A COMPARISON OF THE SODIUM 2,6-DICHLOROBENZENONEINDOPHENOL AND THE DIAZOTIZED 4-METHOXY-2-NITROANILINE COLORIMETRIC METHODS FOR DETERMINING REDUCED ASCORBIC ACID CONTENT OF FROZEN PEAS

by

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TABLE OF CONTENTS

INTRODUCTION

The amount of reduced ascorbic acid in fruits and vegetables has been used as one method of indicating quality. A number of methods have been used for determining reduced ascorbic acid. However, since there appeared to be no one entirely satisfactory method for measuring ascorbic acid content, it seemed desirable to compare two methods considered applicable to a large number of routine laboratory analyses for reduced ascorbic acid.

The sodium 2,6-dichlorobenzenoneindophenol method depends directly upon the oxidation-reduction reaction, whereas the diazotized 4-methoxy-2-nitroaniline method depends somewhat on the enediol character of ascorbic acid rather than directly upon its reducing power. The diazotized 4-methoxy-2-nitroaniline method has been found to be highly specific for ascorbic acid. Also, since readings with a Klett-Summerson photoelectric colorimeter must be made within 15 seconds with the sodium 2,6-dichlorobenezenoneindophenol method, an apparent advantage of the diazotized 4-methoxy-2-nitroaniline method would be the longer time allowance of 10 minutes for reading the instrument.

The purpose of the present investigation was: (1) to compare values for reduced ascorbic acid in individually frozen peas as determined by the sodium 2,6-dichlorobenzenoneindophenol and the diazotized 4-methoxy-2-nitroaniline methods and (2) to determine any variations that exist in the reduced ascorbic acid content within one grade of frozen peas from different

packages, analyzed either in the morning or afternoon.

REVIEW OF LITERATURE

Determination of Reduced Ascorbic Acid

Many methods of ascorbic acid analysis have been used from early times. Since the number of methods has been so great and the number of modifications even greater, this review has been limited to indophenol methods, with emphasis on those using a photoelectric colorimeter, and the diazotized 4-methoxy-2-nitroaniline method. The study reported was concerned only with reduced ascorbic acid of peas, therefore, the review was further limited to methods of determining the reduced ascorbic acid content of fruits and vegetables.

One of the chief concerns of the person analyzing food for reduced ascorbic acid is the prevention of oxidation, since ascorbic acid itself is a strong reducing agent. The oxidation reaction is catalyzed by oxygen, copper, iron, silver, light, and such fluorescent materials as riboflavin (Rosenberg, 1951). Musulin and King (1936) stated that nearly all tissue containing vitamin C also contains enzymes which catalyze aerobic oxidation of vitamin C in injured tissue.

Acids are used during tissue extraction to retard the oxidation. An eight percent hot acetic acid was used by Bessey and King (1933) for extracting plant tissue. Musulin and King (1936) demonstrated the value of using a two percent metaphosphoric acid solution with either four to eight percent acetic

or trichloroacetic acid. Metaphosphoric acid helped to protect the ascorbic acid from oxidation. However, Bessey (1938) called attention to the instability of metaphosphoric acid. He stated that although metaphosphoric acid reacts slowly with water to form the more acid orthophosphate it was satisfactory for use for 15 days when held at 5°C. Higher temperatures caused a more rapid change. Bessey (1938) and Morell (1941) used a three percent metaphosphoric acid buffered between pH 3.5 and 3.7. King (1941) indicated metaphosphoric acid was superior to either acetic or trichloroacetic acid used in early work for ascorbic acid extraction. He also postulated that something other than pH was involved in the protective action of an acid, since at the same pH metaphosphoric acid was superior to sulfuric, which was superior to hydrochloric acid.

Loeffler and Ponting (1942) used one percent metaphosphoric acid for the extracting medium and no buffer was required. They indicated 0.4 percent oxalic acid could be substituted for metaphosphoric acid. Ponting (1943) compared 13 acids for stabilizing effect on ascorbic acid solutions and found metaphosphoric acid and oxalic acid were superior to the others tested. It was concluded that oxalic acid could be made in larger quantities and had the advantages of being more stable, more easily obtained, and less expensive than metaphosphoric. Oxalic acid also seemed to be slightly more effective in preventing ascorbic acid oxidation than metaphosphoric if copper were present.

