## CAROTENE

# Effect of Antioxidants on Determination in Dehydrated Alfalfa Meal

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*N, N'*-Diphenyl-*p*-phenylenediamine, added to dehydrated alfalfa meal to stabilize carotene, caused noncarotenoid color formation during analysis, which resulted in erroneous carotene values. This was confirmed by a preliminary bioassay with chicks. The color produced resulted from a reaction of the compound with the magnesium oxide adsorbent. The amount of interference varied with the method of analysis. A few other compounds which were studied for possible antioxidant activity also contributed noncarotenoid color in some manner. Thus, it may be difficult in many cases to determine carotene accurately in meals to which stabilizers have been added.

THE POSSIBILITY OF STABILIZING CAROTENE in alfalfa meal by the use of antioxidants has received considerable study in recent years. A large number of chemicals have been screened for this purpose (*6*), and a few have been reported to possess appreciable antioxidant activity.

The first chemical to be patented and used commercially for stabilizing carotene in alfalfa meal is *N,N'*-diphenyl-*p*-phenylenediamine (*3*). Tests in this laboratory, however, indicated that the presence of this compound on alfalfa meal interfered with the determination of carotene. This is a report of studies which were conducted to investigate possible interference due to the use of certain antioxidants.

### Experimental Work

The chemicals under investigation were applied to alfalfa meal at a rate of 4 pounds per ton (0.2%). Each chemical (0.45 gram) was placed in a DeVilbiss No. 15 nasal atomizer and was dissolved with 10 ml. of acetone and 10 ml. of Skellysolve B. As it is customary industrially to apply antioxidants in an oil solution, 1.8 grams of Wesson oil were added. One-half pound of alfalfa meal was placed in the drum of a small rotary mixer which was made by mounting a 4-gallon metal can on a wheel, which in turn was attached to an axle bent upward at an angle of 30° from the horizontal. The atomizer was connected to a source of air pressure by means of rubber tubing. The nozzle of the atomizer was inserted through a small hole in the center of the lid of the can, and the solution was sprayed on the meal while the mixer was rotated at 37 r.p.m. The sprayed meal was transferred to a sheet of wrapping paper and was placed in a darkened room for 2 hours to permit the solvent to evaporate. The meal was mixed, and carotene was determined by the method of Silker, Schrenk, and King (*5*).

**Table I. Effect of Certain Antioxidants on Determination of Carotene in Alfalfa Meal**

| | Antioxidant | Carotene, Mg./100 G. |
|---|---|---|
| 1. | None | 26.8 |
| | 2,5-Di-*tert*-butylhydroquinone | 26.7 |
| | *N,N'*-Diphenyl-*p*-phenylenediamine | 30.9 |
| 2. | None | 19.1 |
| | 2,5-Di-*tert*-butylhydroquinone | 19.7 |
| | *N,N'*-Diphenyl-*p*-phenylenediamine | 23.5 |
| 3. | None | 21.9 |
| | 2,5-Di-*tert*-butylhydroquinone | 21.9 |
| | *N,N'*-Diphenyl-*p*-phenylenediamine | 27.3 |

In Table I are shown the carotene values obtained with untreated meal and meal treated with two antioxidants. The presence of *N,N'*-diphenyl-*p*-phenylenediamine caused appreciably high carotene values in each instance, when compared with the values for the untreated meals. Di-*tert*-butylhydroquinone had no influence, with the possible exception of the second sample. According to these data, the diamine-treated meals had apparent carotene contents 15 to 25% greater than the untreated meals. These results were obtained with three lots of the diamine, and it is probable that others would behave in a similar manner.

