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CAROTENE

Determination in Alfalfa Meal Treated with *N,N'*-Diphenyl-*p*-phenylenediamine

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The AOAC method for determination of carotene in dehydrated alfalfa meal is not suitable for use with alfalfa meal treated with *N,N'*-diphenyl-*p*-phenylenediamine, as the passage of the diamine through the magnesium oxide adsorbent results in the production of a yellow color. A method is presented which employs tricalcium phosphate as the adsorbent, and which can be used when the diamine is present. By means of this method the potency of *N,N'*-diphenyl-*p*-phenylenediamine as a carotene stabilizer was evaluated.

BEAUCHENE *et al.* (*1*) have shown that the presence of *N,N'*-diphenyl-*p*-phenylenediamine on alfalfa meal interferes with the determination of carotene when the AOAC method of analysis is used. The magnesia of the adsorbent caused alteration of the diamine in some manner, producing a yellow color which contaminated the carotene fraction and caused abnormal carotene values.

N,N'-diphenyl-*p*-phenylenediamine has been approved by the U. S. Department of Agriculture for use on alfalfa meal as a carotene stabilizer (*4*). In view of the results of Beauchene *et al.*, it is probable that this chemical is less efficient as an antioxidant than was indicated by previous work (*2, 3, 8*). It is of interest, therefore, to have available a method that will accurately evaluate the degree of carotene preservation in alfalfa meal treated with this substance. Furthermore, because diamine-treated meal may be moving in commercial channels and thus would be subject to quality control, a method for carotene determination is needed which will not be influenced by the presence of the antioxidant. This investigation was initiated for the purpose of developing such a method.

Experimental Work

The specific problem involved is the selection of an adsorbent that will not react with the diamine to produce a color. At the same time, the adsorbent must separate the carotene adequately from the noncarotene pigments of the meal.

Mitchell, Schrenk, and Silker (*6*) reported that powdered tricalcium phosphate would adsorb chlorophyll and xanthophylls from a Skellysolve B solution, but that carotene was adsorbed weakly and could be removed easily from the adsorbent by washing with Skellysolve B. This adsorbent was investigated as a substitute for magnesium oxide in the AOAC method of analysis. By adding tricalcium phosphate to a solution of the diamine in Skellysolve B and shaking the mixture vigorously for a few minutes, it was determined that it did not react with the diamine to produce a color.

The quantitative aspects of the proposed adsorbent were studied by analyzing untreated alfalfa meal samples for carotene by the AOAC method and by a modification of the AOAC method which permitted the use of tricalcium phosphate as the adsorbent. The modification consisted of transferring the extract obtained by the AOAC extraction procedure to a 600-ml. beaker and diluting with Skellysolve B to about 300 ml. The extract was concentrated to 30 to 40 ml. on a steam plate to drive off most of the acetone. This was the procedure used by Wall and Kelley (*10*) for eliminating acetone from the extract, and is necessary to prevent elution of noncarotene pigments by the acetone during adsorption. The concentrated extract was drawn through a 9-cm. column of a 1 to 1 mixture by weight of powdered tricalcium phosphate and Super Cel. The column was washed with Skellysolve B until the eluate was colorless. Less than 100 ml. of Skellysolve B was required to accomplish this. The eluate was diluted to 250 ml. with Skellysolve B and color intensity was measured at 4360 A. with a Beckman DU spectrophotometer.

Carotene values obtained by the two

Table I. Carotene Content of Untreated Alfalfa Meals

(As obtained by AOAC method and by a modification which permitted use of tricalcium phosphate as adsorbent)

Sample	AOAC Method, Mg./100 Grams	Calcium Phosphate Method, Mg./100 Grams
1	21.9	20.7
2	22.3	21.8
3	18.4	17.5
4	21.6	21.0
5	21.8	20.4
6	16.8	16.2
7	12.9	12.5
8	11.6	11.0
9	6.5	5.8

Table II. Comparison of Different Lots of Powdered Tricalcium Phosphate

(Carotene content of alfalfa meal by AOAC method was 22.3 mg./100 grams)

Source	Manufacturer's Description	Carotene, Mg./100 Grams
Baker and Adamson	Reagent	21.6
Monsanto Chemical Co.	Conditioner grade	20.8
Victor Chemical Co.	NF	20.4
Victor Chemical Co.	TCP	20.0

methods are presented in Table I. Tricalcium phosphate method gave slightly lower values than the AOAC method. It was pointed out by Quackenbush (*7*) that in the later stages of elution in the AOAC method some noncarotene pigment is removed from the adsorbent. Hence, the values obtained by the phosphate procedure in this study may be the more accurate. It would appear that the combination of weaker adsorbent and milder eluting agent resulted in the retention of more of the noncarotene pigments on the adsorbent than occurred with the AOAC method. Absorption spectra of the carotene solutions obtained by the two procedures were identical except for slight differences in the magnitude of absorption. This was to be expected, for the presence of such small amounts of noncarotene pigments would be difficult to detect by absorption studies on the solutions.