However, when copper was not present, metaphosphoric acid was slightly more effective than the oxalic.

Bessey (1944) indicated that the functions of acetic acid and metaphosphoric acid in ascorbic acid analysis are: (1) to maintain necessary acidity, (2) to inhibit oxidation catalyzed by certain metallic ions, (3) to inactivate enzymes, and (4) to precipitate protein and liberate protein-bound ascorbic acid.

Lantz (1951) used 0.5 percent oxalic acid or one percent metaphosphoric acid for extracting ascorbic acid from peaches. No differences were found in ascorbic acid values of metaphosphoric acid and oxalic acid extracts of the same peaches. This confirmed the findings of Ponting (1943).

Morse (1953) indicated that earlier workers had established the reaction between ascorbic acid and oxygen in the presence of copper as a first order reaction. He indicated oxalic acid and metaphosphoric acid were among a number found effective in inhibiting catalytic destruction of ascorbic acid by copper. Oxalic acid was listed as a powerful inhibitor. Schmall, et al. (1953) and (1954) used a 0.5 percent oxalic acid solution for extraction of ascorbic acid.

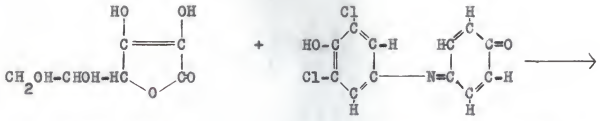
Barker and Mapson (1959) found there was less oxidation of ascorbic acid if: (1) extraction was carried out at -3°C rather than at room temperature, (2) oxygen was excluded by use of a stream of nitrogen, (3) a four percent metaphosphoric acid solution was used rather than a two percent, (4) blending was done immediately without allowing the food to stand in the

metaphosphoric acid, even at temperatures of -70° and -179°C.

Freebairn (1959) found both metaphosphoric acid and oxalic acid inferior to a trichloroacetic acid solution containing (ethylene dinitrilo) tetraacetic acid (EDTA) for preventing oxidation of ascorbic acid in plant materials.

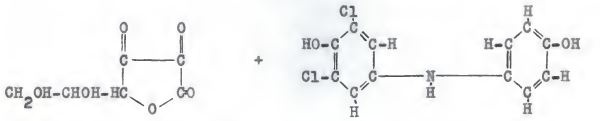
Indophenol Methods. One of the early successful methods of ascorbic acid analysis, using the oxidation-reduction indicator, 2,6-dichlorophenolindophenol with fruits and vegetables was developed by Bessey and King (1933). Dye titration was compared with iodine titration and with animal assay as methods of ascorbic acid analysis. Ascorbic acid values for dye titration tended to be lower than for iodine titration. However, there was a close relationship between the values from dye titration and the range of values supplied by animal assay.

The general reaction of ascorbic acid with indophenol dye was illustrated by Eddy (1949).



L-Ascorbic Acid
(reduced form)

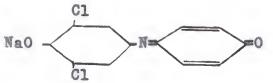
2,6 Dichlorophenolindophenol
(red in acid; blue in alkali)



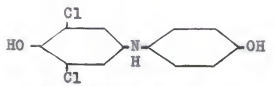
L-Dehydroascorbic Acid
(oxidized form)

Leuco-Indophenol Dye
(colorless)

Several studies were carried out by the United States Public Health Service on oxidation-reduction indicators. Gibbs, et al. (1925) indicated dichloro compounds were among the more stable of the indophenols, were easy to prepare and purify for use, and retained their brilliant blue color in mildly acid solutions. They studied seven different dichloro-substitution products of phenol indophenol. Two of the seven are illustrated.

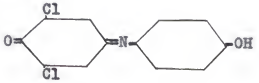


Phenol Indo-2,6-Dichlorophenol

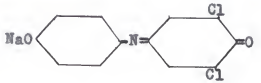


Leuco Derivative of Above

There has been considerable confusion in the naming of indophenol compounds. The nomenclature of indophenols was discussed by Gibbs, et al. (1928). The First Decennial Index of Chemical Abstracts (1907-1916) builds a name upon "benzenone" which is represented by the structure  . Therefore, the following compound has the scientific name 2,6 dichlorobenzenoneindophenol and the common name 2,6 dichlorophenolindophenol.



