Stability of α-Tocopherol during Alfalfa Dehydration and Storage

A. L. Livingston, J. W. Nelson, and G. O. Kohler

Losses of α-tocopherol during commercial-scale alfalfa dehydration ranged from 5 to 33%. The larger losses occurred at meal moisture levels of less than 3%. Although α-tocopherol was lost rapidly, the total related isoprenoid reducing compounds actually increased in dehydrated alfalfa meal during storage.

Dehydrated alfalfa has been known for many years as an excellent source of vitamin E (Brown, 1953; Cabell and Ellis, 1942) and has, therefore, been used in poultry feeds for the prevention of encephalomalacia in chicks (Singsen *et al.*, 1955), and to increase fertility in laying hens (Jensen and McGinnis, 1956). Recently, there has been interest in the feeding of alfalfa to dairy cows as a source of vitamin E in milk to prevent oxidized flavor (Dunkley *et al.*, 1960). Although alfalfa has been a valuable source of vitamin E for poultry and animal feeds, there has been only a limited amount of data published on the stability of vitamin E during alfalfa processing and storage. Kohler *et al.* (1955) compared the rates of loss of carotene and vitamin E in dehydrated cereal grass and found that vitamin E was more stable than carotene. Charkey *et al.* (1961) found a wide variability in the α-tocopherol content of dehydrated and sun-cured alfalfa from various sources. Recent studies at this laboratory have shown that large losses of xanthophyll may occur during alfalfa dehydration (Livingston *et al.*, 1966, 1968) and storage (Knowles *et al.*, 1968). These losses were correlated with meal moisture and the outlet temperature of the dehydrator.

The present report describes the stability of α-tocopherol during alfalfa dehydration and storage. Since the related tocopherols and reduced isoprenoid quinones may also possess antioxidant and biological activity, they have also been measured and included in this study.

EXPERIMENTAL

Reagents. α-Tocopherol. Pure *dl*-α-tocopherol was obtained from Hoffmann-La Roche, Inc.

Absolute Ethanol. Reagent grade ethanol was purified by distilling from 0.2% by weight of potassium permanganate and potassium hydroxide.

Ferric Chloride Solution. A $1.0 \times 10^{-3}M$ concentration in absolute ethanol was prepared daily directly in an amber flask.

Orthophosphoric Acid Solution. $0.1M$ in absolute ethanol.

Acetic Acid Solution. One-to-one dilution of glacial acetic acid with distilled water.

Hexane. High purity hexane (Phillips) was redistilled prior to use.

Ethyl Acetate. Reagent grade ethyl acetate was redistilled prior to use.

4,7-Diphenyl-1,10-phenanthroline (bathophenanthroline, Fisher Scientific Co.) solution. A $6.0 \times 10^{-3}M$ concentration in absolute ethanol, prepared daily directly in an amber bottle.

Procedure. Fresh alfalfa from a single field was dehydrated at two nearby industrial plants, one employing an Arnold alfalfa dehydrator and the other a Stearns-Roger dehydrator. Details on the operation of the dehydrators and the collection of fresh and dehydrated alfalfa samples have been previously described (Livingston *et al.*, 1968).

Following freeze-drying, the samples of fresh alfalfa were ground so as to pass through a No. 40 screen and analyzed for moisture by drying in a forced draft oven at 110° C. for 24 hours. Grinding and moisture determination of the dehydrated meal samples were carried out in the same manner as the freeze-dried meals. The samples of freeze-dried meals served as initial reference meals to which the dehydrated meals were compared in ascertaining losses.

Solvent extraction of α-tocopherol and related isoprenoid compounds was carried out by an overnight soak of 1- or 2-gram samples of the alfalfa meals under an inert atmosphere with 50 ml. of a mixture of hexane-acetone (7 to 3), and 0.5 ml. of 50% aqueous acetic acid. The following day the samples were adjusted to 100 ml. with hexane and thoroughly mixed. Ten-milliliter aliquots were then concentrated to near dryness on a rotary vacuum evaporator. The entire aliquot was taken up in 1 ml. of hexane-acetone (1 to 1) and streaked on a 20 × 20 cm. plate coated with silica gel G (0.25 mm. thick, activated for 30 minutes at 110° C.). A small quantity of a related alfalfa extract was spotted on each side of the streak to be used as a reference. The chromatoplates were developed according to the procedure of Polesello and Vistarini (1965) in hexane–ethyl acetate (92.5 to 7.5). The developed plates were dried under a stream of nitrogen and the developed reference spots sprayed with ferric chloride–bathophenanthroline reagent. The band corresponding to α-tocopherol and the two bands corresponding to the related reduced isoprenoid quinones were scraped off and transferred to sintered glass funnels. The α-tocopherol and related isoprenoid compounds were eluted with three 2-ml. portions of absolute alcohol into 10-ml. amber-colored volumetric flasks.