Uniformity of Tricalcium Phosphate as Adsorbent Alfalfa extracts were chromatographed on four lots of powdered tricalcium phosphate, as shown in Table II, to determine variability of adsorbents obtained from different sources. The data indicate that the lots of adsorbent tested were reasonably uniform in adsorbability. The Victor phosphates when mixed in equal amounts with Super Cel gave columns from which carotene was eluted more slowly than from the others. However, when the mixture consisted of 1 part by weight of phosphate and 2 parts of Super Cel, speed of elution was improved without affecting the carotene content of the eluates.

Mann (*5*) proposed the use of bone meal as an adsorbent for the determination of carotene. However, a sample of bone meal tested in this study was less retentive than powdered tricalcium phosphate, and carotene values obtained with it were appreciably higher than values obtained with the AOAC method.

The rate of flow of the eluting agent through the Monsanto phosphate–Super Cel column (1 to 1) was considered the most desirable, and all subsequent work was done with this brand and proportion of phosphate.

Studies with Diamine-Treated Meal Alfalfa meal was sprayed, as described prevously (*7*), with Wesson oil at the rate of 16 pounds per ton and with *N,N'*-diphenyl-*p*-phenylenediamine at rates ranging from 0 to 0.2%. The meals were analyzed for carotene both by the AOAC method and by the tricalcium phosphate procedure immediately after spraying, and again after storage at 37° C. With the AOAC method, the volume of eluting agent required for the untreated extracts was used also for eluting the carotene from the treated samples. This was necessary because the color which is produced from the diamine during its passage through the magnesia obscures the point at which carotene has been eluted, and greater discrepancies will occur between the samples if washing is continued. The data are presented in Table III.

The use of tricalcium phosphate in the absence of the diamine again gave values which were slightly lower than those obtained with the AOAC method, both initially and after storage. With the diamine-treated meals, the AOAC method gave apparent initial carotene values which were considerably higher than the values obtained with the corresponding untreated meals. Hence, the AOAC method is not suitable for use with alfalfa meal containing this antioxidant. With tricalcium phosphate as the adsorbent, however, excellent agreement was found between the treated and untreated meals on initial analysis.

From the data of Table III it is apparent that the diamine does increase carotene stability in alfalfa meal. To determine its relative potency, its activity was compared with that of 6-ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline, which is considered to be one of the more active antioxidants for alfalfa carotene (*8*). Alfalfa meal was treated with various amounts of each antioxidant, as described above. Storage was at 37° C. for 6 weeks. Analyses were made on 2-gram samples by both the AOAC and tricalcium phosphate procedures. The results are presented in Table IV.

It is evident again that the calcium phosphate method was in good agreement with the AOAC method for both the untreated meal and the meal treated with the dihydroquinoline. It is apparent from a comparison of the percentages of carotene destruction after 6 weeks of storage that the diamine-treated meals were less stable than the dihydroquinoline-treated meals at a given level.

These data indicate that application of the diamine at a rate of 0.015%, which is currently the commercial practice (*9*), will not result in adequate carotene stability in alfalfa meal. In order to attain the stability desired by the dehydrated alfalfa producer, enough of the diamine will be required that it will have an appreciable effect on carotene determination by the AOAC method. Thus, if *N,N'*-diphenyl-*p*-phenylenediamine is to be used as an antioxidant, attention must be given both to the level at which it should be used and to the selection of an analytical method that will give reliable results in the presence of the diamine.

Table III. Determination of Carotene in Alfalfa Meal Treated with *N,N'*-Diphenyl-p-phenylenediamine

Antioxidant Level, %	Weeks at 37° C. 0	3	6	Weeks at 37° C. 0	3	6
	Carotene, AOAC method, mg./100 grams			Carotene, calcium phosphate method, mg./100 grams		
0	21.7	13.8	9.5	20.9	12.8	8.7
0.02	23.0	16.8	13.0	21.0	14.8	11.3
0.05	24.7	19.2	16.0	21.1	15.5	12.3
0.1	27.7	22.4	19.2	21.0	16.4	13.3
0.2	32.8	27.2	24.4	21.0	16.8	13.4