Since sodium salts are more stable than the acid form of the dye, the sodium salt form is preferred. Cohen, et al. (1924) stated salts were easier to handle than the acid form of the dye. Rosin (1955) illustrated the formula for the sodium salt of 2,6-dichlorobenzenoneindophenol:



Stone (1940) stated that standardized aqueous solutions of the oxidation-reduction indicator, sodium 2,6 dichlorobenzenoneindophenol had been used for some time in ascorbic acid analysis. Bessey and King (1933) were among investigators mentioned who used this compound.

A number of workers prepared a solution of sodium 2,6-dichlorobenzenoneindophenol dye for photoelectric methods of ascorbic acid analysis. The dye was designated by various names. It was called 2,6 dichlorophenolindophenol by Bessey (1938) and Morell (1941), 2,6 dichlorobenzenoneindophenol by Robinson and Stotz (1945), and sodium 2,6 dichlorobenzenone-indophenol by Rubin,et al. (1945).

Several workers (Bessey, 1938), (Robinson and Stotz, 1945) and (Rubin,et al., 1945) indicated the dye solution could be kept for 10 days to two weeks if refrigerated. Esan (1959) stated that the instability of 2,6 dichlorobenzenoneindophenol is inherent in the dye and is caused also by inadequate care in manufacturing dyes for analytical purposes. He emphasized that if a good product and pure water were used to prepare a dye solution it would be stable for many weeks.

The 2,6 dichlorophenolindophenol dye used by Bessey (1933) required daily restandardization with lemon juice and an occasional check with pure ascorbic acid. The dye was standardized by using an iodine and a dye titration of the lemon juice. Using the values obtained, the amount of vitamin C in plant tissue was calculated.

Menaker and Guerrant (1938) raised objections to the practice of titrating lemon juice or ascorbic acid against a standard iodine solution as a means of dye standardization. They pointed out certain problems such as: (1) iodine solutions are instable, (2) fresh lemon juice or ascorbic acid must be

prepared daily, (3) substances in lemon juice interfere with the apparent amount of ascorbic acid in solution, and (4) it is difficult to obtain a satisfactory end point. They recommended the use of sodium thiosulfate solution instead of iodine solution. Sodium thiosulfate was more stable than iodine, produced a sharper end point of titration, and eliminated one step of standardization, since in the earlier methods the iodine standard required checking with sodium thiosulfate. Rubin, et al. (1945) used this method of dye standardization.

A photoelectric method, that made possible the ascorbic acid analysis of highly colored tissue extracts and eliminated the subjective reading of an end point, was developed by Bessey (1938). The sample to be analyzed was prepared as in the method of Bessey and King (1933) except that a metaphosphoric acid was employed as the extracting acid rather than acetic. The extract was centrifuged and a specified amount, which contained a quantity of ascorbic acid insufficient to reduce the dye completely, was added to test tubes containing a 2,6 dichlorophenolindophenol dye solution. Readings were taken in a photoelectric colorimeter after 15 seconds and again after 30 seconds. A crystal of ascorbic acid was added to the tubes and a third reading taken after the dye was completely reduced, to use in correcting for turbidity of the sample.

Davis (1939) suggested a liquifier made by Waring Corporation would simplify the grinding of fruits and vegetables and reported its use in his study (1942). Morell (1941) altered

the method of Bessey (1938) by using a Waring Blendor, filtering in place of centrifuging the blended material and using standard ascorbic acid solutions for standardizing the dye.

Loeffler and Ponting (1942) developed an indophenol method to be used on a wide variety of fresh, dehydrated, and frozen fruits and vegetables in the Western Regional Research Laboratories. The method of preparing plant extracts used by Morell (1941) and a modification of the colorimetric method of ascorbic acid analysis of Evelyn,et al. (1938) were combined. The method of Evelyn,et al. was modified by using a one percent metaphosphoric acid in place of glacial acetic acid for the extracting medium. Loeffler and Ponting also chose to add the dye to the test tube before it was put into the colorimeter and to take readings in 15 seconds instead of five, 10, 20, and 30 seconds. The most important contribution of the Loeffler and Ponting method was the elimination of the need for buffering the extracting medium. With a one percent metaphosphoric acid solution the pH (2.4 to 3.1 before addition of dye) was sufficiently low to prevent ascorbic acid losses in blending and still was high enough to prevent fading of the dye.

