Stabilization of Carotenoids by Ethoxyquin in Harvested Fresh Alfalfa

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Chopped fresh alfalfa samples were sprayed with aqueous solutions of ethoxyquin sulfate or an aqueous ethoxyquin emulsion, then exposed to the air at 32° C. for 0 to 49 hours before freeze-drying or oven-drying. Some of the dried samples were subjected to an accelerated storage stability test. Samples were analyzed for total carotene and xanthophyll and three individual xanthophylls, lutein, violaxanthin, and neoxanthin. The ethoxyquin treatments protected carotenoids in fresh alfalfa during the time from cutting to dehydrating and provided additional protection during subsequent storage of the meals.

In the course of recent investigations into losses of carotenoids during alfalfa dehydration (Livingston *et al.*, 1968), we were impressed by the magnitude of the apparent loss of carotene and xanthophyll in fresh alfalfa from the time of cutting until actual entry into the dehydrator. These losses may approximate 25% in commercial practice, and a means of preventing or reducing such loss would be valuable from the standpoint of both the vitamin content and the pigmentation potency of the dehydrated alfalfa meal.

Various workers have studied carotenoid loss in fresh plant material, such as bean leaves (Bernstein and Thompson, 1947), beet leaves (Friend and Nakayama, 1959), kale and collards (Ezell and Wilcox, 1962), and alfalfa (Booth, 1960; Van der Veen and Olcott, 1967; Walsh and Hauge, 1953). Walsh and Hauge identified carotene losses in fresh alfalfa leaves macerated with water as enzymatic and photochemical (but could not measure these separately) and autoxidative (very minor below 40° C.). They found that the enzymatic process was accelerated as pH decreased to 5 and temperature increased to approximately 43° C., but could be inactivated by autoclaving or steam blanching. Carotene loss was more rapid in suspensions than in intact tissue. Friend and Nakayama, studying buffered sucrose suspensions of beet leaf chloroplasts, demonstrated that most of the carotenoid loss was oxidative and was quantitatively in the order: β-carotene > violaxanthin $\gg$ lutein. The work of Booth on fresh alfalfa leaf, ground with water, showed that the enzyme involved is in the chloroplasts, found only in company with chlorophyll, and activated when the plant cells are damaged. Loss of carotene in the aqueous alfalfa preparation was approximately 17% during a 30-minute holding period, but boiling for 1 minute prevented nearly all carotene loss during the same holding time. Van der Veen and Olcott reported that fresh alfalfa held at room temperature following harvest lost 76% of its β-carotene during the first 24 hours. The usual industrial methods of handling fresh alfalfa between cutting and dehydration are, unfortunately, favorable for this oxidative, enzymatic destruction of carotenoids and generally no measures are taken to prevent such loss.

Ethoxyquin (6-ethoxy-2,2,4-trimethyl-1,2,-dihydroquinoline), an antioxidant now commonly added to alfalfa after dehydration for preservation of carotene during storage, has been found effective also in preserving xanthophylls in dehydrated alfalfa during storage (Knowles *et al.*, 1968). The present experiments were conducted to test the value of aqueous ethoxyquin sprays in reducing loss of carotene and xanthophylls in chopped fresh alfalfa. A limited test of possible residual protection during heated-air dehydration was made, and residual effect upon storage stability was also investigated.

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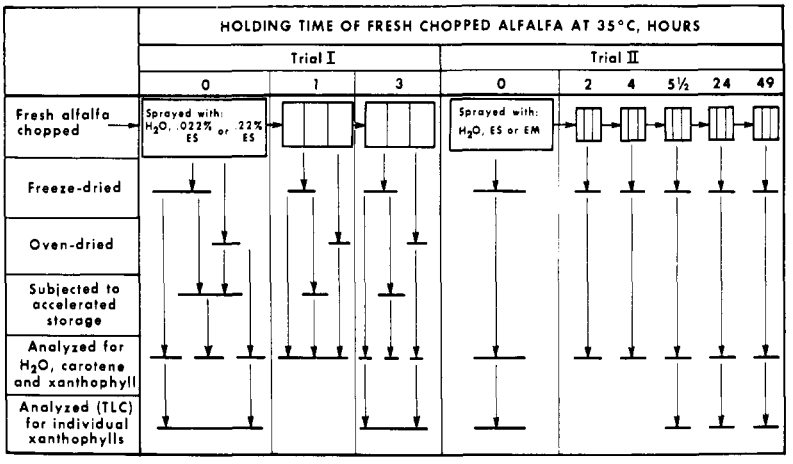