Table IV. Stabilizing Ability of 6-Ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline and *N,N'*-Diphenyl-p-phenylenediamine

(Applied to alfalfa meal at varying levels. Samples stored at 37° C. for 6 weeks)

Antioxidant	Level, %	Carotene, Mg./100 Grams: AOAC Method Initial	Final	Loss, %	Calcium Phosphate Method Initial	Final	Loss, %
None		21.5	11.7	46	21.1	11.0	49
Dihydroquinoline	0.02	21.9	15.5	29	21.3	14.6	31
	0.05	22.2	17.2	23	21.4	16.2	24
	0.1	22.2	17.7	20	21.4	16.9	21
	0.2	22.5	18.3	19	21.4	17.5	18
Diamine	0.02				21.6	13.3	38
	0.05				21.8	14.8	32
	0.1				22.3	16.0	28
	0.2				22.2	16.9	24

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Enzyme and Flavor Components of Fruits Isolated

ENZYMES

Course of Action of Polygalacturonase on Polygalacturonic Acids

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In studying the biochemical processes that take place during ripening of fruits, further information on pectic enzymes acting on pectic substances was desired. The course of action of a purified fungal polygalacturonase on polygalacturonic acid was followed by qualitative and quantitative paper chromatography. Crystalline brucine salts of galacturonic di- and trigalacturonic acids were isolated and used as standards to prove the reaction course. These and other oligogalacturonic acids appeared in the very early stage of hydrolysis and ultimately all were converted to galacturonic acid. The rates and apparent course of hydrolysis of polygalacturonic acids were independent of the molecular weights of substrates within the limits of 1900 to 35,000.

THAT PECTIC ENZYMES produce a series of oligouronides from pectic substances is shown by recently published chromatographic studies by Jermyn and Tomkins (*9*), Altermatt and Deuel (*1*), Ayers *et al.* (*3*), Dingle *et al.* (*4*), and Roelofsen (*21*). While much preliminary information can be gained from paper chromatographic studies, isolation and characterization of materials are required as final proof. Phaff and Luh (*19*) and Altermatt and Deuel (*1*) obtained preparations of galacturonic, di-, and trigalacturonic acids that analyzed as monohydrates. The latter workers (*2*) used a combination of ion exchange and lead precipitation to obtain tetragalacturonic acid. None of these oligouronic acids were distinctly crystalline.

In studies of the action of purified polygalacturonase (PG) on polygalacturonic acids the hydrolysis products were separated by paper chromatography on heavy filter paper and crystallized as their brucine salts (*12*). These salts and the free acids regenerated from them were characterized by chemical analyses and used as final proof of the reaction course of polygalacturonase on polygalacturonic acids.

A purified fungal polygalacturonase prepared by the method of Jansen and MacDonnell (*8*) was shown to hydrolyze the glycosidic linkage of pectic acid in such a manner that the rate of hydrolysis was 17 times faster up to 50% hydrolysis than from 50% on. It was suggested that the rapid stage of the reaction might be concerned with the formation of digalacturonic acid, and the second slow stage with the hydrolysis of digalacturonic acid to galacturonic acid. It was found that galacturonic, di- and trigalacturonic, and probably tetragalacturonic acids appeared in the very early stages of hydrolysis. Tetragalacturonic acid was present up to about 72% hydrolysis, but traces of trigalacturonic acid were demonstrated when 95% of the bonds were hydrolyzed. Traces of digalacturonic acid were still present at 97% hydrolysis. These results suggest a somewhat random attack of polygalacturonic acid by polygalacturonase. No differences in initial rates and apparent course of hydrolysis of polygalacturonic acid substrates with polygalacturonase were observed within the limits of molecular weights of 1900 to 35,000.

Methods and Materials

Polygalacturonic acids of several molecular weights were prepared from commercial citrus pectin (Pectinum N.F. VII, manufactured by Sunkist Growers, Ontario, Calif.).

Enzyme-de-esterified polygalacturonic acid was made with orange pectinesterase (PE) by the method of MacDonnell, Jansen, and Lineweaver (*14*). Pectin in 1% solution plus orange pectinesterase was maintained at pH 6.0 by adding dilute sodium hydroxide over a period of an hour. No further additions of alkali were then necessary to maintain pH 6.0 and hydrochloric acid was added to pH 1.5. The precipitated polygalacturonic acid was pressed free of most of the solution, washed once in an equal volume of water, pressed, and washed in 60% ethyl alcohol to remove hydrochloric acid. After the washings were essentially free of chloride ion, the polygalacturonic acid was further dehydrated with 95% ethyl alcohol, air-