THE EFFECT OF ANTIOXIDANTS ON THE STABILITY OF
VITAMIN A IN A VITAMIN-MINERAL PREMIX

by

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INTRODUCTION

Vitamin A is of nutritional importance in human and animal food products. It is necessary for optimal growth, vision, and reproductive performance of practically all higher animals. Its nutritional importance in the maintenance and production of livestock and poultry has been realized for many years. The instability of this vital nutrient has led to numerous investigations into the problems associated with loss of vitamin A potency in feeds and foods. Aspects of food production, manufacturing, and storage have been studied as possible areas for retarding the process of vitamin A deterioration. Loss of vitamin A is believed to be due to oxidation, which is accelerated and catalyzed by ultraviolet and visible radiation, moisture, elevated temperature, and metallic ions. Vitamin A loss occurs particularly in the presence of high humidity.

Developments in animal and human nutrition have brought about changes in the sources of vitamin A used in feeds and foods. Fish liver oil and foods high in provitamin A (carotene), which were once the major supplemental sources of vitamin A, have been replaced for the most part by synthetic forms of vitamin A such as retinol and retinyl palmitate or acetate. Antioxidants, that occur naturally or as commercial additives, inhibit vitamin A deterioration. For the past 30 years manufacturers have used microencapsulation of vitamin A into various matrices such as casein, fat, wax, and gelatin to protect vitamin A from such environmental factors as light, oxygen, moisture, and feed or food constituents.

Vitamin-mineral premixes are used widely as vehicles for the vitamin A enrichment of feeds by the formula feed industry. Premixes are

mixtures containing concentrates of vitamins and minerals with some diluent and/or carrier. They facilitate uniform dispersion of micro-ingredients into larger batches of feeds and foods.

The nutritional value of the final feed or food product is diminished if vitamin A activity is unstable. The guaranteed vitamin A value of feeds, foods and premixes would be maintained better if an appropriate antioxidant could be incorporated to increase the stability of vitamin A.

Factors affecting vitamin A loss in vitamin-mineral premixes have not been studied adequately. Most current information regarding this problem was compiled as manufacturer's inhouse investigations. Results of these studies are used in sales promotions and are not readily available to the public.

This study was designed to determine the effect of three commercial antioxidant preparations on the stability of vitamin A in a poultry vitamin-mineral premix. Stability of vitamin A in the premix was tested under the especially stressful conditions of high humidity and elevated temperature. Three storage conditions used in this study were: high temperature-high humidity, high temperature-low humidity, and ambient room temperature.

REVIEW OF LITERATURE

Vitamin A in Feeds and Foods

In the past 50 years there has been a change in animal production feeding practices. The use of fresh feeds has been supplemented by the use of mixed feedingstuffs (Nadai and Brubacher, 1970). The need for larger quantities and convenient sources other than that provided by natural ingredients led to the formulation of synthetic and stabilized products of vitamin A. The instability of vitamin A has been recognized for many years, and many investigators have searched for methods to stabilize this nutrient.

In the 1930's alfalfa meal, alfalfa leaf meal, yellow corn, and other provitamin A-containing products and liver oils were major sources of vitamin A utilized by feeders and manufacturers of commercial feeds. Alfalfa meal and other leafy vegetable sources of vitamin A contain carotene, a provitamin compound which is converted into retinol in vivo and stored as an ester in the liver. β-carotene, the most widely distributed provitamin, is and has been an important source of vitamin A activity in animal and human nutrition. Cryptoxanthin (possessing half the vitamin A activity of β-carotene), a provitamin found in yellow corn, is important nutritionally because of the large human and animal consumption of corn. The α- and γ-carotene forms also are found widely in plants but, because of smaller concentrations, are less important nutritionally (Green, 1970).

The stability of carotene was studied concurrently with the stability of vitamin A in several early works. Fraps and Kemmerer (1937), Record et al. (1937), Fraps and Treichler (1933), Almquist et al. (1938),

Bolin (1939), and Bethke et al. (1939) were among those who tested the stability of carotene in feeds. Fraps and Treichler (1933) reported a gradual loss of vitamin A activity in alfalfa leaf meal and dried green sweet peppers during storage at room temperature. It was concluded that the destruction of vitamin A activity varies with the length of storage period, temperature and type of feed ingredients. Guilbert (1935) found a decrease in alfalfa carotene of 30 to 50% during room temperature storage with practically no loss at -5 to 0°C in samples stored for 8 weeks. Others have found that carotene was destroyed especially in the presence of high storage temperatures (Kane and Shinn, 1937; Smith, 1939; Taylor and Russell, 1938).

In food products, such as edible fats and oils, the stability of vitamin A incorporated as fish oil was studied. Dastur (1937) and Doctor and Banerjee (1939) found that light was more active than heat in destroying vitamin A (from fish oil) in ghee. Feigenbaum (1946) fortified margarine with fish liver oil vitamin A concentrate containing 300,000 I.U. per gram incorporated at 30 I.U. per gram of margarine. He found that temperatures over 40°C depleted vitamin A content.

Holmes and Corbet (1936) and Baxter and Robeson (1940) isolated crystallized products of vitamin A from fish liver oils. Experimental work utilizing these types of vitamin A products in foods and feeds is limited.

During the late 1940's and early 1950's dry vitamin A preparations were made commercially. These products were advertised as more stable than vitamin A oils. They consisted of fish oil sprayed on carriers such as vegetable flours, mineral products and milk powder (Kläui et al., 1970). Burns and Quackenbush (1951) studied several dry vitamin A

products which contained 150 to 1500 micrograms of vitamin A or carotene per gram. Four different storage tests were made, which included comparisons between these products and fish oil products. Samples were placed in screw cap bottles and stored at room temperature in a dark room. Vitamin A was most stable when incorporated into products containing vegetable meals or natural antioxidants.

Reid et al. (1955) compared the stability of an encapsulated product (a vitamin concentrate sealed in microcrystalline wax) and a fortified fish liver oil. Stability characteristics of the two products was observed in several types of mixed feeds and premixes. Samples were stored under warehouse (76-93°F) and cold room (42°F) conditions for 16 weeks. Biological availability and stability of vitamin A was measured by chick and poult growth and vitamin A in livers. The stabilized concentrate was more effective than the fish liver oil in its retention of vitamin A potency as evaluated chemically and biologically.

Crystalline vitamin A esters were used in feeds to a limited degree during the early 1950's. Castano et al. (1951) found that crystalline vitamin A acetate was more effectively utilized by turkey poults than pure crystalline carotene and vitamin A from "black cod" liver oil. Levels of vitamin A added to feeds ranged from 500-24,000 I.U./kg. The feeding period lasted for 8 weeks. They used growth rate, liver storage, and blood plasma levels of vitamin A as indicators of utilization. The results demonstrated that the lowest liver storage occurred with crystalline carotene and the greatest liver storage occurred when vitamin A acetate was fed to turkey poults and chicks (Castano et al., 1951). Gurcay et al. (1950) found that a higher concentration of pure crystalline carotene, than vitamin A acetate, was needed to promote liver

storage in turkey poults. Crystalline vitamin A acetate, black cod liver oil, and crystalline carotene were used as vitamin A sources in the turkey poults' feed. Levels of 500 I.U./kg to 24,000 I.U./kg were used for each source of vitamin A. In the two forgoing tests vitamin A that was added at the higher levels may have interfered with obtaining useful test results.

A contemporary stabilization method for vitamin A involves the micro-encapsulation of this nutrient into a matrix. Substances used for matrices include gelatin, casein, vegetable gum, and cellulose. Upon micro-encapsulation, air is excluded. This limits the interaction between vitamin A and oxygen as well as other substances used in the feed products. Encapsulated vitamin A is considered the most stable form of this nutrient at the present time.

