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Antioxidant Activity of Ethoxyquin and the Effect of Storage Temperature on the Stability of Carotene in Dehydrated Alfalfa Meal which was treated with Ethoxyquin

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(Plate 1; Text-fig. 1; Tables 1~4)

NEED FOR THIS STUDY

Dehydrated forages, principally dehydrated alfalfa meal, are used widely as "vitamin concentrates" for addition to mixed poultry and live stock feeds. These products supply carotene, xanthophylls, α-tocopherols, vitamin K, vitamin C, and B-complex. Of special interest is carotene, for this compound is unstable during storage period, and may be lost from feed by oxidation. Many factors accelerate the oxidation of carotene in dehydrated forages: type of fatty acids present, temperature fluctuations, exposure to light (sun), surface area of materials exposed to air (oxygen). These several factors may hasten the acceleration of cell nutrients and thereby produce an adverse effect on carotene content. From this respect it is necessary to prevent the formation of oxidation in dehydrated meal.

Oxidation prevention methods applicable to most edibles, such as colored or opaque wrapping, airtight packing, are impractical for alfalfa meal. The only practical means of oxidation inhibition is a chemical (antioxidant) preservative. Several chemical stabilizers have been studied for preserving fat-soluble vitamins in dehydrated forages. KEPHART in 1949 proposed the use of DPPD*[1)1)] and two diphenylamine derivatives. DPPD was used in dehydrated alfalfa meal and mixed feeds for several years until adverse toxicity reactions were observed and its use was disallowed by the Federal Food and Drug Administration.

There are many other antioxidants such as DBH,*[2)] BHT,*[3)] and BHA.*[4)] But these are not effective in dry feed. THOMPSON showed that several phenylenediamines and dihydroquinolines particularly ethoxyquin preserved carotene in alfalfa meal. Ethoxyquin is presently allowed at the rate of 150 parts per million (0.015%)[2)].

The purpose of the work described below was undertaken to determine quantitatively the preservativeness of ethoxyquin and the effect of storage temperatures on the retention of carotene in dehydrated alfalfa meal which was treated with ethoxyquin. This is an important problem, especially, from the standpoint of consumers. They need to know how carotene in dehydrated forages is affected by the ordinary storage methods, lest it lose its strength after purchase. The studies presented here were carried out at the Kamo Dairy Farm, Miyuki Farm and University Reseach

*1)=N, N^1-Diphenyl-p-phenylenediamine
*2)=2, 5-Ditert-butyl-hydroquinone
*3)=Butylated-hydroxy-toluene
*4)=Butylated-hydroxy-anisole

Laboratory, Fukuyama, from June, 1963 until Augst, 1964.

SAMPLES AND EXPERIMENTAL PROCEDURE

Treated (ethoxyquin added) dehydrated alfalfa meal (pelleted) were obtained from a U. S. dehydration firm through U.S.F.G.C.*[5] Samples were recieved within one month of production. They were immediately stored in closed containers at 2–3°C temperature and kept there during the period of the stability test. Half of the samples were stored in closed containers under room temperature in the laboratory room. Both samples were stored under the same conditions except for the storage temperatures.

Carotene was assayed at the beginning and at the end of every two months. Moisture content of the representative samples at the outset was 6.5 percent (Exactly weighted samples of from 1 to 2 grams were placed in moisture dishes, heated 4 hours at 100° to 110°C. Then they cooled in a desicator and weighed). The carotene was analyzed by the method determined by the association of Vitamin Chemist, Inc. described as follows.

METHOD OF CAROTENE ASSAY[3]

Briefly, the method follows the following line. The color and fat are extracted from a sample. A known quantity is saponified and the total carotinoid coloring is extracted by a solvent. Xanthophyll, lutein, and the like are extracted by a solvent immishible in the first solvent, leaving the carotene, which may then be estimated colorimetrically.

1. Extraction

All samples were pulverized to a 40-mesh powder or finer, and weighed representative samples were rehydrated by covering them with a small quantity of hot water for 5 to 10 minutes to insure complete extraction.

Materials were saponified by blending with 12% alcoholic KOH for 30 minutes at room temperature. Then, about 20 *ml* acetone were added to extract and stirred for at least 30 seconds. This extraction was continued by stirring with successive 20*ml* portions of acetone and removing the solvent each time until the extract and residue were colorless.