Figure 1. Diagram of experimental procedure

EXPERIMENTAL

Treatments. In the first (Trial I) of two investigations, freshly harvested alfalfa plants were chopped in a Hobart food cutter to approximately $^3/_4$-inch pieces, and the entire lot was thoroughly mixed, then divided into three 500-gram lots (Figure 1). The portions of alfalfa, in 1-gallon glass jars, were sprayed with 20 ml. of water (control), aqueous ethoxyquin sulfate solution (ES) (Monsanto Chemical Co., St. Louis, Mo.) $\cong$ 0.022% or 0.22% of dry solids of alfalfa. A fine air sprayer was used and the samples were well mixed during the treatment. A 100-gram sample of each lot was immediately placed in a plastic bag and frozen between layers of dry ice; a second 100-gram sample of each was placed in a covered screen tray and dehydrated for 1 hour in a forced-air oven at 121° C.; the remainder of each lot was held in the open jar in a 32° C. constant-temperature room. Samples were again taken from these jars for freezing and oven-drying after 1 and 3 hours. The frozen samples were then dried in a Virtis vacuum freeze-dryer. Moisture content of all dried meals was approximately 3%.

Accelerated Storage Stability Test. Two-gram samples of all freeze-dried meals and of the zero-holding-time oven-dehydrated meals were placed in open vials, loosely covered to permit air circulation, in a forced-draft oven maintained at 75° C. They were stored thus for 7 days, which is approximately equivalent to 6 months' storage at ambient temperature (Thompson, 1950). Although these meals undoubtedly dried somewhat during this storage period, lowering carotene retention (Bailey *et al.*, 1949), the extent of drying was approximately the same in all samples. Therefore the measured stability differences can be attributed to treatment.

Analyses. All dried samples, including those stored at 75° C., were ground to pass through a 40-mesh screen in a Wiley mill and were analyzed in duplicate for carotene and xanthophyll by the method of Kohler *et al.* (1967). Moisture in the ground meal was determined by drying duplicate 1- to 2-gram samples at 100° C. for 24 hours in a forced draft oven. The 0-time and 3-hour samples of both freeze-dried and oven-dehydrated alfalfa, untreated and treated with 0.22% ethoxyquin sulfate, were analyzed also by thin-layer chromatography (TLC) for the major xanthophylls and their isomers by the method of Nelson and Livingston (1967).

A second investigation (Trial II) was conducted, similar to the first, with the following differences: Sprays applied were water (control), ethoxyquin emulsion in water (EM) (Monsanto) $\cong$ 0.15% on dry weight of alfalfa, or ethoxyquin sulfate solution in water, 0.22% (equivalent to 0.15% ethoxyquin); time of holding chopped alfalfa at 32° C. covered the range, 0, 2, 4, $5^1/_2$, 24, and 49 hours; oven-dehydration was not carried out; all but the 2-hour and 4-hour samples were analyzed by TLC for individual xanthophylls; no accelerated storage trial of the dried meals was conducted.

RESULTS AND DISCUSSION

Retention of Total Carotene and Total Xanthophyll during Holding at 32° C. before Freeze-Drying. The values in Table I indicate preservative effects of ethoxyquin upon both carotene and xanthophyll, with increased effect at the higher treatment level. The per cent increase in carotenoid retention due to treatment was greater during the longer holding periods. In the preliminary trial (I) the higher ES level apparently prevented all loss of carotene or xanthophyll during 3 hours' exposure. In Trial II carotene and xanthophyll retention in the untreated control decreased smoothly as a function of exposure time, with carotene showing definitely lower stability than xanthophyll (Figures 2 and 3). Both ethoxyquin emulsion and sulfate solution afforded protection of carotene and xanthophyll. The per cent retention means for EM- and ES-treated samples are significantly different from the controls at greater than 98% confidence levels determined by Student's *t* test. Ethoxyquin sulfate was somewhat more effective than EM, especially during the longer holding periods. For the entire second trial, the two treatment effects were significantly different from each other at 93 and 95% confidence levels for carotene and xanthophyll, respectively. The increase in retention of carotene due to ethoxyquin spraying was greater than the corresponding effect on xanthophyll during $5^1/_2$ hours; thereafter, xanthophyll retention was enhanced more than carotene retention. The results of both holding trials in-