Stephenson (1953) compared two stabilized vitamin A products with a feeding oil containing an antioxidant. The stabilized products included a wax-coated natural vitamin A and a gelatin coated synthetic vitamin A acetate. Each product was mixed with a conventional poultry mash at 2,000 I.U. per pound. This level was based upon National Research Council recommendations for chicks. Comparative feeding values after 12 weeks of storage in a poultry broiler house indicated all three vitamin A supplements supported optimum growth. Davies and Worden (1953) and Halverson and Hendrick (1955) reported that mixed in feeds vitamin A as a dry concentrate was much more stable than as an oil supplement.

The micro-encapsulation of vitamin A brought up questions as to the relative utilization of the vitamin when enclosed in matrices of wax, gelatin, ethyl cellulose, gum acacia, and hard fat. Olsen et al. (1959) studied the utilization by chicks of several dry products in which

vitamin A was encapsulated as acetate in gelatin, palmitate in gelatin, starch and sugar; oil in microcrystalline wax, and oil in hydrogenated fat (a dry product). Their results based on storage of vitamin A in livers indicated that vitamin A in gelatin-coated products (250,000 I.U. per gram) was better utilized than vitamin A in wax products (250,000 I.U. per gram), fat coated (10,000 I.U. per gram) or vegetable protein (5,000 I.U. per gram) stabilized products. Brubacher (1962) used a tracer technique to observe absorption efficiencies of gelatin stabilized vitamin A and unstabilized vitamin A. Their results were comparable to those of Olsen et al. (1959).

Adams (1974) found no difference in the stability of three vitamin A palmitates and two vitamin A acetates as dry protected products in a pelleted feed. In one phase of his study the concentration and type of antioxidant present and particle size distribution had more influence on the stability of the vitamin A than did the type of ester.

Stability of Vitamin A in Feeds and Foods

Vitamin A in feeds and foods is susceptible to loss of nutritional value due to oxidation by atmospheric oxygen accelerated by radiation in the ultraviolet and visible ranges, metallic ions, heat, and humidity. Particle size, composition of the product and processing methods are other factors that may affect vitamin A stability (Kläui et al., 1970).

In early reports Bethke et al. (1939) found that with the addition of meat scraps and dried skim milk a greater loss of natural vitamin A (cod liver oil) occurred in a basal chick diet. Holder and Ford (1939) found no such loss with incorporation of meat scraps.

The effect of minerals on the destruction of vitamin A in feeds has been studied. Miller et al. (1942) added manganese sulphate (0.5%) to a commercial feed containing cod liver oil at 1,870 U.S.P. units per gram as the source of vitamin A. They found no vitamin A remaining after storage for 56 days. The same ration with no manganese had 78% of the vitamin A remaining. Creek et al. (1960) found that vitamin A was unstable with the addition of manganese sulfate to a complete ration.

The use of free trace mineral additives to commercial rations increased during the 1950's. Halverson and Hendrick (1955) studied the stability of three vitamin A sources in vegetable type diets containing various combinations of minerals. Vitamin A supplements were added to the ration at levels of 0.5, 0.3 and 1.5 percent. Supplements were incorporated into the diet by premixing with portions of the diet before blending with the batch. Samples were placed in paper sacks and stored for 150 days at a temperature of 37 ± 1.5°C. Vitamin A was determined by the Carr-Price colorimetric reaction. The researchers concluded that a greater loss of vitamin A occurred when a combination of trace minerals that included limestone, manganese salt, and iron, copper and cobalt salts were present.

The effect of storage time and storage temperature as deteriorative factors for natural vitamin A was studied by Fraps and Treichler (1933). The stability of vitamin A in various dried food products was determined after storage for 19 months. After 11 months of storage all samples showed more than a 50% decrease in vitamin A content. They concluded that the storage period was an important factor in the stability of vitamin A and suggested that there might be decreased losses if products were placed in cold storage. Fraps and Kemmerer (1937) studied the

stability of cod liver oil in feed mixtures. They found that after storage for 2 weeks at an average temperature of 66-96°F, there was a loss of 34 to 74% vitamin A at 6°C, 73 to 89% at room temperature, and 73 to 100% at 35°C. They concluded that storage at 6°C provided better stability of vitamin A than storage at higher temperatures.

Baird et al. (1939) stored chick rations in burlap bags at summer temperatures for 25 weeks. The vitamin A source was cod liver oil. Results indicated an increase in vitamin A destruction as the storage period increased. Reid et al. (1955) studied the stability of vitamin A in a cod liver oil and in a stabilized vitamin A concentrate (sealed in microcrystalline wax) blended into various feeds and premixes. Although the stabilized vitamin A retained its potency better than that of the fish oil source, both lost vitamin A activity as storage time increased. Upon storage at a lower temperature (42°F), vitamin A in the fish liver oil showed a decreased rate of loss. Wall and Kelley (1951) found similar results when a poultry mash was supplemented with various vitamin A and carotene concentrates. Results suggested that with decreased storage temperature vitamin A stability was increased.

Holmes and Pigott (1942) studied the effect of light on medicinal cod liver oils and on an oleo vitamin A and D preparation dissolved in three types of edible vegetable oils. Samples were stored in glass bottles with screw cap closures and placed in direct sunshine at an approximate temperature of 86°F during the day and 50°F at night. Vitameter assay was made before and after exposure. The cod liver oil source retained vitamin A potency better than the vegetable oil mixtures.

Davies and Worden (1953) studied the effect of particulate size, temperature, light, and air on the stability of cod liver oil vitamin A

source used in a typical feedingstuff. They concluded that ration of coarse particulate size lost vitamin A potency more rapidly than the same ration of medium particulate size under the various storage conditions. They suggested that vitamin A as cod liver oil be incorporated by spray-gun immediately before feeding. They also found that cubing a ration increased stability of vitamin A palmitate. Nir et al. (1980) compared the stability of protected vitamin A in pelleted and unpelleted feeds by both biological and analytical procedures. They found that pelleting decreased the vitamin A recovery to a slight degree but did not affect the biological potency. They suggested that consideration be given to the effects of hydrothermic treatment on vitamin A stability in the processing procedure of pelleted rations.

<div align="center">Antioxidants as Stabilizing Agents for
Vitamin A in Feeds and Foods</div>

Natural Sources of Antioxidants

The use of natural antioxidants as vitamin A stabilizers was studied by Miller (1935). He reported that cottonseed meal may function as a preservative for vitamin A as indicated by increases in chick weights during a feeding study. Holmes et al. (1936) found that vitamin A in halibut liver oil could be protected by the combination of lecithin and hydroquinone as antioxidants. Feigenbaum (1946) reported that vitamin A from fish liver oil in margarine prepared from vegetable oils and hardened fats could be protected during processing by the addition of a commercial soya bean lecithin, added at 0.25% levels. The processing temperatures did not exceed $40°C$. He reported that after six weeks the original vitamin A content of 30 I.U. per gram remained. Presently

tocopherol and ascorbic acid are two natural antioxidants added in the stabilization of encapsulated vitamin A products used in foods and feeds (Bauernfeind and Cort, 1974).

Commercial Sources of Antioxidants

Burns and Quackenbush (1951) found that the highest stability of vitamin A in dry commercial products was exhibited by those that contained vegetable meals or antioxidants, e.g. butylated hydroxyanisole (BHA). Hydroquinone used in feeds at 0.1% prevented the destruction of vitamin A as cod liver oil during the first week of storage (Fraps and Kemmerer, 1937). Fritz et al. (1956) used BHA and diphenyl-para-phenylene diamine (DPPD), Matterson et al. (1955) used DPPD, and Monson et al. (1957) used DPPD in feeds containing vitamin A in oil. They concluded that the antioxidants preserved the vitamin A in the feed mixtures.