Colorimetric determination was carried out by a modification of the method of Tsen (1961) and consisted of pipetting into each sample 1 ml. of the bathophenanthroline solution, followed by 0.5 ml. of the ferric chloride solution. After exactly 2 minutes, 0.1 ml. of an orthophosphoric acid solution was added. The absorbance of the solution was measured at 535 mμ in a Cary spectrophotometer against

Western Regional Research Laboratory, Agricultural Research Service, U. S. Department of Agriculture, Albany, Calif. 94710

a blank of the reagents used in the color development. Concentrations of α-tocopherol were calculated by employing a calibration curve previously prepared with known solutions of pure α-tocopherol.

The quantitative determination of the two related reducing bands was made by combining the eluents from the two bands and determining their concentration in the same manner as the α-tocopherol band. The concentration was then calculated as α-tocopherol equivalents.

Determination of α-tocopherol in alfalfa meals previously treated with the antioxidant ethoxyquin (6-ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline, EQ) required an added purification step to remove the EQ which interfered with the analysis. This consisted of washing 25-ml. aliquots of the alfalfa extracts with six 25-ml. portions of 0.3*N* sulfuric acid in separatory funnels. The washed extracts were dried over sodium sulfate and readjusted to 25 ml. with small portions of hexane. Ten-milliliter aliquots were then taken for concentration and TLC analysis in the same manner as that employed for nonwashed samples.

Storage stability trials were carried out by storing 2-gram samples of the alfalfa meals in open shell vials in a constant temperature room at 90° F.

A large portion of the dehydrated alfalfa meal produced in the United States is treated with the antioxidant EQ. It is, therefore, essential that methods for determination of α-tocopherol in alfalfa include a procedure to remove the EQ and its degradation products which are not removed by usual chromatographic procedures and interfere with colorimetric analysis for α-tocopherol. Solutions of pure α-tocopherol as well as an extract of alfalfa with and without added EQ were examined by the above sulfuric acid wash procedure. The results demonstrated that all of the interfering EQ was removed without loss of α-tocopherol or isoprenoid reducing compounds (Table I).

Purity of the α-tocopherol band was ascertained by rechromatography of the eluted band in four additional solvent systems. Rechromatography of the two more polar reducing bands, as described by Polesello (1967a), gave 4 to 5 additional bands. One of these has been identified as solanachromene, the cyclic isoprenoid of plastoquinone (Polesello, 1967a).

Table I. Removal of Ethoxyquin from Alfalfa Extracts by Sulfuric Acid Wash

Sample	Absorbance at 535 Mμ[a]	
α-Tocopherol only	0.46	
Ethoxyquin plus α-tocopherol	1.12	
Ethoxyquin plus α-tocopherol plus H_2SO_4 wash	0.45	
	α-Tocopherol Band	Related Reducing Band
Alfalfa extract	0.76	0.30
Alfalfa extract plus ethoxyquin	1.22	1.64
Alfalfa extract plus ethoxyquin plus H_2SO_4 wash	0.75	0.30

[a] Average of duplicate analyses; absorbance determined following addition of bathophenanthroline and ferric chloride reagents.

RESULTS AND DISCUSSION

Column chromatographic procedures for the purification of α-tocopherol frequently do not allow complete recovery of α-tocopherol or else elute the related reduced isoprenoid quinones in the α-tocopherol fraction (Dicks-Bushnell, 1967). Accordingly, a modification of the TLC procedure of Polesello and Vistarini (1965) was employed for the purification of α-tocopherol.

Precision of the method from the overnight extraction through the color measurement was determined for a typical alfalfa meal sample and is presented in Table II. Recovery of added α-tocopherol (Table III) is similar to that which Booth (1963) found using paper chromatography as the method of purification of α-tocopherol. The precision of the method for the related reducing bands was the same as that for the α-tocopherol band. Examination of the extracted meal residue, by filtration of the solvent, and re-extraction of the meal confirmed that no additional α-tocopherol or related reducing compounds remained in the extracted meal.

Loss of α-tocopherol during dehydration in the Arnold dryer was greater at the lower meal moisture level and the higher output temperature (Table IV). At the two conditions studied in the Arnold dryer there was little if any loss of the related reducing compounds. Studies at this laboratory have found that during column chromatographic purification of α-tocopherol employing Florisil and benzene, the α-tocopherol and related reducing compounds are frequently eluted in the same fraction. This, of course, would give misleading results as to the real α-

Table II. Precision of Method for α-Tocopherol in a Typical Sample of Alfalfa Meal

Determination	Mg./100 Grams[a]
1	18.5
2	19.4
3	16.5
4	16.9
5	16.8
6	16.6
7	16.5
8	19.5
Mean	17.7
Std. dev.	1.24
Coeff. of variation, %	7.0

[a] Dry basis.