The flask was rinsed with the same portions of petroleum ether and water. The extracts were shaken gently for at least 30 seconds and the layers allowed to separate. It is important to avoid vigorus shaking to prevent the formation of stable emulsions.

The water-acetone layer was drown into a second separatory funnel. This water-acetone layer was re-extracted. The same portion of water was poured through the combined petroleum ether extracts. It was washed about 3-5 times.

2. Purification (chromatography)

The absorption tube was attached to a filter flask and a plug of non-absorbent

*5) = U.S.Feed and Grain Council

cotton or glass wool was packed into the constriction of the absorption tube. Vacuum was applied and enough absorbent added to make the column 10*cm* in length. Samples were purified by Al_2O_3 chromatography. The adsorbent was pressed down firmly once or twice with a plunger.

One *cm* of Na_2SO_4 was placed over the top of the column. The column was wetted by washing it with 20-25*ml* of petroleum ether.

3. Absorption and Elution

The extract to be chromatographed was poured onto the column and suction was applied. The flask was rinsed with 5-to-10 *ml* portions of eluent (We used 10% acetone in the petroleum ether), pouring each rinsing onto the column before the previous one had been entirely absorbed into the Na_2SO_4. The column was washed continuously with eluent, adding the successive portions of eluent when the preceding one was just barely visible above the Na_2SO_4. If the column was allowed to become dry, air would be pulled through and destruction of carotene by oxidation would occur.

The rate of elution and the distinctness of the bands may also be varied by changing the percentage of acetone in the petroleum ether from 0 to 10%. Preliminary saponification of the sample with 10% alcoholic KOH often aids in securing chromatogram with the materials as dehydrated products. The washing was continued until the carotene pigment had moved off the column and the filtrate was colorless. The contents of the filter flask was transferred to a volumetric flask and diluted with eluent. The concentration of carotene should be 1 to 4 *mcg* per *ml*.

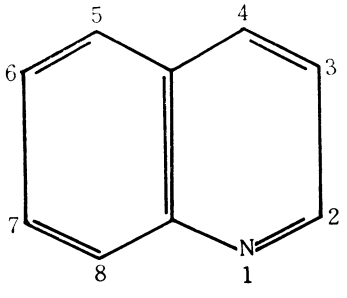
4. Determination of Concentration

The intensity of color at 450 *mμ* in the final solution of carotene was determined.

SYNOPSIS

The antioxydant, ethoxyquin has been recognized since 1951 as having the capability to protect carotene and α-tocopherol against oxidation, and has shown that ethoxyquin is a most effective antioxidant for fat-soluble pigments such as xanthophylls, carotene and α-tocopherol. Chemically, ethoxyquin is 1,2-dihydroxy-6-ethoxy-2,2,4-trimethylquinoline, a dark yellow-to-black liquid, and this is one of the derivatives of quinoline.

The quinoline nucleus is as follows:[4]



There are many other derivatives of quinoline such as 4, 8-Dimethyl-2-hydroxyquinoline, 5,7-Dichloro-8-hydroxyquinoline, 6,7-Dimethoxy-2,2,4-trimethyl-1,2-dihydroxyquinoline, etc.

E. M. BICKOFF, A.L. LIVINGSTON, J. GUGGOLZ, and C.R. THOMPSON as reported in Table 1[4] about the antioxidant effectiveness of quinoline and a number of its derivertives.

The most effective antioxidant studied was 6-Ethoxy-2,2,4-trimethy-1,2-dihydroquinoline (compound No. 38).

