dietary groups were 0.16 ± 0.02 and 0.14 ± 0.06. The lactic dehydrogenase activity was markedly lower than corresponding succinoxidase activity values. As the heart obtains a great amount of its energy from lactic acid, it was believed that the high dietary energy ration would give a greater concentration of available lactic acid, resulting in an increased capacity of the lactic dehydrogenase enzyme. As this did not occur in this study to a significant extent, it may indicate that the heart has a considerable capacity to maintain its needs under greatly varying dietary intakes of foods that may supply the lactic acid metabolite.

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ALFALFA CAROTENE

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

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The AOAC method for the determination of carotene in alfalfa has been modified to eliminate interference from *N,N'*-diphenyl-*p*-phenylenediamine, a carotene antioxidant. The diamine causes the formation of a yellow color when chromatographed on magnesia as in the official method, interfering with the colorimetric determination of carotene. After chromatography, this color can be removed from the carotene eluate without affecting the carotene by adding dilute alcoholic stannous chloride.

When carotene is determined by the AOAC method (*1*), *N,N'*-diphenyl-*p*-phenylenediamine used to stabilize carotene in alfalfa (*6*) causes high results (*2*). When an acetone-hexane solution containing this diamine is eluted through magnesia as in the AOAC method, the colored product has an absorption spectrum similar to carotene. Beauchene and others (*2*) state that a correction factor for the interference is impractical because it would depend on the amount of the diamine present, size of column, and amount of eluate. When a solution of *N,N'*-diphenyl-*p*-phenylenediamine only is eluted through magnesia, a large amount is adsorbed in a black band at the top of the column. The colored reaction product is slowly eluted as the chromatogram is developed, but apparently not all the diamine is eluted even with high acetone concentrations.

Mitchell and Silker (*7*) avoided this interference by using tricalcium phosphate as an adsorbent. Booth (*3*) used aluminum oxide for the adsorbent and light petroleum (b. p. 80° to 100° C.) to extract the carotene from the leaf meal, taking advantage of the low solubility of the diamine in light petroleum in the absence of acetone. These adsorbents do not form the colored reaction product. Brew (*4, 5*) removed the reaction product from the eluate in the AOAC method by washing the eluate with 85% phosphoric acid. For the purpose of quality control it would be better if the AOAC method could be used with only a slight modification. The method of Mitchell and Silker requires removal of the acetone from the carotene extract by evaporation before it is chromatographed on the tricalcium phosphate.

It appeared to this author that the yellow color was caused by an oxidation product of the diamine, probably *N,N'*-diphenyl-*p*-quinonediimine. It had been found in previous experiments in removing the colored reaction product from the eluate that concentrated phosphoric acid produced a pink color (*5*) and concentrated hydrochloric acid produced a blue color in the acid layer; therefore, *N,N'*-diphenyl-*p*-phenylenediamine was oxidized with a solution of benzoyl peroxide in hexane and found to undergo similar color reactions. A 1% alcoholic solution of stannic chloride also produced a deep blue color upon addition to the diamine-magnesia eluate and to the oxidized diamine in the absence of excess unreacted benzoyl peroxide. The unoxidized diamine, oils, and magnesia eluates of untreated alfalfa extracts gave negative results.

The question arose, whether the diamine in an oil can autoxidize and give the same interference for a sample treated with the oil, even though the carotene extract is not eluted through magnesia. A 2-year-old laboratory sample of a crude vegetable oil treated with the diamine that gave a positive test for the autoxidized diamine by the above color reactions was used to test this possibility. A sample of dehydrated alfalfa meal was treated with the oil and analyzed for carotene by eluting the carotene extract through tricalcium phosphate and Hyflo Supercel (1 + 1 by weight) as in the method of Mitchell and Silker. The oxidized diamine showed up in a narrow band trailing behind the carotene, giving no interference for this adsorbent in the complete absence of acetone. A trace of acetone caused the oxidized diamine to move down with the carotene tailings.

A similar band formed when a few milliliters of a diamine eluate from a magnesia chromatographic column were

added to an alfalfa carotene extract and then evaporated and chromatographed as above. Further evidence that the diamine can autoxidize to form the same compound as in the magnesia eluate was shown by chromatographing a hexane solution of 0.5 ml. of the oil sample and a few milliliters of the diamine-magnesia eluate on tricalcium

Table I. Determination of Carotene

	$SnCl_2$ Used	None Used
Meal 1		
A. Untreated		27.9
B. Control (1% oil)	27.8	27.8
C. 0.1% *N,N'* - diphenyl-*p*-phenylenediamine	28.2	33.2
Meal 2		
A. Control	18.1	18.0
B. 0.1% *N,N'* - diphenyl-*p*-phenylenediamine	18.3	22.0
C. 5% oil and 0.1% *N,N'*-diphenyl-*p*-phenylenediamine	18.3	
Meal 3		
A. Control	26.0	25.9
B. 0.1% *N,N'* - diphenyl-*p*-phenylenediamine	26.3	35.4
C. Sample (B) by method of Mitchell and Silker		23.9
Meal 4		
A. Control	22.1	22.1
B. 0.025 *N,N'* - diphenyl-*p*-phenylenediamine	22.4	22.6
C. Sample (B) by method of Mitchell and Silker		19.7

phosphate after evaporation to remove any acetone. Only a single narrow yellow band formed. The higher concentration of the magnesia-oxidized diamine formed so sharp and narrow a band that it can even be used as a marker for any slight amount of autoxidized diamine with this adsorbent.