**Source of Noncarotene Pigment** During chromatography of the extracts of meal treated with *N,N'*-diphenyl-*p*-phenylenediamine it was observed that more eluting agent was needed to attain colorless eluates than was needed for extracts of untreated meal. To study this, 1-gram samples of treated and untreated meals were analyzed by the method of Silker *et al.* The adsorption columns were washed with 235 ml. of 4% acetone in Skellysolve B. These eluates were made to a volume of 250 ml. and were designated as "initial eluates." The columns then were washed with 35 ml. of the eluting agent. Duplicates of these washings were combined and the volumes were reduced to 25 ml. under reduced pressure. These solutions were designated "final eluates." In addition, an amount of the diamine equivalent to that in 1 gram of the treated meal was dissolved in Skellysolve B and the solution was chromatographed, two eluates again being collected. Absorption spectra of the eluates were obtained by means of a Model DU Beckman spectrophotom-

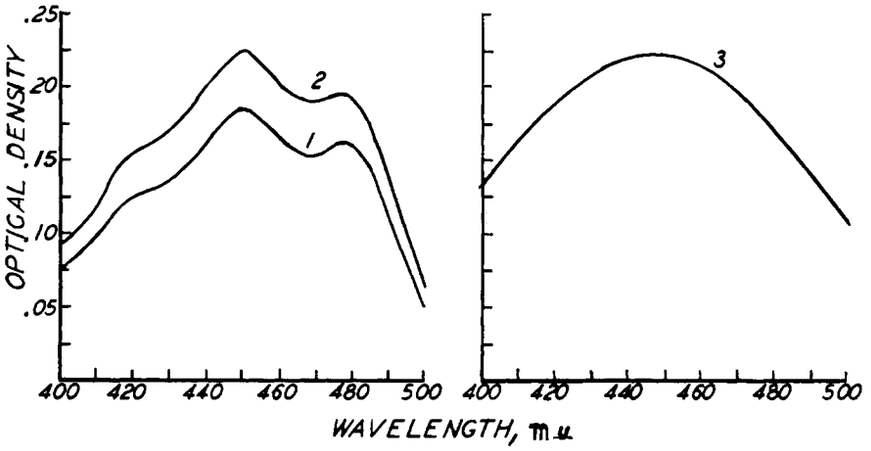


**Figure 1. Absorption spectra obtained from eluates of untreated meal and meal treated with N,N′-diphenyl-p-phenylenediamine**

1 Initial eluate from untreated meal. 2 Initial eluate from treated meal. 3 Tailings from treated meal

eter. The absorption spectra are presented in Figures 1 and 2.

At a given wave length the absorbance (optical density) of the initial eluate of the treated meal was greater than that of the initial eluate of the untreated meal, but the shapes of the two curves were similar. Thus, the extraneous color caused by the antioxidant was not sufficient to alter appreciably the shape of the curve or to shift the location of the maxima. The final eluate of the untreated sample did not contain any color, so that an absorption spectrum was not obtained. The final eluate of the treated meal was yellow, but its spectrum differed considerably from that of the initial eluate of the untreated meal. Hence, the pigment in the final eluate of the treated meal was not carotene.

The absorption spectra of the initial and final eluates obtained by chromatographing the solution containing only the diamine, although of different absorption intensities, were similar in shape (Figure 2), indicating that the constituents of the two eluates were identical. Furthermore, the spectra of the final eluates of the antioxidant solution and of the treated meal were similar. Thus, the extraneous color obtained from the treated sample was not a carotenoid and was not derived by interaction of the antioxidant with some constituent of the alfalfa meal, as neither carotenoids nor meal were present during chromatography of the antioxidant solution.

Because the diamine solution before chromatography was essentially colorless, it was apparent that the yellow constituent was produced during passage of the solution through the adsorbent. In an effort to determine the cause of the color, a small amount of the diamine was dissolved in a solution of 4% acetone in Skellysolve B. A yellow color developed when the active ingredient of the adsorbent, magnesium oxide, was added. When either Skellysolve B or acetone was omitted, color still developed. Hence, the color was due to reaction of the diamine with the adsorbent and not with either of the solvents.