Table 1. Efficiency of Antioxidants Stabilizing Carotene in Alfalfa Meal

Compound No.	Name of Compound	Effect on Carotene Stability Carotene Remaining* in Alfalfa Meal, %	
		2 Weeks 65°C	18Mo., 25°C
Control	None	22	13
1	Quinoline	24	.....
2	8-Aminoquinoline	20	.....
3	4, 8-Dimethyl-2-hydroxyquinoline	22	15
4	8-Hydroxyquinoline	22	8
5	5, 7-Dichloro-8-hydroxyquinoline	4	2
6	Isoquinoline	24	.....
7	6-Methoxyquinoline	27	16
8	6-Phenylquinoline	27	14
9	6-Ethoxy-2, 4-dimethylquinoline	27	15
10	2, 6-Dimethylquinoline	28	16
16	6, 7-Dimethoxy-2, 2, 4-trimethyl-1, 2-dihydroquinoline	48	29
17	8-Ethyl-2, 2, 4-trimethyl-1, 2-dihydroquinoline	42	41
18	8, 2, 2, 4-Tetramethyl-1, 2-dihydroquinoline	53	41
20	8-Methoxy-2, 2, 4-trimethyl-1, 2-dihydroquinoline	40	37
21	6, 7, 8-Trimethoxy-2, 2, 4-trimethyl-1, 2-dihydroquinoline	43	44
28	8-Ethoxy-2, 2, 4-trimethyl-1, 2-dihydroquinoline	38	40
30	6-phenyl-2, 2, 4-trimethyl-1, 2-dihydroquinoline	48	45
33	6, 6, 2, 4-Tetramethyl-1, 2-dihydroquinoline	53	50
35	6-n-Butoxy-2, 2, 4-trimethyl-1, 2-dihydroquinoline	59	58
38	6-Ethoxy-2, 2, 4-trimethyl-1, 2-dihydroquinoline	59	60
40	6-Hydroxy-2, 2, 4-trimethyl-1, 2-dihydroquinoline	38	20

* % of initial carotene retained after 2 weeks' storage at 65°C and after 18 months' storage at 25°C.

Table 1 shows the comparative effects of antioxidants on the various compounds tested for carotene in alfalfa meal in the accelerated storage test. After 2 weeks' storage at 65°C the control sample of meal retained only 22% of its original carotene. The 6-ethoxy-2, 2, 4-trimethyl-1,2-dihydroquinoline was the most effective antioxidant in stability of any of the derivatives.

In January, 1961, ethoxyquin was approved by the FDA*[6] for use in animal

*6) =Food and Drug Administration, Department of Health, Education and Welfare, U.S.A.

feeds. The maximum quantity of the addition permitted to be used and to remain in the dehydrated forage crop shall not exceed 0.015 percent. (0.3 pound per ton in dehydrated forages).

It may be added to dehydrated forage prepared from[2]:

Alfalfa	Medicago Sativa
Barley	Hordeum Vulgare
Clovers:	
Alsike clover	Trifolium hybridum
Crimson clover	Trifolium incarnatum
Red clover	Trifolium pratense
White clover (including Ladino)	Trifolium repens
Yellow sweetclover	Melilotus officialis
White sweetclover	Melilotus alba
Coastal Bermudagrass	Cynodon dactylon
Fescue	Festuca sp.
Oats	Avena sativa
Orchardgrass	Dactylis glomerata
Reed canarygrass	Phalaris arundinacea
Ryegrass (annual and perennial)	Elymus sp. and Lolium perrenne
Wheat	Triticum aestivum

Or any mixture of such forage crops, for use only as an animal feed.
Recently, ethoxyquine is mostly used in dehydrated alfalfa meal to retain more carotene, and α-tocopherol and also to increase storage life.

RESULTS AND DISCUSSION

During 1963-1964 our research laboratory studied the carotene stability in dehydrated alfalfa meal which was treated with ethoxyquin and stored under inert gas until sent from the dehydrated feed firm in the U.S.A.

Test results in Table 2, 3 and 4 showed the effectiveness of ethoxyquin and the effect of storage temperature. After storage for 30 days the carotene content was determined. When untreated samples were stored at room temperature about 28–30°C for 30 days, the carotene content was reduced to about one third of the amount present originally. On the other hand, samples treated with ethoxyquin retained 75.40%. From the results obtained in this study (Table 2) it can be seen that ethoxyquin will significantly increase the retention of carotene during storage. Losses occured even in the treated samples though greatly reduced. If samles were not treated with ethoxyquin, the storage ability of carotene greatly declined.

Table 2. Efficiency of Ethoxyquin for Stabilizing Carotene in Alfalfa Meal

	%Carotene Retention 30 days at 29°C
Untreated Pellets	63.16
Treated Pellets	75.40

Comments: Samples stored in loosely covered vials at room temperature in our laboratory.

The storage tests showed that the temperature had effect on carotene stability (Table 3, 4). Carotene was analyzed initially and at the end of every two months. The assays of alfalfa meal were treated with the recomended level (150 parts per million) of ethoxyquin and stored at 2-3°C for fourteen months as shown in Table 3.