A very sensitive qualitative test used for the presence of the oxidized diamine can be made as follows: Add 5 ml. of concentrated hydrochloric acid to 95 ml. of acetone and mix. Add 5 ml. of this solution freshly made to 10 or 20 ml. of the eluate. About 0.5 ml. will separate in the acid layer, which will be blue if there is any interference. The test is not so sensitive when hydrochloric acid is not previously mixed with acetone.

In course of these experiments it was found that alcoholic stannous chloride would reduce the yellow colored product. The possibility of the use of this reagent was immediately tested to eliminate the interference in the AOAC method for carotene, which was to be used exclusively in quality control. Pursuing this idea, alcoholic stannous chloride was added to numerous carotene eluates. Allowing for dilution of the eluate by the reagent, the absorbance of the carotene solutions was not affected within the hour that may be required to read the absorbance on a spectrophotometer. Neither was there further loss of absorbance within 1 hour in carotene solutions that had contained the oxidized diamine, after the initial loss due to reduction of the interfering yellow color. There was considerable loss of carotene in some eluates containing stannous chloride after they were allowed to stand overnight.

The AOAC method was modified as follows:

Reagent. Add 0.1 gram of $SnCl_2.2H_2O$ to 50 ml. of absolute ethyl alcohol. Make up fresh daily.

Procedure. Follow the AOAC method of extraction and chromatography, but before making to volume add 5 ml. of the reagent to the eluate. Shake, make to 100-ml. volume according to the regular procedure, and immediately read on the spectrophotometer.

Experimental Work

It was found that 5 ml. of the reagent, equivalent to 10 mg. of stannous chloride, was more than adequate to reduce all the oxidized diamine ordinarily encountered in an analysis. In an eluate containing only the oxidized diamine, the yellow color was removed, leaving a clear solution with 100% transmittance.

The method was tested using alfalfa treated with 1% soybean oil and various levels of the diamine. The diamine was dissolved in the oil by heating slightly, and 99 grams of dehydrated alfalfa meal were gradually blended into 1 gram of the oil, by adding the meal in small increments to the oil and thoroughly mixing manually with a spatula, thus avoiding the formation of lumps. At higher levels of the diamine, where it was difficultly soluble in the oil, a volume of acetone equal to the oil was added. After the alfalfa meal had been blended into the oil-acetone solution, the alfalfa was spread out to air-dry to remove the acetone. The meal was then analyzed for carotene. Typical results are shown in Table I. Results using stannous chloride are slightly higher than with untreated controls, but they are closer to the AOAC method in absence of diamine than the Mitchell and Silker method.

Absorption spectra were also made of carotene eluates with and without fresh stannous chloride and *N,N'*-diphenyl-*p*-phenylenediamine. No difference could be found in the shape of the curves, as shown in Figure 1.

Stronger concentrations of stannous chloride in alcohol than that given above are likely to produce a turbidity in the eluate, particularly when isopropyl alcohol, methanol, or anhydrous denatured alcohol is used. There was no difficulty with cloudy eluates, however, even with isopropyl alcohol, when 0.2% alcoholic stannous chloride was used.

The reagent deteriorated rapidly when allowed to stand a day or so at room temperature, although it was stable for over a month when stored at −10° C. in a freezer. The old reagent no longer completely decolorized the oxidized diamine and actually caused as much as 10% increase in the absorbance of pure carotene solutions. It is therefore recommended that the reagent be made up fresh daily.

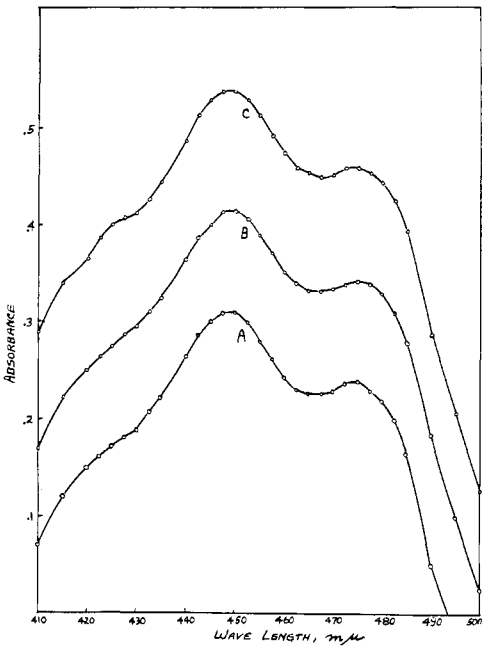

Figure 1. Spectral absorption curves obtained on Beckman B spectrophotometer for carotene eluates

A. Eluate from untreated alfalfa
B. Similar eluate containing alcoholic stannous chloride
C. Eluate from dehydrated alfalfa treated with 0.025% *N,N'*-diphenyl-p-phenylenediamine

Color interference removed with stannous chloride. Absorbance scale is only relative

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