It was noted that during chromatography of the solution of the diamine in Skellysolve B, color appeared in the eluate very quickly. Thus, carotene could not be eluted during analysis of a meal without eluting some of the noncarotene pigment also. It seems improbable that *N,N′*-diphenyl-*p*-phenylenediamine can be evaluated as a carotene antioxidant by methods employing magnesia as the adsorbent and acetone as the eluting agent. A correction factor would be of questionable value, as its magnitude would depend on the concentration of the antioxidant on the alfalfa meal, the dimensions of the adsorption column, and the amount of eluting agent.

**Other Antioxidants** It seemed desirable to know if other chemicals which may be used as antioxidants would interfere with the determination of carotene. An important consideration is the possibility of the development of interference due to changes that may take place during storage of the treated meal.

The chemicals selected for study have been reported to possess appreciable antioxidant activity (*2*, *6*). The chemicals were applied as described earlier. Carotene determinations were made before storage by the method of Silker *et al.* and by the official method of the Association of Official Agricultural Chemists (*1*). The samples again were analyzed by the two methods after storage at 37° C. for 3 and 6 weeks. The data are shown in Table II.

The initial carotene values obtained by the two methods were in good agreement for all the samples except the meal treated with *N,N′*-diphenyl-*p*-phenylenediamine. With the latter, the greatest interference by the chemical occurred with the official method. This difference in the apparent carotene contents obtained by the two methods persisted during storage, with a tendency toward greater interference by the official method as storage progressed. This behavior can be explained by considering the techniques of the two methods. Elution of carotene from the adsorption column in the Silker *et al.* method is accomplished with 4% acetone in Skellysolve B, causing the carotene to move slowly down the column as a distinct band. With the official method, elution is accomplished with 9% acetone in Skellysolve B. This stronger eluting agent causes rapid elution of the carotene without band formation. Under these conditions more of the interfering substance was removed also.

The slower elution employed by the method of Silker *et al.* also permitted the detection of a noncarotene pigment which developed during storage of the sample treated with *N,N′*-di-4(2,6-di-

**Figure 2. Absorption spectra obtained from eluates of solution of N,N′-diphenyl-p-phenylenediamine**

1 Initial eluate. 2 Tailings

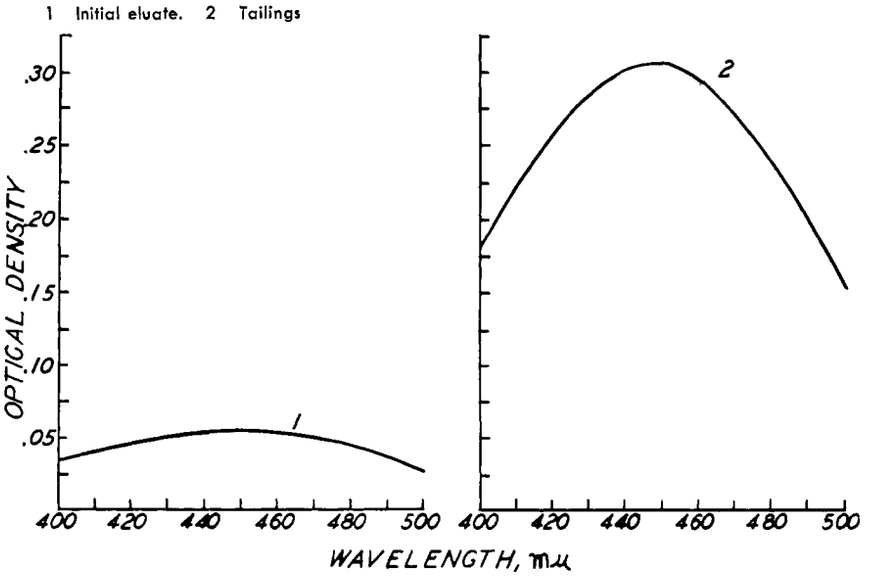

**Table II. Determination of Carotene in Antioxidant-Treated Alfalfa Meals by AOAC and Silker, Schrenk, and King Methods**

(Storage at 37° C. Carotene expressed as mg./100 g.)