Table 3. Retention of Carotene in Alfalfa Meal

Period	Milligrams of Carotene per 100 grams	Units of Vitamin A per pound	%Carotene Retention
Initial	16.472	124,588	100
Two Month	14.935	112,963	90.67
Four 〃	12.096	91,444	73.40
Six 〃	10.762	81,400	65.34
Eight 〃	10.150	76,771	61.62
Ten 〃	9.155	67,245	55.79
Twelve 〃	8.912	67,007	54.10
Fourteen 〃	8.370	63,308	50.81

Comments: Material stored in an refrigerator at 2—3°C.

Samples which were stored at 2-3°C temperature retained 50.81 percent of the original carotene after fourteen months. On the other hand, the results showed that the dehydrated alfalfa meal stored at room temperature in the laboratory retained 32.85 percent of carotene (Table 4).

Table 4. Retention of Carotene in Alfalfa Meal

Period	Milligrams of Carotene per 100 grams	Units of Vitamin A per pound	%Carotene Retention
Initial	16.472	124,588	100
Two Month	14.127	106,852	85.76
Four 〃	10.272	77,694	62.29
Six 〃	8.352	63,172	50.71
Eight 〃	8.185	61,908	49.70
Ten 〃	7.641	57,794	46.39
Twelve 〃	6.878	52,023	41.76
Fourteen 〃	4.788	32,338	32.85

Comments: Samples held at room temperature in laboratory.

The difference of carotene retention was about 17.96 percent at the end of the tests in the samples stored at low and high temperatures. The average loss for each two months was 2.56 percent more for the samples stored at room temperature.

Losses occured even at low temperatures though at a greatly reduced rate and retained carotene more efficiently than the higher storage temperatures.

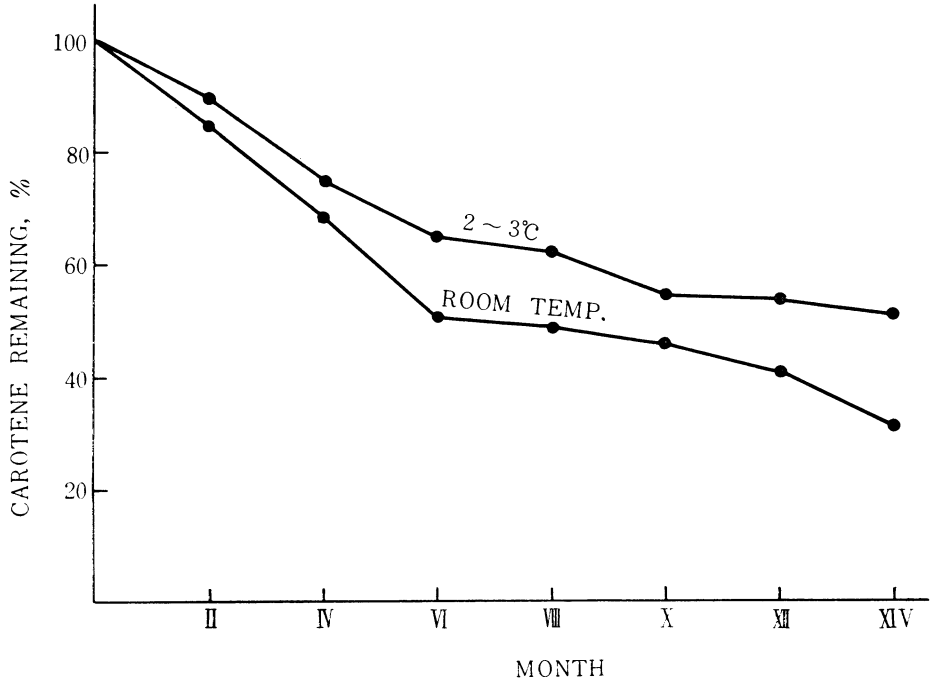


Fig. 1. Effect of Storage Temperature and Period on Stability of Carotene in Alfalfa Meal

The analytical data obtained in the study at every two months are shown in Fig. 1. It can be seen that the storage length affects the carotene contents of treated alfalfa meals. Loss of carotene increases with the storage length, and also the higher the temperature, the less carotene it will contain. Furthermore, the rate of loss is greater during hot summer weather than cold winter seasons.