Siedler and Schweigert (1954) studied the effect of adding the antioxidant BHT (0.02%), citric acid (0.01%) and propyl gallate (0.005%) to inedible pork fat which was to be used in dry dog meal. After one year of storage at room temperature, the stabilized fats added at the 6% level retarded vitamin A loss. In a later study Siedler et al. (1956) studied the effectiveness of citric acid, BHA, butylated hydroxytoluene (BHT), DPPD, Santoquin (ethoxyquin), and combinations of antioxidants-- BHA plus 2,5,-di-tert-amylhydroquine (DAH) and BHA plus BHT. Poultry-type feeds were compounded to include choice white grease and tallow mixture (1:1), which was stabilized with 0.02% of each antioxidant, and contained vitamin A added at 2250 U.S.P. units per gram. All antioxidants used were effective in stabilizing the vitamin A with santoquin (0.02%) being the most effective antioxidant of those tested.

Mechanism of Antioxidant Action

When fat containing substances react with oxygen, free radicals may
be formed. Free radicals contain unpaired electrons which contribute to
their highly reactive characteristics, in biological systems. During
oxidation, free radicals act as catalysts to accelerate the continued
breakdown of fat in a chain reaction which causes the formation of
peroxides. Vitamins A and E, and the provitamin (β-carotene) are
susceptible to attack by free radicals (oxidation), which results in
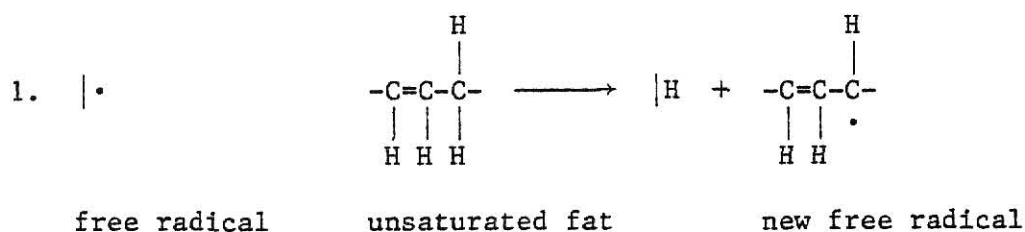their loss of potency.

The idea that oxidation could be inhibited by the use of antioxidants
as chain breaking substances has been accepted for years. Antioxidants
have been characterized as free radical scavengers because they inhibit
free radical formation. Shelton (1959) suggested that the antixoidant
mechanisms occurring in fats could be grouped into four categories:
(1) hydrogen donation by the antioxidant, (2) formation of lipid-
antioxidant reaction product, (3) electron donation by the antioxidant,
and (4) addition of the lipid to the aromatic ring of the antioxidant.
Stuckey (1968) proposed that the antioxidant mechanism involved a
combination of the above factors. Ethoxyquin is believed to exert its
effect by giving up the hydrogen attached to nitrogen and then two
residual molecules combine to form a dimer (Heller's dimer).[1] Most
studies of the free radical oxidation-antioxidant mechanisms have been
related to unsaturated fats, with only limited studies devoted to anti-
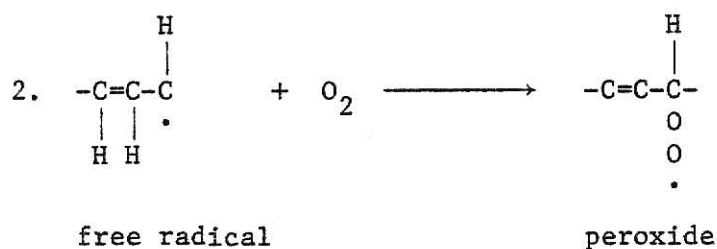oxidant protective mechanisms involved in the stabilization of vitamin A.

[1] Dr. William Shermer, Senior Research Specialist, personal communica-
tion, 1983, Monsanto Co., St. Louis, Mo.

Vitamin A and carotene have unsaturated ring structures and conjugated unsaturated chain linkages (Stuckey, 1962). The oxidation of vitamin A as catalyzed by free radicals may be similar to the oxidative reactions in fats. Siedler and Schweigert (1954), Buxton (1947), and Dugan et al. (1950) all found that vitamin A loss in a fish liver oil paralleled the increase in peroxide value of fat. Siedler and Schweigert (1954) reported an increased stability of vitamin A (in fish liver oil) and decrease in peroxide values when antioxidants were used to stabilize fat in a commercial feed. Budowski and Bondi (1960) found that an antioxidant (DPPD) addition to a paraffin solution containing carotene, vitamin A and an unsaturated fat, lengthened the induction period (period before rapid deterioration occurs) of the fat and vitamin A and conversely decreased the rate of autoxidation.
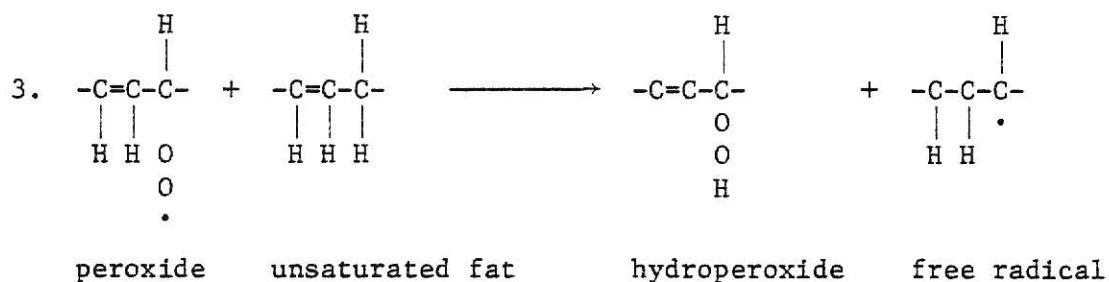
Presently the free radical theory is the most accepted explanation of oxidation. The scheme (Diamond Shamrock, 1980) may be characterized as follows:

1.
$$ |\cdot \quad + \quad \underset{\substack{| \ | \ | \\ H\ H\ H}}{-C=C-\overset{\overset{H}{|}}{C}-} \quad \longrightarrow \quad |H \quad + \quad \underset{\substack{| \ | \\ H\ H}}{-C=C-\overset{\overset{H}{|}}{C}-}\cdot $$

free radical   unsaturated fat   new free radical

(A hydrogen atom is removed from the fat creating a new free radical.)

2.
$$ \underset{\substack{| \ | \\ H\ H}}{-C=C-\overset{\overset{H}{|}}{C}}\cdot \quad + \quad O_2 \quad \longrightarrow \quad -C=C-\overset{\overset{H}{|}}{\underset{\substack{O \\ O \\ \cdot}}{C}}- $$

free radical   peroxide
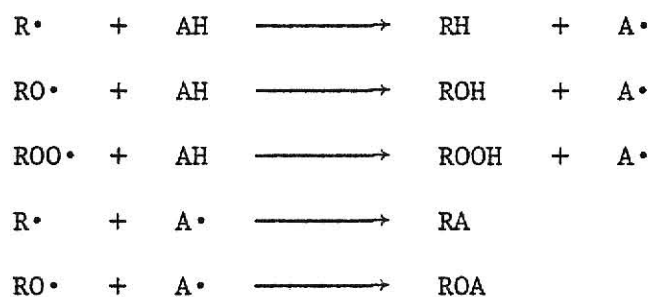
(Oxygen may combine with the new free radical to form a peroxide.)

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        H                H                              H                    H
        |                |                              |                    |
 3.  -C=C-C-    +     -C=C-C-      ───────────→    -C=C-C-       +      -C-C-C-
      |  | |            |  | |                          |                  | |  •
      H  H O            H  H H                          O                  H  H
           |                                            |
           O                                            H
           •
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peroxide   unsaturated fat   hydroperoxide   free radical

(Oxygen also may become a free radical initiator.)