Table III. Recovery of α-Tocopherol from a Known Alfalfa Extract

Mg./100 Grams[a]		
Added	Recovered	Recovery, %
39.5	35.5	90
39.5	38.0	96
39.5	36.2	92
39.5	36.3	92
39.5	36.3	92
39.5	36.2	92
39.5	36.2	92
39.5	35.4	90
Av. 39.5	36.3	92

[a] Dry basis.

tocopherol content of alfalfa samples and the effects of drying conditions on these samples. Thus at both meal moisture levels in the Arnold dryer, there was a greater loss of α-tocopherol than the related reducing compounds.

In trial 1 with the Stearns-Roger dryer, the α-tocopherol loss was consistently higher than in trial 2. This higher rate of loss was not correlated with the meal moisture, but was apparently due to a longer period of drying in the retention chamber. In four of the six drying conditions studied in the Stearns-Roger, there was a greater loss of the related reducing compounds than α-tocopherol. In trial 2, the largest loss of α-tocopherol occurred at the lowest meal moisture level. Conversely, there was a correlation of the loss of the related compounds with increasing meal moisture levels.

Storage losses of α-tocopherol in the untreated dehydrated alfalfa meals ranged from 54 to 73% and could be related to the meal moisture level (Table V). In contrast, the freeze-dried meals lost only 3 to 28% of their initial α-tocopherol; this rate of loss was apparently not correlated with meal moisture. The addition of EQ to the dehydrated alfalfa meal resulted in a substantial reduction in loss of α-tocopherol; however, the addition of EQ to the freeze-dried meal had little apparent effect, since the natural antioxidants apparently afforded almost complete stability to the particular sample treated. Although in certain meals there was a large loss of α-tocopherol during storage, the total amount of the related reducing compounds actually increased in both dehydrated and freeze-dried meals. In certain samples, there was more than a doubling of the total quantity present. In the present study the individual compounds were not identified; however, Polesello (1967a) identified solanachromene as one of the reducing compounds which increased during storage, apparently being formed from plastoquinone. Since solanachromene has also been found to be an antioxidant for carotene (Polesello, 1967b), the actual total antioxidant activity of a freeze-dried alfalfa meal may well remain relatively constant during storage. Knowles *et al.* (1968) found the antioxidant ethoxyquin gave no additional protection to xanthophyll stability during storage over that afforded by the natural antioxidants already present in freeze-dried alfalfa. Suitable drying conditions might, therefore, be found which could enhance or preserve the inherent antioxidant activity of forage, providing a source of vitamins and nutritives as rich as the fresh plant material.

Table IV. Stability of α-Tocopherol and Related Compounds during Alfalfa Dehydration

Dryer	Outlet Temperature of Dryer, ° F.	Dehy. Meal Moisture, %	α-Tocopherol, Mg./100 Grams[a]			Related Reducing Compounds, Mg./100 Grams[a]		
			Fresh freeze-dried meal	Dehy. meal	Loss, %	Fresh freeze-dried meal	Dehy. meal	Loss, %
Arnold	300	9.2	18.1	17.2	5	9.3	10.9	(Increase)
	330	2.3	22.8	18.1	21	13.9	13.6	2
Stearns-Roger	250	12.2	18.8	13.7	27	10.1	5.0	50
(Trial 1)	250	7.1	17.5	12.9	26	11.5	3.8	67
	250	2.5	20.6	13.8	33	9.4	7.9	16
Stearns-Roger	275	7.1	19.6	18.7	5	14.7	9.2	35
(Trial 2)	275	3.1	19.0	18.1	5	13.9	11.0	21
	275	1.5	21.9	17.8	19	9.1	9.1	(None)

[a] Dry basis, average of duplicate analysis.

Table V. Stability of α-Tocopherol and Related Reducing Compounds during Storage of Alfalfa[a]

Sample	Meal Moisture, %	α-Tocopherol, Mg./100 Grams[b]			Related Reducing Compounds, Mg./100 Grams[b]	
		Initial	12 weeks	Loss, %	Initial	12 weeks
Dehydrated meals						
1	12.2	13.7	3.7	73	5.0	8.7
2	9.2	17.2	4.9	72	10.9	12.9
3	7.1	12.9	3.7	71	3.8	9.1
4	2.5	13.8	6.3	54	7.9	12.1
5[c]	2.5	13.8	9.0	35	7.9	21.2
6	8.3	18.1	7.6	55	13.6	18.4
Freeze-dried meals						
1	8.4	18.8	14.5	23	10.1	21.4
2	5.4	17.5	16.9	3	11.5	20.7
3	5.4	20.6	19.3	6	9.4	25.6
4[c]	5.4	20.6	19.7	4	9.4	25.0
5	3.3	18.1	13.1	28	9.3	25.3

[a] Stored 12 weeks at 90° F.
[b] Dry basis, average of duplicate analyses.
[c] Added 0.150% ethoxyquin.

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