At the end of the tests the carotene content was as follows: low temperature-stored meals, 8.370 *mg%*, high temperature-stored samples, 4.788 *mg%*.

CONCLUSION

Carotene, one of the most important precursors of vitamin A, is the component which perhaps has received most attention. Since the discovery of carotene by WACKENRODER in 1831, the distribution of carotenoids in nature has been much investigated.[5] Extensive studies have shown that all green parts of forages contain this pigment but this is only present in small amounts in forage crops. Then, its' importance should not be minimized.

Unfortunately, carotene is not a stable compound and may be lost from feed by oxidation.

Therefore it is important to prevent oxidation with respect to the content and

stability of carotene. According to studies ethoxyquin is the antioxidant preservative that most efficiently protects dehydrated alfalfa meal from oxidation loss during storage.

The datas obtained from these studies strongly sugested that the storage temperatures are one of the important factors in the retention of carotene of dehydrated meals even if it is treated with the stabilizer, ethoxyquin. But the destruction of carotene decreased with treated materials. Storage periods also had effects on the carotene contents, as the longer the period the less amounts it will contain.

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エトキシキンの抗酸化性およびエトキシキン処理脱水アルファルファミール中のカロチンの安定性におよぼす貯蔵温度の影響

高崎鉄之助

Dehydrated alfalfa meal は乾燥中の栄養の損失が少く特に脂溶性ビタミン類(カロチン，α-トコフエロール，ビタミンK等) の多い製品がえられるが，貯蔵中酸素に最も弱いカロチンは Alfalfa meal が酸素にふれる時間と温度に比例して酸化は増大してくる．故に酸化分解防止の途は低温貯蔵 (Cold Storage)，ガス貯蔵 (Inert Gas Storage) および有効な抗酸化剤 (Antioxidant) 利用の三点にある．特に最近は Alfalfa meal の野外貯蔵輸送等実際に使用直前まで之等損失を如何に最少限にとどめるかについて多数の抗酸化剤の研究が行われている．これら抗酸化剤中エトキシキン (Ethoxyquin) が1959年2月アメリカにおいて農務省食料薬事局によって認定され，1961年この化学製品の一般使用が開始され輸送中のビタミンの損失は大いに抑制された．

本実験結果は明らかにエトキシキンの効果を示し又低温貯蔵は更にその効果を倍加することを示している．即ち夏季高室温のもとで1ケ月間エトキシキン処理および無処理の試料を貯蔵した結果を見ると共にカロチンの損失を生じたが処理した場合の 24.60％に対し無処理のものは 36.84％の損失となり明らかに抗酸化剤エトキシキンの効果が認められた．

又貯蔵温度がエトキシキン処理のカロチン含量におよぼす影響を見ると，貯蔵14ケ月後低温貯蔵では当初含量の50.81％を保有し高温貯蔵のサンプルは 32.85％に減少して両者間に17.96％の差を生じた．この事実は例え抗酸化剤添加処理製品でも含有カロチンの保存上低温不変の条件下に貯蔵することの重要性を示している．更に貯蔵期間の延長と共に両者 (高，低温貯蔵) 何れの場合もカロチン含量は漸減することが見られたが，低温貯蔵では月平均 3.5％，室温(高温)ではこれが 4.7％であった．かかる貯蔵温度の相違により14ケ月後の Dehydrated alfalfa meal 中のカロチン含量は前者 8.37 mg％ (63,308A(I.U./lb))，後者 4.788mg％(32,338A(I.U./lb)) となり両者間に3.582mg％の甚だしい差が見られた．

Both pelleting alfalfa meals were used in the tests. The right group is a sample of pellets which were dehydrated and had ethoxyquin added. The pellets at the left show field cured and untreated samples. The right samples still have a bright green color and good aroma after a considerable period in storage.

They have 16.472 mg% (124,588 I.U./lb) of carotene. The left lot, the field-cured, untreated control samples, have either a trace of green color or are slightly discolored, and have a carotene content of only 2.463 mg% (18,629 I.U./lb).

Note the color of the pellets. If a pellet contains a large amount of carotene it usually has bright green color as seen in the photograph. This is because it's dehydrated and treated with stabilizer, ethoxyquin.