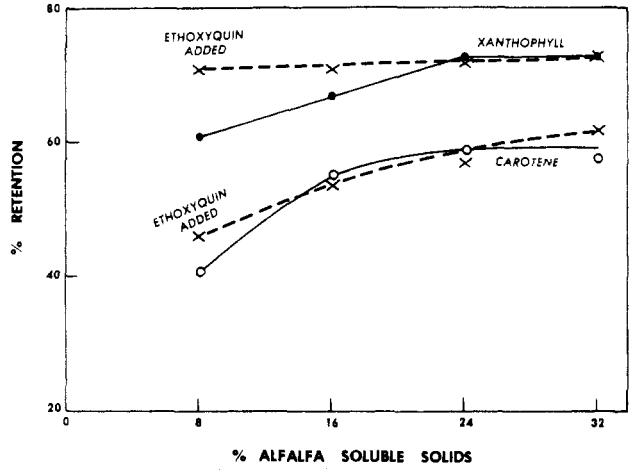

Figure 3. Effect of alfalfa soluble solids and ethoxyquin (0.05%) on retention of carotenoids in Pro-Xan (dried in tunnel dryer) after 12 weeks at 38 °C. Initial levels with 8% soluble solids: 1138 mg of xanthophyll/kg; 939 mg of carotene/kg.

mized in any fat added since the coupled oxidation of carotenoids and unsaturated fatty acids increases with the degree of unsaturation of the fatty acids (Budowski and Bondi, 1960; Holman, 1950). The high concentration of linoleic and linolenic acid in the lipids of leaf protein concentrates contributes to the sensitivity to oxidation of the carotenoids in these concentrates. The lower initial carotene and xanthophyll contents and reduced stability of these carotenoids in the Pro-Xan samples listed in Table I, compared to those (dried in fluidized bed dryer) used in the experiment shown in Figure 1, are probably due to the coupled oxidation of unsaturated fats and carotenoids during prolonged drying (20 h at 55 °C).

It seemed that adding tallow, containing ethoxyquin, to alfalfa juice and then coprecipitating the tallow and protein might protect the carotenoids in Pro-Xan during processing and drying. However, it can be seen in Figure 1 that this procedure afforded less protection than did addition of tallow with ethoxyquin after drying. Some ethoxyquin may have been lost during processing.

Addition of Alfalfa-Soluble Solids. The effects of alfalfa-soluble solids at 1, 2, 3, and 4 times the normal level of 8%, with and without 0.05% ethoxyquin, are shown in Figure 3. In the absence of ethoxyquin, the soluble solids which contain natural antioxidants (Ben Aziz et al., 1968)

Table III. Effect of Moisture Content on Carotenoid Stability in Pro-Xan[a] Stored in Air at 38 °C for 12 Weeks

		initial values, mg/kg		retention of initial value, %	
moisture, %	ethoxyquin, %	carotene	xanthophyll	carotene	xanthophyll
10	0	860	1105	38	62
6	0	882	1105	43	65
10	0.05	922	1153	46	72
5	0.05	944	1142	60	79

[a] Dried in fluidized bed dryer.

increased the stability of carotene and xanthophyll up to the level of 24% soluble solids. In the presence of ethoxyquin, stability of xanthophyll was as high at all levels as with 24% soluble solids alone and was essentially unaffected by level of soluble solids; stability of carotene, though higher than without ethoxyquin at the 8% soluble solids levels, was unchanged at higher levels by ethoxyquin. In the absence of ethoxyquin, Witt et al. (1971) and Livingston et al. (1980) achieved less improvement in carotenoid stability by addition of soluble solids, possibly because their samples had suffered more heat exposure on drying and lost more of the natural fat-soluble antioxidants (Hudson and Mahgoub, 1980; Livingston et al., 1968).