| Antioxidant | Length of Storage, Weeks: Silker, Schrenk, and King 0 | 3 | 6 | AOAC 0 | 3 | 6 |
|---|---|---|---|---|---|---|
| None | 21.9 | 9.1 | 6.4 | 21.9 | 9.1 | 6.5 |
| 2,5-Di-*tert*-butylhydroquinone | 22.0 | 16.3 | 14.5 | 22.2 | 16.6 | 14.8 |
| *N,N'*-Diphenyl-*p*-phenylenediamine | 27.3 | 18.1 | 16.3 | 28.5 | 20.1 | 18.5 |
| *N,N'*-Di-4(2,6-dimethylheptyl)-*p*-phenylenediamine | 22.2 | 17.5 | 15.1[a] | 22.2 | 17.9 | 15.7 |
| 6-Ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline | 22.6 | 16.6 | 14.6 | 22.3 | 16.8 | 14.8 |
| *N,N'*-Di-2-naphthyl-*p*-phenylenediamine | 21.8 | 10.3 | 8.2 | 22.3 | 10.5 | 8.4 |

[a] Noncarotene pigment detected and discarded.

**Table III. Storage Conditions and Final Analyses of Antioxidant-Treated Meals Used in Chick Growth Studies**

| Antioxidant | Storage: Weeks | °C. | Method of Analysis | Final Carotene, Units/Lb. |
|---|---|---|---|---|
| **Experiment 1** | | | | |
| None | 12 | 25–28 | Silker, Schrenk, and King | 75,000 |
| 2,5-Di-*tert*-butylhydroquinone | 12 | 25–28 | | 119,000 |
| *N,N'*-Diphenyl-*p*-phenylenediamine | 12 | 25–28 | | 145,000 |
| **Experiment 2** | | | | |
| None | 5 | 30–35 | AOAC | 95,000 |
| 2,5-Di-*tert*-butylhydroquinone | 9 | 30–35 | | 99,000 |
| *N,N'*-Diphenyl-*p*-phenylenediamine | 16 | 30–35 | | 153,000 |

methylheptyl)-*p*-phenylenediamine. This pigment was detected definitely after 6 weeks of storage. It formed a narrow lavender band on the column and moved downward ahead of the carotene during elution. It was eluted and discarded before the carotene appeared in the eluate. With the official method, no distinct bands formed and the separation was not accomplished. Hence, slightly higher values were obtained with the official method, and perhaps the discrepancy would have become greater with a longer period of storage. In view of this experience, it is felt that the method of Silker *et al.* is the better of the two methods for studying the antioxidant activity of various chemicals, as it may afford an opportunity to detect noncarotene bands which could not be observed with the official method.

Another observation which may have significance was concerned with the meal treated with 2,5-di-*tert*-butylhydroquinone. With both methods of analysis it was noted that the adsorption columns below the xanthophyll bands were light yellow-green in color after the carotene had been eluted. Although this colored substance was not removed with further elution of the columns, it is possible that some such noncarotene material was eluted along with the carotene. If so, there was not enough contamination to cause changes in the shape of the absorption curve of the carotene solution. However, the growth studies reported below suggest that the meal may have contained less carotene than was indicated by carotene analyses.

**Chick Growth Studies** Growth studies should be the final basis for evaluating carotene stabilizers if it is suspected that noncarotene pigments cause interference with carotene determinations. Chick growth experiments were conducted with alfalfa meal treated with 2,5-di-*tert*-butylhydroquinone and *N,N'*-diphenyl-*p*-phenylenediamine. The meal was sprayed at the rate of 16 pounds of Wesson oil and 4 pounds of antioxidant per ton of meal, as described earlier. The meal used as a control was sprayed with Wesson oil only. Table III summarizes the storage conditions, methods of analysis, and final carotene content of the meals. In experiment 2 an attempt was made to reduce the carotene content to about 100,000 units per pound. This was done because most of the alfalfa meal which is purchased by feed mixers contains this amount of carotene, as measured by the AOAC method of analysis. However, it became apparent that the diamine-treated meal would not decrease to this level in a reasonable length of time, and storage of it at room temperature was discontinued. All meals were placed in cold storage at −23° C. to minimize further loss of carotene (*4*, *8*). The final carotene determinations shown in Table III were made at the time the growth tests were started.