It has been suggested that antioxidants intercept the oxygen before it attacks foodstuff constituents (i.e. fats or fat-soluble vitamins) leading to the formation of free radicals, with the antioxidant being destroyed in the process (Kläui et al., 1970). The following equations illustrate the reaction sequence of a free radical combining with an antioxidant (Dugan, 1976).

$$R\cdot \quad + \quad AH \quad \longrightarrow \quad RH \quad + \quad A\cdot$$

$$RO\cdot \quad + \quad AH \quad \longrightarrow \quad ROH \quad + \quad A\cdot$$

$$ROO\cdot \quad + \quad AH \quad \longrightarrow \quad ROOH \quad + \quad A\cdot$$

$$R\cdot \quad + \quad A\cdot \quad \longrightarrow \quad RA$$

$$RO\cdot \quad + \quad A\cdot \quad \longrightarrow \quad ROA$$

(R· - free radical, A - antioxidant, O - oxygen, H - hydrogen)

Final products of this chain of reactions consist of antioxidants and lipid molecules.

MATERIALS AND METHODS

Antioxidants

Santoquin, the commercial name for ethoxyquin; Endox, a trade name
for a commercial mixture containing EDTA (disodium ethylenediamine
tetraacetate); BHA (butylated hydroxyanisole), phosphoric acid, mono-
and diglycerides as emulsifiers and inert carriers such as cobmeal and
oat hulls; and BHT (butylated hydroxytoluene) were the three antioxidant
products used in this study. All antioxidants were dry products.[1]
Structures and chemical names for BHT and Ethoxyquin are given in
Figure 1.

Preparation and Storage of Samples

Eight 50-lb bags of poultry layer vitamin-mineral premix were
received from the Dale Alley Company. Composition of the premix is given
in Table 1. Three antioxidants, Santoquin (ethoxyquin) or (EQ), butylated
hydroxytoluene (BHT) and Endox (EN), were incorporated into the premix at
levels of 0.1% and 2.5%. Samples with no antioxidant added were used as
controls. A 50-lb bag of premix was selected randomly for addition of
each antioxidant and the control for a total of seven treatments (Table
2), leaving one untreated bag as a reserve.

To facilitate thorough mixing of antioxidant into premix, the
indicated quantities of antioxidants (Table 2) were hand mixed with 3 kg

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[1] Dale Alley Co., St. Joseph, MO, provided the Endox and BHT, and
Monsanto Chemical Co., St. Louis, MO provided the ethoxyquin (trade
name, Santoquin).

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CH₃
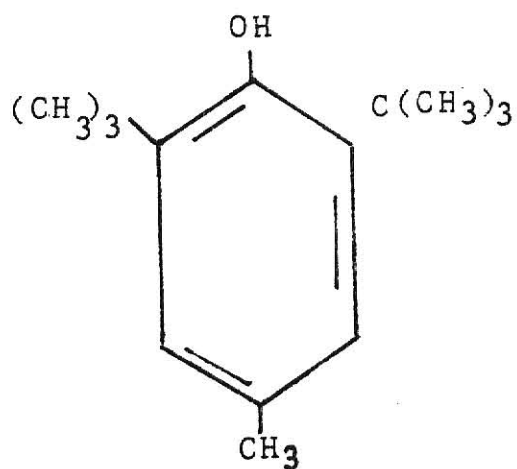
BHT
Butylated hydroxytoluene
2, 6- di- tert. butyl-4-methyl-phenol

H
N   CH₃
         CH₃

C₂H₅O

CH₃
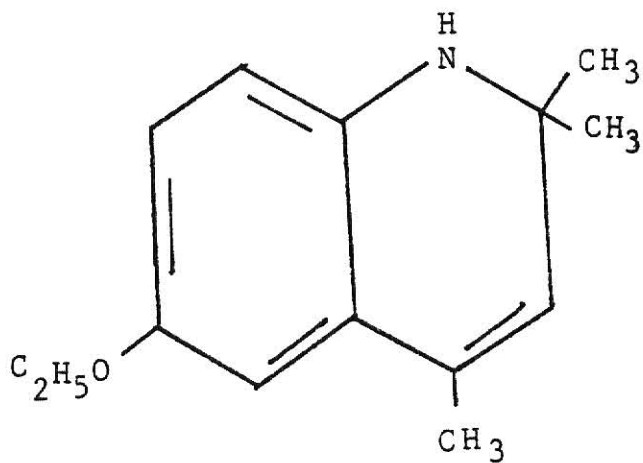
Santoquin
Ethoxyquin
1,2-di-hydro-6-ethoxy-2,4,4,-trimethylquinoline

Fig. 1-Structures and chemical names of two experimental antioxidants.

Table 1—Composition of poultry layer vitamin-mineral premix for the study of the effects of antioxidants on vitamin A stability

Feed constituent	Minimum guarantee per pound	
Manganese	1.7	%
Iron	2.4	%
Copper	.14	%
Iodine	.039	%
Zinc	2.4	%
Selenium	.004	%
Vitamin A	1,200,000	USP units
Vitamin D_3	400,000	USP units
Vitamin E	1,000	I U
Menadione	200	Mgs.
Riboflavin	805	Mgs.
d-Pantothenic acid	1,200	Mgs.
Niacin	2,500	Mgs.
Choline	80,000	Mgs.
Vitamin B_{12}	2	Mgs.
Pyridoxine	205	Mgs.
Thiamine	202	Mgs.
d-Biotin	20	Mgs.
Folic acid	60	Mgs.

Ingredients

Vitamin A Acetate,[a] d-Activated Animal Sterol, dl-alpha Tocopheryl Acetate, Menadione Sodium Bisulfite Complex, Riboflavin Supplement, Calcium Pantothenate, Niacin, Choline Chloride, Vitamin B_{12} Supplement, Pyridoxine Hydrochloride, Thiamine Hydrochloride, Biotin, Folic Acid, Manganese Oxide, Iron Sulfate, Copper Sulfate, Calcium Iodate, Zinc Oxide, Sodium Selenite, Calcium Carbonate and Mineral Oil.

[a] Manufactured by Hoffman-La Roche Inc., Nutley, New Jersey. Contains vitamin A acetate encapsulated in gelatin, sugar, modified food starch and glycerin compounded with 7.5% ethoxyquin as preservative.

Table 2-Amount (g) of antioxidants added at 0.1% and 2.5% to 50 pound bags of a vitamin-mineral premix

Treatments	Ethoxyquin[a]	BHT[b]	Endox[c]
No antioxidant	--	--	--
0.1% Endox	--	--	23
0.1% BHT	--	23	--
0.1% Ethoxyquin	34	--	--
2.5% Endox	--	--	568
2.5% BHT	--	579	--
2.5% Ethoxyquin	860	--	--

[a] Santoquin is the Monsanto Chemical Company brand name for the compound ethoxyquin. It consists of 66% ethoxyquin absorbed on a vermiculite carrier.

[b] Butylated hydroxytoluene product was 98% BHT and 2% inert material. Supplied by Dale Alley Company, St. Joseph, Mo.

[c] Endox is a commercial mixture of EDTA, BHA, phosphoric acid, and monoglycerides, manufactured by Diamond Shamrock Corporation, Cleveland, Ohio.

of premix in a stainless steel bowl. Then this was added to the rest of a 50 pound batch in a mixer.[1] The mixing rate was 25-27 rpm for 15 min.

After mixing, each 50-lb batch was emptied into a container and distributed randomly into seven subsamples. Six subsample sets of approximately 5 lbs each were placed in triple ply paper bags, used commercially for premixes. Three bagged samples were stored in an environmentally controlled room with high temperature (108.3 ± 1.5°F) and high humidity (78 ± 6.9% RH) (HT-HH). Another identical set of three bagged samples was stored at ambient room temperature (79.4 ± 2.8°F) (RT) with average humidity of 59.1 ± 9.0%. A third set of three samples (approximately 3½ lbs) was stored in amber 5 lb glass containers, and sealed with tight lids and parafilm.[2] These samples, labeled as high temperature-low humidity (HT-LH), were stored in the same temperature controlled room as those stored under HT-HH conditions. Samples were stored for 5 months. Another sample, approximately 1½ lbs, was used for determination of initial vitamin A content. A similar sample set was stored in closed glass containers and kept in a freezer (0°C) as a reserve sample. Duplicate vitamin A analyses were performed at 0-, 1-, 3- and 5-month intervals. The AOAC (1980) Method, 43.008, Vitamin A in Mixed Feeds, Premixes, and Foods was used to determine vitamin A content.