Table I. Retention of Carotenoids in Ethoxyquin-Treated Fresh Chopped Alfalfa[a]

	Holding Time at 32° C., Hours	Carotene						Xanthophyll					
		Control		ES, 0.022%		ES, 0.22%		Control		ES, 0.022%		ES, 0.22%	
		Mg./kg.	%	Mg./kg.	%	Mg./kg.	%	Mg./kg.	%	Mg./kg.	%	Mg./kg.	%
Trial I	0	419	100	419	100	419	100	837	100	837	100	837	100
	1	392	94	407	97	432	103	727	87	802	96	837	100
	3	346	83	388	93	427	102	645	77	771	92	841	101
Oven-dried	0	348	85	368	88	390	93	368	45	405	49	407	49
		Control		EM, 0.15%[b]		ES, 0.22%		Control		EM, 0.15%[b]		ES, 0.22%	
		Mg./kg.	%	Mg./kg.	%	Mg./kg.	%	Mg./kg.	%	Mg./kg.	%	Mg./kg.	%
Trial II	0	315	100	315	100	315	100	705	100	705	100	705	100
	2	293	93	297	94	297	94	654	93	661	94	670	95
	4	238	76	289	92	286	91	568	81	643	91	650	92
	5½	225	71	269	85	278	88	555	79	623	88	648	92
	24	161	51	183	58	209	66	432	61	496	70	555	79
	49	121	38	156	50	178	57	348	49	441	63	498	71

[a] All analyzed following freeze-drying except for oven-dried samples.
[b] Equivalent ethoxyquin content of 0.22% ES.

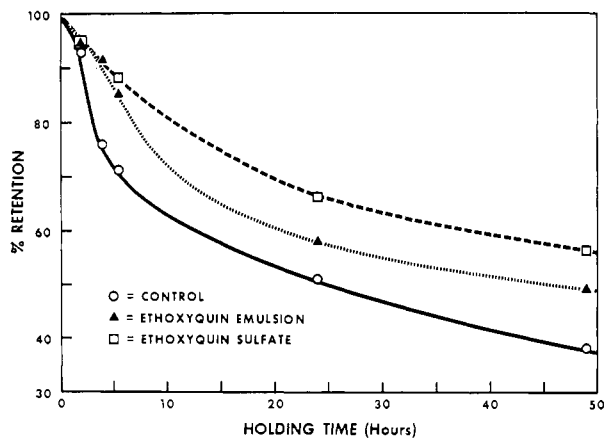

Figure 2. Retention of carotene in fresh chopped alfalfa at 32° C.

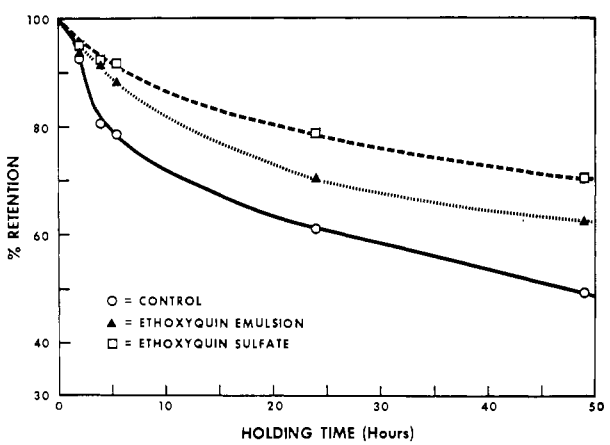

Figure 3. Retention of xanthophyll in fresh chopped alfalfa at 32° C.

dicate that 19 to 21% more carotene and 13 to 30% more xanthophyll were retained during 3 or 4 hours' exposure after fresh chopped alfalfa was sprayed with aqueous ethoxyquin emulsion or ethoxyquin sulfate solution. During the longest holding period 47% more carotene and 43% more xanthophyll were retained in the ES-treated chopped alfalfa than in the control.