A number of workers have used the Loeffler and Ponting (1942) method, or a modification of it. Some of these were Storvick,et al. (1950), Lindquist,et al. (1950), Potgieter and Greenwood (1950), Hummel and Okey (1950), Paul and Ferley (1954), Charles and Van Duyne (1954), Huguenard,et al. (1955), Dietrich, et al. (1955), Fisher and Dodds (1955), Tinklin and Filinger

(1956), Dietrich,et al. (1957 a,b), Guadagni,et al. (1957 a,b, c), Guadagni and Nimmo (1957), Guadagni and Kelly (1958), Dietrich,et al. (1959 a,b), and Boggs,et al. (1960).

Even though indophenol methods have been used widely for ascorbic acid analysis, there are substances other than ascorbic acid that may interfere with obtaining accurate values for ascorbic acid. Mottern,et al. (1932) stated that there are many compounds having no vitamin C activity that will reduce indophenol dye. Bessey and King (1933) indicated that it was essential in interpreting results of the indophenol method to consider the chemical nature of the substance tested, because any substance with a reduction potential lower than the dye might introduce error. King (1941) stated that sulfhydryl compounds and carbohydrate decomposition products are likely to interfere, but natural products do not contain enough of the interfering substances to cause a large error. Furthermore, any error may be decreased by taking readings at 15 second intervals on the photoelectric colorimeter.

An indophenol-xylene extraction method was used on plant materials by Pepkowitz (1943). He found less interference with this method than with the Bessey (1938) method. The indophenol-xylene extraction method with peroxide and formaldehyde modifications to prevent interference was discussed by Robinson and Stotz (1945). After considerable work with a number of foods they indicated the unmodified indophenol methods were not satisfactory for ascorbic acid analysis when foods had been heated

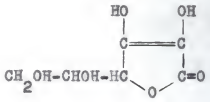
for long periods or stored for long periods. Miller (1947) used the indophenol-xylene extraction method with the formaldehyde modification to study interference in the indophenol method. She found no interference when fresh vegetables and fruits were analyzed. However, interference was noted in all processed foods studied.

Dodds, et al. (1948) compared the indophenol method of Loeffler and Ponting (1942), the indophenol-xylene method with the formaldehyde modification of Robinson and Stotz (1945), and the 2,4 dinitrophenylhydrazine method of Roe and Oesterling (1944) in analyses of sweet potatoes. The results of the various indophenol methods were similar, but the 2,4 dinitrophenylhydrazine method gave variable results. This was in contrast to the findings of Guadagni and Kelly (1958). They found good agreement between the 2,6-dichlorophenolindophenol method and the 2,4-dinitrophenylhydrazine method and used the former for determination of reduced ascorbic acid. In addition to giving comparable results the 2,6-dichlorophenolindophenol method was more rapid than the 2,4-dinitrophenylhydrazine method. Noble and Hanig (1948) determined the reduced ascorbic acid and dehydroascorbic acid content of six different raw and cooked vegetables, including peas, using indophenol and dinitrophenylhydrazine methods. The indophenol method of Morell (1941) with the correction for color and turbidity of Bessey (1938) was used to determine the reduced ascorbic acid.

Diazotized 4-Methoxy-2-Nitroaniline Method. Schmall, et al. (1953) described the diazotized 4-methoxy-2-nitroaniline method of ascorbic acid analysis. It was mentioned also by the Heinz Nutritional Research Division (1956, p. 38) as a chemical method for ascorbic acid determination. Peters, et al. (1960) used the method for determining the ascorbic acid content of a number of colored South Pacific foods, whereas, the 2,6-dichlorophenolindophenol titration method was used for colorless solutions.