Analysis of Samples

The procedure for handling samples initially for the vitamin A determination and at the end of each storage period was as follows:

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[1] Cube tumble mixer used, could handle up to a 50 kg batch per mix.

[2] Five to ten g of indicating drierite (anhydrous $CaSO_4$ crystals) in small paper bags was placed in the sample containers to monitor humidity.

Samples were poured from storage containers into a cone formation on a large piece of wrapping paper. The cone shaped batch was quartered and two opposite quarters were removed. The remaining portion was mixed on the paper by rolling for approximately two min. This was quartered and analytical samples taken by the random removal of portions from the four sections. Sampling was as follows: Two 10-g portions were used for vitamin A analysis and approximately 1 lb was placed in glass containers and stored in a freezer as a reserve.

The AOAC Method (1980) is a photoelectric colorimetric determination of vitamin A with the Carr-Price ($SbCl_3$) reagent. A brief outline of the method follows: A 10-g sample was hydrolyzed under gentle reflux (alkaline digestion) for 30 min. The hydrolyzed solution was diluted to a volume of 260 ml with an alcohol-water solution (3:1) and mixed thoroughly. After the suspended matter settled, 20.0 ml of solution was removed and vitamin A was extracted with 20.0 ml of hexane. An aliquot (1.0-10.0 ml, depending on vitamin A concentration), was evaporated under vacuum by air aspirator and by gently shaking of the tube in a H_2O bath at 60-65°F to remove the solvent. The solvent-free residue was then dissolved in 1 ml $CHCl_3$. Measurement of vitamin A was made using an Evelyn Photoelectric Colorimeter.[1] The intensity of a transitory blue color correlated with content of vitamin A. Vitamin A was calculated as in the AOAC method (1980).[2] Duplicate determinations were made on each sample.

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[1] Electro-Nite Co., Rubican Instruments Division, Comply and Decatur Roads, Philadelphia, Penn., 19154.

[2] Details of the method, reagent and materials are in AOAC Methods of Analysis (1980).

Since samples stored at HT-HH conditions had a low vitamin A level after one month, additional samples were taken from room temperature storage samples and placed under HT-HH conditions. Samples were analyzed weekly for 3 weeks to check on the rate of loss of vitamin A under HT-HH storage conditions.

RESULTS AND DISCUSSION

The effects of selected antioxidants on vitamin A content of a vitamin-mineral premix stored under room temperature, high temperature-high humidity and high temperature-low humidity storage conditions are discussed below.

Room Temperature Storage

Storage temperature throughout the experiment was $79.4 \pm 2.8°F$ and relative humidity was $59.1 \pm 9.0\%$. Vitamin A content (Table 3) and percentages vitamin A retention (Figure 2) at various storage times are presented.

Generally, vitamin A content decreased rapidly from the first through the third month of storage and then leveled off. The most effective antioxidant under room temperature storage conditions appeared to be the ethoxyquin at the 2.5% level. The 2.5% BHT and 0.1% ethoxyquin levels appeared to be somewhat effective up to the 3 month period. In the samples containing no antioxidant and in all treatments except 2.5% ethoxyquin, vitamin A retention was minimal after 3 months storage.

To estimate vitamin A content at selected storage times, least square equations were obtained by the general linear model procedure as outlined below:

Table 3—Vitamin A (I.U./lb) remaining[a] after storage of a poultry vitamin-mineral premix at room temperature

Treatment	Months of storage			
	0	1	3	5
No antioxidant	1,123,196	915,718	73,548	20,304
Endox 0.1%	1,123,196	966,566	38,590	19,976
Endox 2.5%	1,115,478	894,834	42,222	21,974
BHT 0.1%	1,131,368	917,988	57,658	21,406
BHT 2.5%	1,054,680	931,154	190,226	6,061
Ethoxyquin 0.1%	1,115,478	933,412	193,858	43,493
Ethoxyquin 2.5%	1,107,760	1,099,588	620,618	466,258

[a] Average of two analyses.
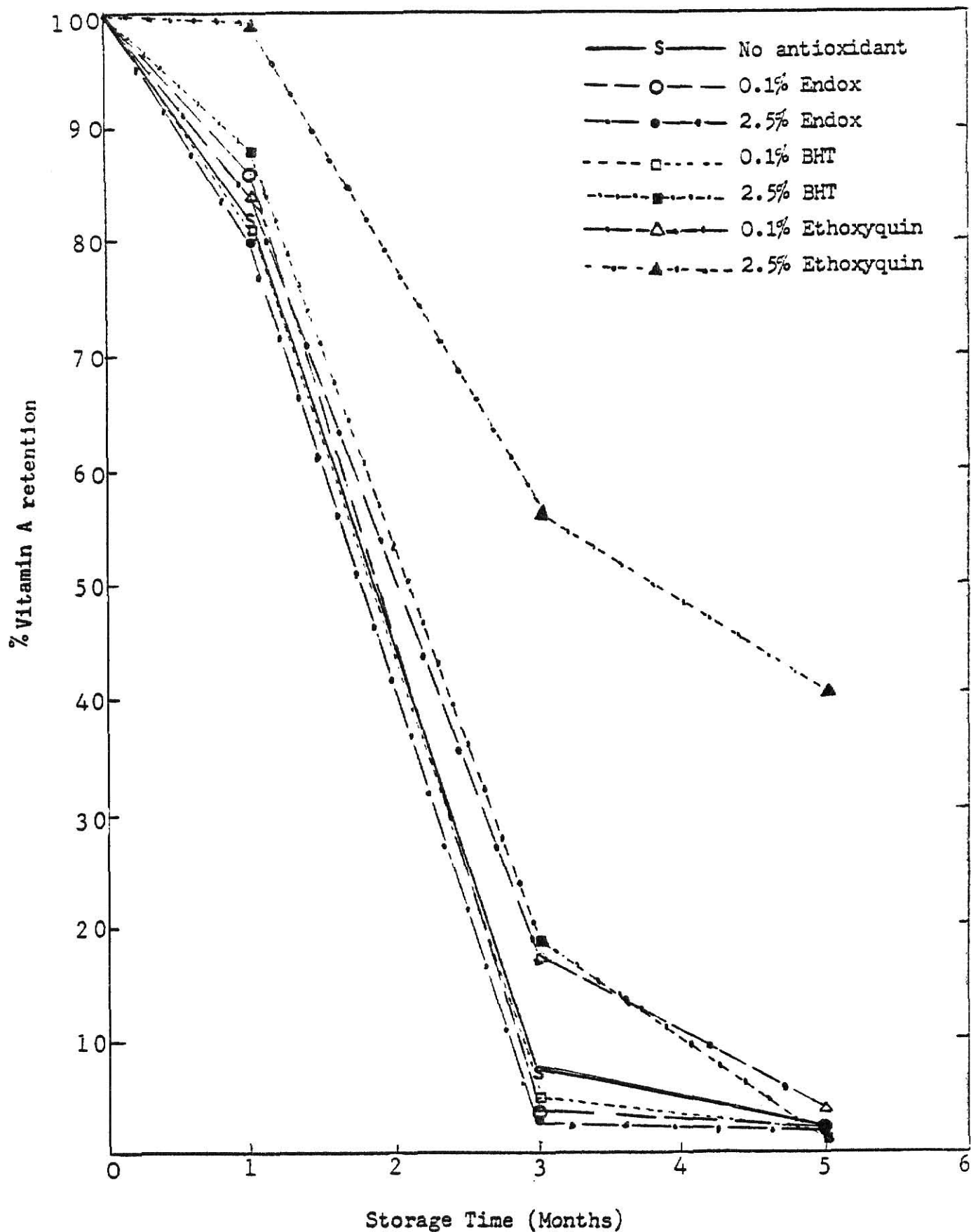
Fig. 2–Percentage vitamin A retention in a poultry vitamin-mineral premix after 1, 3 and 5 months storage at room temperature.