The results of the chick growth tests carried out to determine the relative vitamin A potencies of the antioxidant-treated meals are summarized in Table IV. White leghorn cockerels were used in experiment 1 and Hy-line cockerels in experiment 2. The chicks were fed a carotene-free and vitamin A–free diet for one week and were distributed by random selection into the test groups. During the next 8 weeks each group received the basal diet plus an amount of one of the alfalfa meals which would supply either 250 or 500 units of vitamin A potency per pound of feed, based on

**Table IV. Growth and Condition of Chicks Fed Rations Containing Treated and Untreated Alfalfa Meals**

| Antioxidant | No. of Chicks per Pen[a] | Vit. A Potency, Units/Lb. of Feed | Av. Weight at 9 Weeks, G. | Death Losses[b] | Chicks with Sore Eyes at 9 Weeks[c] |
|---|---|---|---|---|---|
| **Experiment 1** | | | | | |
| None | 20 | 250 | 566 | 4 | 7 |
| 2,5-Di-*tert*-butylhydroquinone | 19 | 250 | 559 | 7 | 6 |
| *N,N'*-Diphenyl-*p*-phenylenediamine | 22 | 250 | 506 | 6 | 10 |
| None | 20 | 500 | 672 | 0 | 5 |
| 2,5-Di-*tert*-butylhydroquinone | 18 | 500 | 644 | 1 | 4 |
| *N,N'*-Diphenyl-*p*-phenylenediamine | 16 | 500 | 619 | 0 | 9 |
| **Experiment 2** | | | | | |
| None | 14 | 250 | 824 | 0 | 1 |
| | | 250 | 851 | 1 | 0 |
| 2,5-Di-*tert*-butylhydroquinone | 14 | 250 | 818 | 1 | 3 |
| | | 250 | 793 | 0 | 2 |
| *N,N'*-Diphenyl-*p*-phenylenediamine | 14 | 250 | 660 | 3 | 4 |
| | | 250 | 711 | 2 | 8 |
| None | 14 | 500 | 1021 | 1 | 0 |
| | | 500 | 1035 | 1 | 0 |
| 2,5-Di-*tert*-butylhydroquinone | 14 | 500 | 1014 | 0 | 2 |
| | | 500 | 1016 | 1 | 1 |
| *N,N'*-Diphenyl-*p*-phenylenediamine | 14 | 500 | 966 | 1 | 2 |
| | | 500 | 888 | 1 | 1 |

[a] Variation in number of chicks in experiment 1 due to discarding of pullets and escaping of some chicks from pens while small.

[b] Losses apparently due to vitamin A deficiency. Ureates in kidneys and other evidences of deficiency found.

[c] Eyes closed, badly inflamed.

the chemical determination of carotene. The basal ration was composed of white corn, wheat bran, solvent-extracted soybean meal, dry skim milk, dry brewer's yeast, bone meal, limestone, salt, manganese sulfate, and a vitamin premix containing adequate levels of all vitamins except vitamin A. The feeds were mixed at frequent intervals, and were never over 14 days old when consumed by the chicks. None of the chicks receiving the basal diet alone lived more than 26 days after hatching.

The data in Table IV show that chicks receiving the meals treated with *N,N'*-diphenyl-*p*-phenylenediamine grew less than chicks fed the untreated alfalfa meal. In general, death losses and condition of the eyes also indicated that the vitamin A potency of the diamine-treated meal was less than was indicated by carotene determinations. Thus, the chick assay data support the observation that noncarotene pigment is eluted from the chromatographic columns during carotene analysis of meal treated with *N,N'*-diphenyl-*p*-phenylenediamine, resulting in high carotene values.