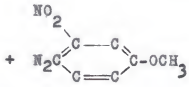
The diazotized 4-methoxy-2-nitroaniline method was found to be highly specific for ascorbic acid by Schmall, et al. (1953). They pointed out that the method depends upon the enediol character of ascorbic acid rather than directly upon the oxidation reduction reaction as in the sodium 2,6-dichlorobenzenoneindophenol method.

The reaction of ascorbic acid with diazotized 4-methoxy-2-nitroaniline has not been completely worked out, but according to Schmall, et al. (1954) the final product has been identified as the deep blue disodium salt of oxalic acid 4-methoxy-2-nitrophenylhydrazide (p. 14).

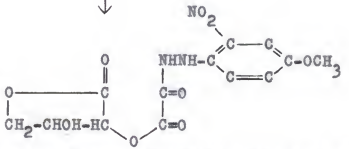


Ascorbic Acid

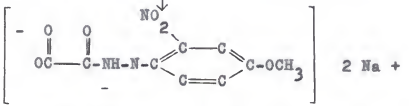
+



4-Methoxy-2-Nitrobenzenediazonium Cation



4-Methoxy-2-Nitrophenylhydrazide of the Oxalate
of D Threonic Acid Lactone



Deep Blue Disodium Salt of Oxalic Acid
4-Methoxy-2-Nitrophenylhydrazide

Factors Affecting the Reduced Ascorbic Acid Content of Peas

The variability in reduced ascorbic acid content of canned and frozen peas determined in the study of Storvick, et al. (1950) was explained on the basis of variability in: (1) fresh material, (2) storage, (3) sampling, and (4) cooking.

Size of Peas. The ascorbic acid content of peas was determined along with that of other vegetables and fruits by Olliver (1938). She found higher values for a given weight of small peas than for large peas. The ascorbic acid values dropped rapidly at first and then gradually as the size of pea increased. However, when ascorbic acid content per pea was determined there was an increase in ascorbic acid as the size of the pea increased. The findings of Tressler, et al. (1936), Todhunter and Sparling (1938), and Todhunter and Robbins (1941) confirmed the fact that small peas are a more concentrated source of ascorbic acid than large peas.

Freezing Rate. Lee, et al. (1946) studied the effect of freezing rate on peas. Slowly frozen products had larger veins of ice than more rapidly frozen peas, but after thawing no differences were determined which could be attributed to the rate of freezing. This was in contrast to the findings of Paul and Ferley (1954) on broccoli. They found ascorbic acid content was decreased slightly as increased insulation from packaging material reduced the speed of freezing.

Storage Temperature. Boggs, et al., (1960) found fluctuation in temperature per se had little or no effect on the ascorbic acid content of frozen peas. However, based on steady temperature, for each five degree Fahrenheit rise from 0° to 25°F, deterioration rates doubled and ascorbic acid losses more than doubled.

Enzymes. Watts and Griswold (1953) indicated that enzymes commonly catalyze the oxidation of ascorbic acid to dehydroascorbic acid. Therefore, inactivation of enzymes by blanching is important for ascorbic acid retention of frozen peas as indicated by Tressler, et al. (1936) and Dietrich, et al. (1955).

Cooking Water. Van Duyne, et al. (1947), using various cooking times and various levels of cooking water with fresh peas, found cooked peas lost more reduced ascorbic acid as the cooking time and water increased. However, most of the reduced ascorbic acid lost from the vegetables (19 to 52 percent based on raw-weight) was present in the cooking water.

EXPERIMENTAL PROCEDURE

Statistical Plan and Methods of Analyses for the Study

The randomized block design with 12 replications of 100-g samples of peas for each method of ascorbic acid analysis is given in Table 1. This design will make it possible to analyze the data by analysis of variance; Fisher's test (Fisher, 1949) for least significant differences, when appropriate; the t-test; and the Bartlett test.

Table 1. Design of the experiment.