Analysis: General Linear Model Procedure

Source	DF	Significance of F-value[a]
Antioxidant treatment	6	NS
Week	1	***
Week^2	1	*
Week × treatment	6	NS
Week^2 × treatment	6	NS

[a] *, **, ***, significance at the 5, 1,and .1% levels respectively, NS indicates nonsignificance.

There was a significant decrease in vitamin A content with time, however the effect of antioxidant was not significant.

The equation that best predicted the vitamin A content during room temperature storage was y = 104921 + Ti + (-57335) (week). Ti values for each treatment are presented in Table 4. The predicted vitamin A content for a vitamin-mineral premix with 2.5% ethoxyquin treatment tended to be the greatest. Due to limited data and lack of replications, estimate values are misleading, in some cases, because of negative numbers.

It was observed that after 7-8 days of storage ethoxyquin at both 0.1% and 2.5% levels migrated through the double-ply storage bags causing a brownish gold discoloration. Ethoxyquin at these levels did not cause an abnormal effect in the Carr-Price determination of vitamin A.

Samples with 2.5% BHT stored 1 mo produced a yellow color in the hexane extract and a blue purple color (instead of the characteristic transitory blue color) upon reaction with Carr-Price reagent. The samples were reanalyzed by first chromatographing on alumina.[1] The yellow colored band was then removed with 4% acetone-in-hexane and vitamin A band with 15% acetone-in-hexane. The eluate was evaporated to dryness,

[1]Alumina Woelm with 5% moisture.

Table 4-Estimated T_i values for an equation for predicting vitamin A content of a vitamin-mineral premix stored under room temperature conditions.

Treatment (%)	T_i[a]
No antioxidant	0
BHT 0.1	−4945
BHT 2.5	−18068
Endox 0.1	11481
Endox 2.5	−11205
Ethoxyquin 0.1	38368
Ethoxyquin 2.5	285364

[a] The least square equation for estimated vitamin A content at different storage times was $y = 104921 + T_i + (-57335)$ (week).

the residue was dissolved in chloroform, and measured for vitamin A
content and recorded. The sample stored five months containing 2.5% BHT
gave the same reaction and the above procedure was performed. No
abnormal reaction was observed with samples containing 0.1% BHT, or
Endox at either level.

<div align="center">High Temperature-High Humidity Storage</div>

High temperature-high humidity storage conditions throughout the
experiments were at 108.3 ± 1.5°F and 78.0 ± 6.9% RH. Vitamin A contents
(Table 5) and percentages vitamin A retention (Figure 3) at various
storage times are presented. Only the 0.1% and 2.5% ethoxyquin samples
were kept on experiment after 3 months, because of the low levels of
vitamin A remaining in the other samples.

To estimate vitamin A content over storage time, least square equa-
tions were obtained by the general linear model procedure outlined below:

<div align="center">Analysis: General Linear Model Procedure</div>

Source	DF	Significance of F-value[a]
Antioxidant treatment	6	NS
Week	1	***
Week^2	1	***
Week × treatment	6	*
Week^2 × treatment	6	**

[a] *, **, *** significance at the 5, 1, and .1% levels, respec-
tively, NS indicates nonsignificance.

There was a significant decrease in vitamin A content with time, and no
significant difference among antioxidant treatments.

Estimated Ti, B_{1i}, B_{2i} values for each antioxidant for an equation
to predict vitamin A content is given in Table 6. The equation that

Table 5-Vitamin A (I.U./lb) remaining[a] after storage of a poultry vitamin-mineral premix at high temperature high humidity

| Treatment | Storage period | | | | | | |
	Weeks				Months		
	0	1	2	3	1	3	5
No antioxidant	1,123,196	545,254	277,394	119,856	85,352	26,695	—
Endox 0.1%	1,123,196	581,574	251,062	144,372	78,542	18,614	—
Endox 2.5%	1,115,478	305,769	153,906	73,548	62,652	8,626	—
BHT 0.1%	1,131,368	410,643	107,144	75,364	64,922	14,528	—
BHT 2.5%	1,054,680	238,809	421,312	80,358	217,466	10,782	—
Ethoxyquin 0.1%	1,115,478	721,406	449,914	342,316	190,226	49,989	35,934
Ethoxyquin 2.5%	1,107,760	856,968	780,426	620,618	526,186	266,725	140,173

a Average of two analyses.
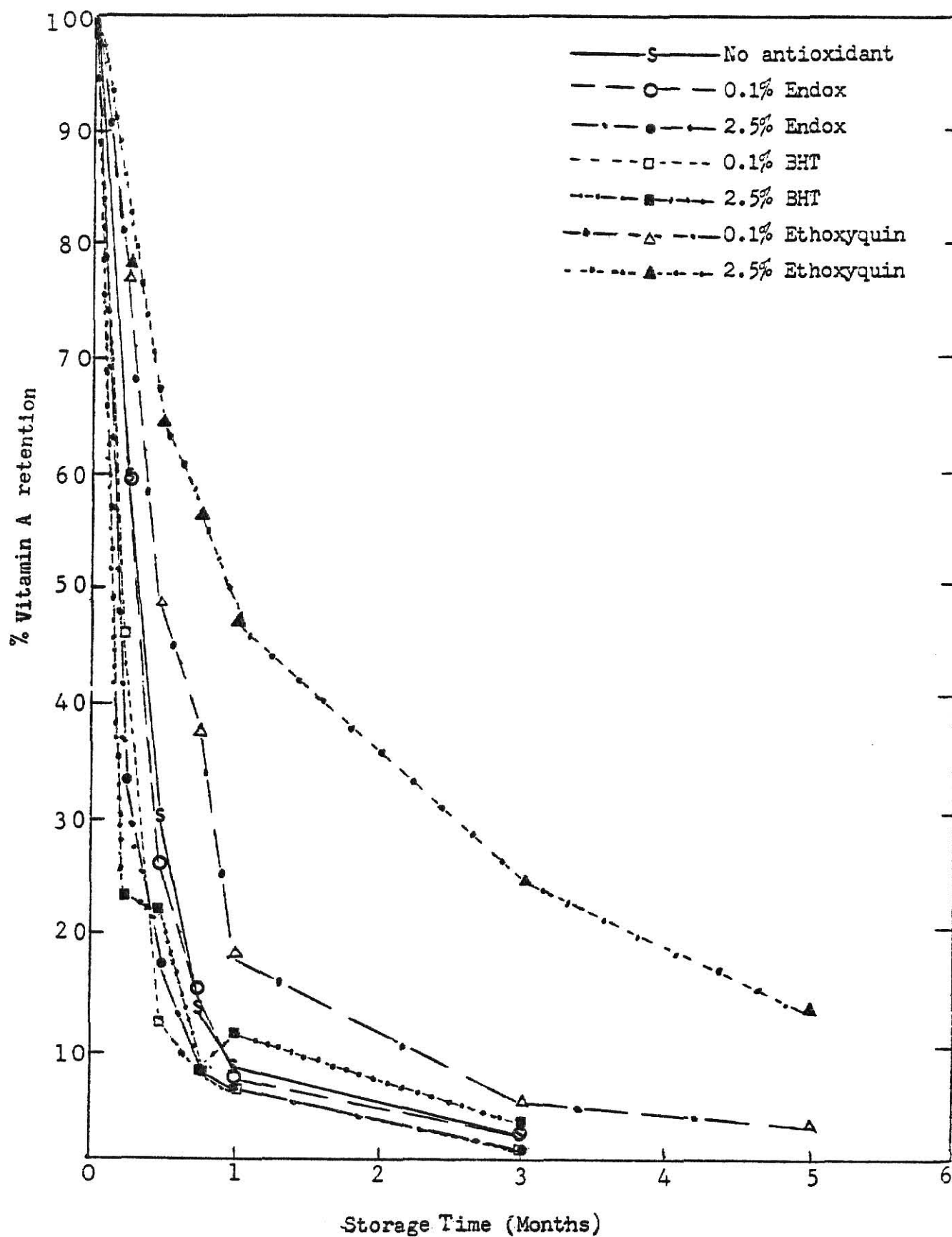
Fig. 3-Percentage vitamin A retention in a poultry vitamin-mineral premix after 1, 3 and 5 months storage at high temperature-high humidity.