Moisture Content of Pro-Xan. In confirmation of earlier data of Livingston et al. (1980), the stability of the carotenoids was found to be greater in Pro-Xan dried to a lower moisture content (Table III). The difference is small but is somewhat larger in the presence of ethoxyquin.

pH Effect. During the preparation of the Pro-Xan samples whose stabilities are plotted in Figure 1, another set of samples was prepared by heat coagulating the protein at the natural pH of 5.8 rather than the usual pH of 8.5. These samples were dried, with and without added ethoxyquin, stored 12 weeks at 38 °C, and found to retain about 5% more carotene and xanthophyll than the samples prepared at pH 8.5. This small increase in stability may be due to a decreased solubility of the natural phenolic antioxidants or increased association with proteins at the lower pH so that more are retained in the coagulated Pro-Xan curd. Pro-Xan prepared at pH 5.8 contained 0.39–0.43% ferulic acid equivalent, while control samples prepared at pH 8.5 contained 0.28% ferulic acid equivalent. However, it is unlikely that practical advantage can be taken of this observation since it is important that the pH be raised to about 8.5 during processing to minimize destruction of alfalfa proteins and carotenoids by natural proteases, lipoxygenases, peroxidases, and oxidases (de Fremery et al., 1972; Arkcoll and Holden, 1973).

Pelleting. Commercial Pro-Xan is pelleted and might be expected to be more stable because less surface is exposed to air. However, no significant difference (±2% retention difference after 12 weeks at 38 °C) was found in the stability of carotenoids in pelleted and granular Pro-Xan from the Valley Dehydrating Co. Witt et al. (1972), similarly, found no change in stability on pelleting Pro-Xan.

Inert Atmosphere Storage. Pro-Xan, sealed under vacuum in glass, retained 96% of its xanthophyll and 87% of its carotene after storage for 15 weeks in the dark at 38 °C. The same Pro-Xan, sealed in filled aluminum foil lined bags and stored under the same conditions, retained 92% of its xanthophyll and carotene. The headspace gas in the bags contained no oxygen after 15 weeks and only about 2% after 3 weeks. Evidently, oxygen in tightly filled containers is soon used up by easily oxidized compounds in Pro-Xan so that the atmosphere becomes essentially

inert. Similar protection of the carotenoids in Pro-Xan (Witt et al., 1971), other leaf protein concentrates (Arkcoll, 1973), and dehydrated alfalfa (Hoffman et al., 1945; Livingston et al., 1955) has been reported, and commercial Pro-Xan is now stored under inert gas (Edwards et al., 1979).

Cold Storage. Temperature effects were not studied, but Witt et al. (1971) reported that after 19 weeks of storage, Pro-Xan containing ethoxyquin retained 96% of its xanthophyll at 2 °C but only 50% at 38 °C.

Light Effects. Leaf protein concentrates should always be stored in the dark since oxidation of carotenoids in the light is strongly catalyzed by the chlorophyll present (Arkcoll, 1973).

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Mutagens in Dried/Salted Hawaiian Fish

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Mutagenic substances were found in dried/salted skipjack, *Katsuwonus pelamis*, Indo-Pacific jackfish, *Caranx ignoblis*, and big-eyed scad, *Trachuroks crumenophthalamus*. These mutagens were detected by *Salmonella* tester strain TA100 in the absence of rat liver microsomes. The mutagens can be obtained as purified materials by extraction with selective solvents, absorption on silica gel, and high-pressure liquid chromatography. The mutagens are not detected in freshly caught fish or fish that is dried/salted soon after being caught. The dried/salted product, which contains mutagens, is prepared from fish that has been frozen at sea and stored for several months or chilled fresh fish that has remained unsold for 6–8 days.

Dietary components are believed to have an important influence in the causation of some cancers (Armstrong and Doll, 1975). Because of this, and because most carcinogens are also mutagens, a systematic search was undertaken for mutagens among unusual food items eaten in Hawaii. We report here that dried, salted fish frequently contain high levels of mutagens as detected by Ames tester strain TA100 in the absence of rat liver microsomes.

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MATERIALS AND METHODS

The dried fish were purchased in local food markets and are common food items sold in the local food section of many supermarkets in Hawaii. The items studied were dried/salted skipjack or striped tuna, *Katsuwonus pelamis* (local name Aku), small Indo-Pacific jackfish, *Caranx ignoblis* (local name Papio), and big-eyed scad, *Trachuroks crumenophthalamus* (local name Akule). All fish were

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