Retention of Total Carotene and Total Xanthophyll during Oven-Dehydration. Carotene retention during oven-drying of treated and untreated alfalfa was much higher than xanthophyll retention (Table I), as previously reported for pilot and commercial scale dehydration (Livingston *et al.*, 1966, 1968). Because of unpreventable variation in the final per cent moisture in the oven-dried 1-hour and 3-hour samples, only the zero-exposure results were considered to show antioxidant treatment effects on the dehydration process. These results demonstrated a small protective effect upon carotenoids during dehydration as a result of ethoxyquin sulfate treatment of the fresh chopped alfalfa; carotene showed a small response to treatment level, xanthophyll did not.

Retention of Total Carotene and Total Xanthophyll during Accelerated Storage. The storage stability of carotene in freeze-dried alfalfa was related to the time of exposure at 32° C. before freeze-drying (Table II); the 1-hour samples showed slight effect but the 3-hour samples retained 17 to 21% less of the remaining carotene during accelerated storage than those frozen immediately. This effect was demonstrated in both treated and untreated samples. Ethoxyquin added immediately after harvest increased the storage stability of carotene in freeze-dried meals, for each holding period, particularly at the higher application rate.

Storage effects on xanthophyll in freeze-dried alfalfa were more equivocal. Increased time of exposure before freeze-drying showed a reasonably certain relationship to lowered storage stability, in the treated samples. Ethoxyquin treatment increased storage stability of xanthophyll

Table II. Carotenoid Retention during Accelerated Storage of Meals from Ethoxyquin-Treated Fresh Alfalfa[a]

Drying Procedure	Holding Time at 32° C., Hours	Carotene						Xanthophyll					
		Control		ES, 0.022%		ES, 0.22%		Control		ES, 0.022%		ES, 0.22%	
		Mg./kg.	%[b]	Mg./kg.	%	Mg./kg.	%	Mg./kg.	%	Mg./kg.	%	Mg./kg.	%
Freeze-dried	0	132	32	148	35	216	52	280	34	315	38	352	42
	1	121	31	145	36	211	49	300	41	284	35	352	42
	3	95	27	112	29	176	41	249	39	271	35	300	36
Oven-dried	0	97	28	112	30	205	53	126	34	137	34	207	51

[a] Stored 7 days at 75° C.
[b] Per cent based upon mg./kg. in dried alfalfa for each treatment and holding time, as recorded in Table I (Trial I).

in the zero-exposure freeze-dried alfalfa meals, but not as greatly as in the case of carotene.

Storage of oven-dehydrated alfalfa treated with ES at the time of harvest, with zero holding time, demonstrated very appreciable protection of carotene and almost as large an effect upon xanthophyll. The enhancement of storage stability of both carotenoids was greater than in the case of treated freeze-dried alfalfa.

Retention of Individual Xanthophylls. In the first trial only the 0.22% ES-treated samples and the untreated samples, both after zero and 3 hours' holding at 32° C., were analyzed by TLC for individual xanthophylls. As shown in Table III, lutein was the most stable of the three major xanthophylls in the untreated alfalfa, neoxanthin and violaxanthin showing identical stabilities. Ethoxyquin sulfate stabilized neoxanthin and lutein fully for the 3-hour holding period, and violaxanthin almost completely. (Oven-dehydration of the untreated zero-holding-time samples caused loss of most of the violaxanthin, two thirds of the neoxanthin, and somewhat less than half the lutein. The ethoxyquin sulfate treatment improved retention of both neoxanthin and violaxanthin to a limited extent but had virtually no effect upon lutein stability during dehydration.)