Table 6-Estimated Ti values for an equation for predicting vitamin A content of a vitamin-mineral premix stored at high temperature-high humidity storage

Treatment (%)	Ti[a]	B$_{1i}$	B$_{2i}$
No antioxidant	0	−348853	22433
BHT 0.1	−68341	−353861	23275
BHT 2.5	−179571	−261826	16346
Endox 0.1	10389	−351535	22527
Endox 2.5	−112600	−335823	22038
Ethoxyquin 0.1	−122271	−156126	5884
Ethoxyquin 2.5	−94	−110040	3450

[a] The least square equation for estimated vitamin A content was
$y = 991512 + T i + B_{1i} (\text{week}) + B_{2i} (\text{week}^2)$.

best explained the change in vitamin A loss during high temperature-high humidity was $y = 991512 + T_i + B_{1i}$ (week) $+ B_{2i}$ (week^2). Predicted vitamin A content of samples containing 2.5% ethoxyquin would tend to be the highest. Estimated negative values indicated under this storage condition may have been caused by limited data and replications.

Sample bags containing BHT at the 2.5% level were discolored to a dirty gold color after one month storage. Bags containing ethoxyquin samples at the 0.1% and 2.5% levels were also discolored but to a lesser degree (light brown or tan) than those containing samples with BHT. Upon extraction of the sample containing 2.5% BHT, a hexane extract of a fairly strong golden color was obtained. Carr-Price reaction resulted in an uncharacteristic lavender color, as with samples stored at room temperature. The sample extracts were chromatographed on alumina (as with samples stored at room temperature) before determining vitamin A. The 2.5% ethoxyquin treatment gave a light brown color to hexane extract. Carr-Price reaction of the sample gave a transitory blue vitamin A reaction; the brownish color apparently did not interfere to a major extent in the final determination, since color formation and fading were normal.

The lavender colors obtained in the samples containing 0.1% and 2.5% BHT were thought to be artifacts or an oxidation product of vitamin A. This theory was checked by using blank samples of 2.5% BHT, 2.5% Endox (for comparison) and 2.5% ethoxyquin in a feed mixture containing starch, casein, ground corn, wheat, sorghum, B vitamins, oil and minerals. The samples were placed in high temperature-high humidity storage for 2 weeks.

Upon extraction with hexane the sample containing 2.5% ethoxyquin gave a yellow brown extract but samples with 2.5% Endox and BHT resulted in a clear extract. These blanks indicated that the antioxidants have

no effect on color, unless they are reacted with oxygen or some premix ingredients.[1]

High Temperature-Low Humidity Storage

Storage temperature throughout the experiment was $108 \pm 1.5°F$. Moisture content was monitored by the enclosure of Drierite packets. Observation of indicating Drierite packets showed only minimal but variable changes in color. Generally, the initial blue color was only slightly faded to a grayish blue color. The color changes in the drierite were possibly due to the initial moisture content existing within the sample jars or ingredients upon sealing. There was no color change which would indicate the presence of a substantial level of moisture.

Vitamin A content and percentage vitamin A retention of each sample treatment at various storage times are presented in Table 7 and Figure 4, respectively. Vitamin A loss appeared to be less rapid under this storage condition than those kept under room temperature and high tempera-ture-high humidity conditions. After 5 months storage the premix contain-ing 2.5% ethoxyquin apparently retained the most vitamin A.

To estimate vitamin A content over storage time least square equa-tions were obtained by the general linear model procedure outline given below.

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[1] The blank mixture was more like a heavy mineralized feed than a vitamin-mineral premix. It did not have several ingredients of the test premix, so were not exactly comparable samples.

Table 7—Vitamin A (I.U./lb) remaining[a] after storage of a poultry
vitamin-mineral premix at high temperature dry storage

Treatment	Months of storage			
	0	1	3	5
No antioxidant	1,123,196	997,892	607,906	509,615
Endox 0.1%	1,123,196	1,054,642	420,404	245,160
Endox 2.5%	1,115,478	963,388	624,477	245,160
BHT 0.1%	1,131,368	1,038,752	823,389	174,336
BHT 2.5%	1,054,680	984,045	769,530	577,488
Ethoxyquin 0.1%	1,115,478	1,081,776	607,906	208,613
Ethoxyquin 2.5%	1,107,760	1,035,080	749,100	870,403

[a] Average of two analyses.
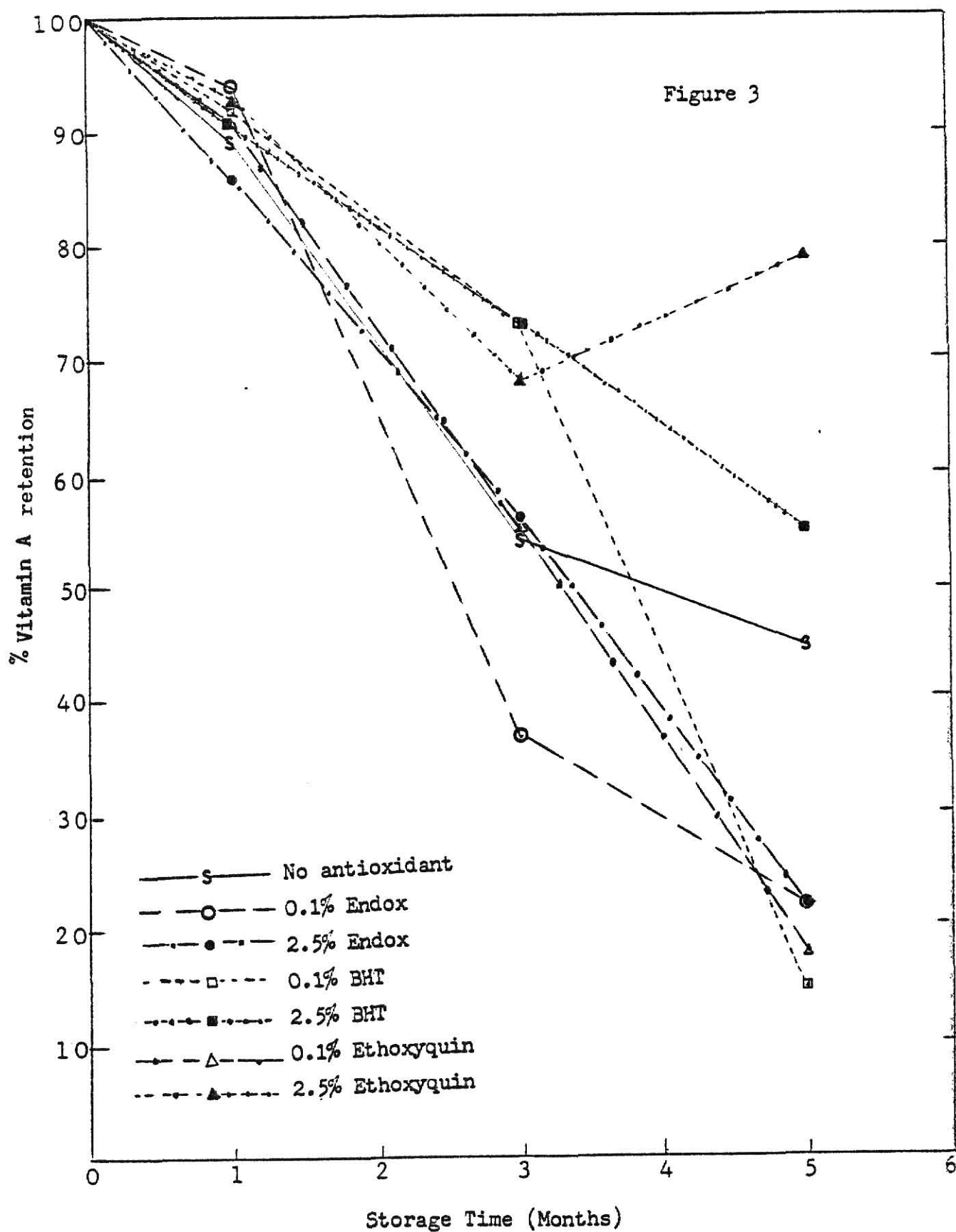
Fig. 4-Percentage vitamin A retention in a poultry vitamin-mineral premix after 1, 3 and 5 months storage at high temperature-low humidity.