The results of the growth tests (gains in weight, deaths, and eye condition) with meals treated with 2,5-di-*tert*-butylhydroquinone were less favorable than those with meals containing no antioxidant. The differences, although small, were consistent, and some interference with carotene determination may have occurred with this antioxidant.


### Acknowledgment

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### Literature Cited


(1) Assoc. Offic. Agr. Chemists, "Official Methods of Analysis," 7th ed., 1950.
(2) Bickoff, E. M., Livingston, A. L., Guggolz, J., and Thompson, C. R., *J. Am. Oil Chemists' Soc.*, **29,** 445 (1952).
(3) Kephart, J. C., U. S. Patent 2,474,182 (June 21, 1949).
(4) Mitchell, H. L., and Silker, R. E., *Trans. Kansas Acad. Sci.*, **55,** 479 (1952).
(5) Silker, R. E., Schrenk, W. G., and King, H. H., *Ind. Eng. Chem., Anal. Ed.*, **16,** 513 (1944).
(6) Thompson, C. R., *Ind. Eng. Chem.*, **42,** 922 (1950).
(7) Thompson, C. R., U. S. Patent 2,562,970 (Aug. 7, 1951).
(8) Wilder, O. H. M., and Bethke, R. M., *Poultry Sci.*, **20,** 304 (1941).



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# TRACE ELEMENT DEFICIENCIES

# Water-Culture Crops Designed to Study Deficiencies in Animals


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**Experiments were carried out in order to obtain diets lacking halogens and sodium, particularly for the study of iodine and fluorine deficiency in rats. A water-cultured crop was obtained with successful elimination of halogens or sodium. With natural foodstuffs devoid of iodine, fluorine, or sodium, the effect on experimental animals can be definitively determined. Conversely, the optimum amount of these halogens or sodium for fertilization can be ascertained.**


WATER CULTURE MIGHT be one solution to the problem of obtaining foodstuffs deficient in essential elements. Small-scale water culture has been used to show the necessity of certain trace elements in plants, but the use of large-scale water culture of the same chemical purity to grow foodstuffs for animals has never been described (*7*). Recently reported were successful studies of diets which were composed of foods obtained by water culture free of fluorine. The effect of these fluorine-free diets on dental caries and bone calcification was strikingly demonstrated by this procedure (*4*). This report deals with the technique evolved during the past 9 years for water culture of plants devoid of halogens or sodium.

### Analytical Methods

Owing to the large quantity of pure water required, rain water was analyzed. Even though halogens pass into the atmosphere from sea spray, the burning of coal, and fluorine from open-hearth furnaces, there was only 0.002 to 0.004 p.p.m. of fluorine in rain water at the experimental farm 25 miles from Philadelphia. This value might be increased by contamination. For instance, when maple leaves were dried and extracted with double-distilled water, 2 p. p. m. of fluorine were extracted from them. Therefore, leaves should not be allowed in the water-collecting system. Rain water collected from an aluminum roof was freed from halogens and sodium by passage through Amberlite ion exchange resins.

Wooden tanks 2 feet wide and 12 feet long, lined with aluminum foil and coated with asphalt varnish, were filled with 200 liters of nutrient solutions each. A porous carbon tube filled with air at 15 pounds per square inch pressure, which was down the middle of each tank, supplied oxygen for the roots. Wooden frames, 2 × 2 feet with bottoms of 1-inch wire mesh, were used to support the root crowns of the plants. Aluminum foil was laid over the wire and on it a litter of last year's crop. A wick made of the previous year's stalks extended into the solution through the middle of the foil and wire mesh, and on top of the wick the seeds were planted.

It was found best to surround the tanks with a glass windbreak and to have the top open to the weather. In order to prevent rain water from entering the tanks, each frame had a roof of aluminum foil, except for the hole through which the plant grew.

For 3 years the nutrient solutions were analyzed frequently and records taken of salts added as they were used up; after that, iron and manganese were added every day and the other salts once a month.

Nutrient salts of reagent purity were used, but the calcium salts were analyzed as though they had been prepared from calcium oxide containing 4 to 6 p. p. m.