In the trials conducted over a longer range of holding times, the untreated samples revealed lutein to be the best preserved of the three major xanthophylls, although it showed progressive loss; neoxanthin ranked next in stability and violaxanthin was the most labile. When treated with 0.22% EM, lutein, neoxanthin, and violaxanthin were apparently entirely stabilized during 5½ hours, after which lutein decreased somewhat, neoxanthin more so, and violaxanthin disappeared most rapidly. Treatment with 0.22% ES also stabilized lutein and neoxanthin for 5½ hours, but by that time a large loss of violaxanthin had commenced which proceeded through 24 and 49 hours. Loss of neoxanthin was very much lower and lutein was best preserved of all.

Examination of the absorption spectra of the individual xanthophylls isolated by TLC suggests that the greater lability of violaxanthin is in part due to isomerization of one or both of its 5,6-epoxide groups to the 5,8 configuration. The spectra were characteristic in the 0- and 3-hour samples of Trial I and in the 0-time samples of Trial II. However, the violaxanthin isolated from the 5½-hour samples revealed a shift of the middle absorption peak toward 445 mμ, and elevation of the peak in the 420-mμ region. This can be attributed to partial isomerization to luteoxanthin and auroxanthin (Knowles *et al.*, 1968). These changes became more pronounced in the 24- and 49-hour violaxanthin fractions, while the neoxanthin and lutein spectra remained characteristic. (In all the oven-dehydrated samples, however, both violaxanthin and neoxanthin showed spectral shifts indicating 5,6 → 5,8-epoxide isomerization.) The total decrease in the major xanthophylls was therefore due both to oxidation and, in the case of neoxanthin and violaxanthin, to isomerization. Ethoxyquin substantially reduced oxidative loss of neoxanthin and more effectively that of lutein. It was much less effective in preventing violaxanthin loss, which is principally due to isomerization.

Enhancement of stability of neoxanthin and lutein, by

Table III. Retention of Individual Xanthophylls in Ethoxyquin-Treated Fresh Chopped Alfalfa[a]

	Holding Time at 32° C., Hours	Control						Ethoxyquin Sulfate						Ethoxyquin Emulsion					
		N		V		L		N		V		L		N		V		L	
		Mg./kg.	%	Mg./kg.	%	Mg./kg.	%	Mg./kg.	%	Mg./kg.	%	Mg./kg.	%	Mg./kg.	%	Mg./kg.	%	Mg./kg.	%
Trial I	0	115	100	211	100	454	100	121	100	207	100	463	100						
	3	84	73	154	73	381	84	121	100	205	99	465	100						
Oven-dried	0	37	33	22	10	264	58	48	40	29	14	269	58						
Trial II	0	99	100	170	100	348	100	106	100	167	100	370	100	93	100	130	100	337	100
	5½	75	76	123	73	291	84	108	100	128	76	388	100	93	100	137	100	346	100
	24	63	64	68	40	260	75	88	83	55	33	346	93	73	79	64	49	275	82
	49	48	49	48	29	218	63	75	71	75	45	306	83	62	67	40	31	282	84

[a] N = neoxanthin, V = violaxanthin, L = lutein and its isomers. All analyzed following freeze-drying except oven-dried samples.

either ethoxyquin treatment, therefore, was appreciable for every exposure period but the effects on violaxanthin retention were generally smaller and less consistent. In the preliminary holding trial, all lutein was retained in the treated alfalfa during 3 hours, 19% more than in the unteated. During the first 5½ hours' holding in the second trial, 19% more lutein was retained in the treated samples. The increase in per cent retention of total xanthophylls was only two thirds as great. Thus lutein, the most valuable of the alfalfa xanthophylls, was protected more than the total by this antioxidant treatment.

During commercial dehydration operations, the time from cutting and chopping alfalfa until it enters the dehydrator may vary from perhaps one to several hours—e.g., during a breakdown in equipment or operations. Conditions of temperature and exposure of cut tissue surfaces are then ideal for rapid enzymatic, oxygen-dependent losses of carotene and xanthophylls. Water-based applications of ethoxyquin immediately after chopping appreciably protect these carotenoids during the time from cutting to dehydrating, and have a residual protective effect during subsequent storage of the dehydrated alfalfa meal. Further tests—e.g., application of ethoxyquin sprays during field harvesting operations—appear to be warranted.

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