Analysis: General Linear Model Procedure

Source	DF	Significance of F-value[a]
Antioxidant treatment	6	NS
Week	1	**
Week^2	1	NS
Week × treatment	6	NS
Week^2 × treatment	6	NS

[a] *, **, *** significance at the 5, 1, and .1% levels respectively, NS indicates nonsignificance.

There was no significant difference among antioxidant treatments. There was a significant decrease in vitamin A content with storage time.

Estimated T_i values for each treatment for equations for predicting vitamin A content are given in Table 8. The equation that best explained the change in vitamin A loss during high temperature-low humidity conditions was $y = 1148742 + T_i + (-105)$ (week). Under these storage conditions, samples with ethoxyquin had the greatest predicted vitamin A value. High values seem to indicate some effectiveness of antioxidants. Negative values were also indicated during high temperature-low humidity storage, due to limited data and replications.

BHT levels in this sample set stored at low humidity did not produce an uncharacteristic reaction (purple color) upon photocolormetric determination by Carr-Price reagent; moisture may have caused this reaction.

Over all storage conditions, the 2.5% ethoxyquin appeared to be the most effective antioxidant for maintaining vitamin A content but at the 0.1% level ethoxyquin had no practical value. Pelevin (1978) found that 0.02% level of ethoxyquin was effective in the stabilization of vitamin A in a mixed feed. Astrup (1963) found that ethoxyquin was more effective than BHT in retaining the carotene level in a grass meal.

Table 8-Estimated T_i values for an equation for predicting vitamin A content of a vitamin-mineral premix stored at high temperature-low humidity storage

Treatment (%)	T_i[a]
No antioxidant	0
BHT 0.1	−17691
BHT 2.5	−14861
Endox 0.1	−98801
Endox 2.5	−72526
Ethoxyquin 0.1	−56209
Ethoxyquin 2.5	130933

[a] The least square equation for estimating vitamin A content was $y = 1148742 + T_i + (-105)$ (week).

Vitamin loss in the poultry vitamin-mineral premix was greatest under the storage conditions of high temperature and high humidity. Even the addition of 2.5% ethoxyquin had questionable value under these extreme conditions. Humidity seems to be the predisposing factor contributing to vitamin A degradation. The humidity could play some role in lessening the integrity of the gelatin matrix surrounding the vitamin A acetate (Bauernfiend and Cort, 1974). The stress of elevated temperature amplified the effect high moisture levels. Luther (1980), Parrish and Patterson (1983), and Richter et al. (1982), all found that storage temperature was an important factor in the stability of encapsulated vitamin A products in high mineral containing feed mixtures or premixes. Generally it has been found that the incorporation of additional antioxidants in feeds has increased the stability of even encapsulated stabilized vitamin A products. Waldroup et al. (1981) studied the need for additional antioxidants in a high energy broiler diet in which an encapsulated vitamin A source was used. They recommended that additional antioxidants be considered and that environmental temperatures, storage time, and presence of trace minerals are all sources of oxidative stress.

Under the high temperature-low humidity storage conditions, vitamin A content in the premix was higher than in premixes stored under other conditions. Comparable data from other workers is not available. Our data suggest that storage under low moisture conditions would be an effective way to reduce vitamin A loss in vitamin-mineral premixes.

Ethoxyquin migrated through storage bags. It is not known if this absorption would affect the potential antioxidative ability of this antioxidant.

After one month storage, 0.1% and 2.5% levels of BHT resulted in an uncharacteristic coloration after saponification and also in the Carr-Price reaction. If not removed, this pigmentation will interfere with the determination of vitamin A. Therefore this pigment was separated by alumina column, before determining the vitamin A value. Intermediate products of oxidation may have been the source of these pigments. In 1938, Robinson (1938) obtained similar pigments in vitamin A concentrates heated in air or pure oxygen. He suggested that these products were a result of hydroxyl groups of vitamin A which were oxidized to aldehydes and ketones and finally to polymerides. Updated information concerning these pigments is not available.

There appears to be little readily available information on the mechanism involved in the antioxidant protection of vitamin A under conditions existing as in the present experiment. The reaction was assumed to be similar to that which occurs in fatty acid oxidation.

SUMMARY

The effects of three commercial antioxidant preparations Endox, ethoxyquin and BHT at 0.1% and 2.5% levels on the stability of vitamin A in a poultry vitamin-mineral premix were studied over a 5-month storage period. Three storage conditions were used, uncontrolled room temperature, high temperature-low humidity, and high temperature-high humidity. The AOAC method 43.008, Vitamin A in Mixed Feeds, Premixes and Foods, was used to determine vitamin A content of sample treatments.

After five months of storage ethoxyquin (2.5%) graphically appeared to be the most effective antioxidant treatment in the stabilization of vitamin A at both room temperature and high temperature-high humidity storage. To estimate vitamin A content at selected storage times, least square equations were obtained by the general linear model procedure. In all treatments there was a significant decrease in vitamin A with time for each storage condition. No significant difference in vitamin A content was found among treatments under any storage conditions. Ethoxyquin (2.5%) tended to have the greatest predicted values among the treatments in all three storage conditions, which indicated some effectiveness of this product.

Vitamin A loss appeared to be less rapid under high temperature-low humidity and most rapid under high temperature-high humidity conditions. Humidity seemed to be a major factor in the deterioration of stabilized vitamin A in the premix used.

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THE EFFECT OF ANTIOXIDANTS ON THE STABILITY OF
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by

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Manhattan, Kansas

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The effects of three commercial antioxidant preparations Endox, ethoxyquin and BHT at 0.1% and 2.5% levels on the stability of vitamin A in a poultry vitamin-mineral premix were studied over a 5-month storage period. Three storage conditions were used, uncontrolled room temperature, high temperature-low humidity and high temperature-high humidity. The AOAC method 43.008, Vitamin A in Mixed Feeds, Premixes and Foods, was used to determine vitamin A content of sample treatments.

After five months of storage ethoxyquin (2.5%) graphically appeared to be the most effective antioxidant treatment in the stabilization of vitamin A at both room temperature and high temperature-high humidity. To estimate vitamin A content at selected storage times, least square equations were obtained by the general linear model procedure. In all treatments there was a significant decrease in vitamin A with time for each storage condition. No significant difference in vitamin A content was found among treatments under any storage conditions. Ethoxyquin (2.5%) tended to have the greatest predicted values among the treatments in all three storage conditions, which indicated some effectiveness of this product.

Vitamin A loss appeared to be less rapid under high temperature-low humidity and most under high temperature-high humidity conditions. Humidity seemed to be a major factor in the deterioration of stabilized vitamin A in the premix used.