Package V	III	I	II	VI	IV
	Randomized Treatments[1]				
$4_a\ A_9$	$7_b\ A_5$	$6_b\ A_1$	$7_b\ A_3$	$4_a\ P_{11}$	$5_b\ A_7$
$5_b\ P_9$	$8_a\ P_5$	$8_b\ A_1$	$4_a\ P_3$	$8_a\ P_{11}$	$2_a\ P_7$
$2_a\ A_9$	$1_b\ A_5$	$2_b\ A_2$	$6_a\ P_3$	$7_b\ A_{11}$	$1_b\ A_7$
$3_b\ P_9$	$2_a\ P_5$	$3_a\ P_1$	$3_b\ A_3$	$1_b\ A_{11}$	$7_b\ A_8$
$7_b\ P_{10}$	$6_a\ A_6$	$7_a\ P_1$	$2_a\ A_4$	$6_a\ P_{12}$	$4_a\ P_7$
$1_b\ P_{10}$	$3_b\ P_6$	$4_b\ A_2$	$1_b\ P_4$	$5_b\ A_{12}$	$8_a\ P_8$
$8_a\ A_{10}$	$5_b\ P_6$	$5_a\ P_2$	$8_a\ A_4$	$3_b\ A_{12}$	$6_a\ P_8$
$6_a\ A_{10}$	$4_a\ A_6$	$1_a\ P_2$	$5_b\ P_4$	$2_a\ P_{12}$	$3_b\ A_8$

[1]Randomized treatments:
Arabic number - number of the 100-g sample of peas
Arabic subscript letter a = sodium 2,6-dichlorobenzenone-indophenol method
b = diazotized 4-methoxy-2-nitroaniline method
A or P = period of analysis (AM or PM)
Arabic subscript number = day of analysis

Purchasing, Preparation, and Storage of Peas

Two-pound packages from one lot of U. S. Grade A Fancy, individually frozen peas were purchased for the entire study from a local market one day in midsummer. The following afternoon each original two-pound package was taken from the freezer, gently tapped to separate the peas, and the contents emptied into a plastic bowl for thorough mixing. From the bowl 100-g samples of peas were weighed on a Toledo scale into polyethylene bags marked to indicate the number of the original package, the

number of the sample, the method of analysis, the day of analysis, and the period of analysis. A beaker and a stainless steel spoon were used to help fill the bags. Weighing was done rapidly to prevent thawing. All samples from one original package were closed, tied with "Twist-em" covered wire, and stored on one shelf of a home freezer at a temperature ranging from -15° to + 20°F.

Analysis of Peas by the Sodium 2,6-Dichlorobenzenoneindophenol Method

A modification of the sodium 2,6-dichlorobenzenoneindophenol method of ascorbic acid analysis developed by Loeffler and Ponting (1942) was used.

Preparation of Sample Extract. A thermostatically controlled gas burner was used for cooking the frozen peas. Each sample of peas was dropped into 80 ml of boiling distilled water in a one-quart aluminum saucepan with a vented lid. When the water returned to a boil they were cooked for one minute with the temperature control set at the highest point. Then the vigorous boiling was reduced by adjusting the temperature control to between 200°F and 225°F and the cooking continued for six minutes. Immediately after cooking, the peas, remaining cooking liquid, and approximately one ml of butyl stearate were blended in a Waring Blendor with one percent metaphosphoric acid. To avoid splashing from the Blendor a Powerstat Variable Transformer was used to control the speed of the Blendor. After

blending five minutes the material was transferred quantitatively to a one liter volumetric flask, made up to volume with one percent metaphosphoric acid, mixed, and filtered through fluted filter paper (Whatman #1) to clear the filtrate. Fourteen ml of the clear filtrate were pipetted into a 50 ml volumetric flask, diluted to volume with one percent metaphosphoric acid, and mixed thoroughly.

Measurement of Ascorbic Acid. For each sample to be analyzed, one test tube was filled with five ml of distilled water and three test tubes with five ml of dye. After taking a reading on the colorimeter for the blank (the dye plus acid), five ml of the sample solution were pipetted into the test tube containing distilled water and the colorimeter was adjusted to zero absorbancy to correct for color of the vegetable. Next, five ml of the sample dilution were pipetted into each test tube containing dye. Readings on the colorimeter, used in calculating ascorbic acid values were checked as described for standardization of the dye (Appendix, p. 43).

Calculation of Reduced Ascorbic Acid Values. The concentration of reduced ascorbic acid in mg per 100 g of peas was found by the following formula:

blank reading - unknown reading = corrected unknown reading

$$\frac{\text{ascorbic acid factor} \times \text{corrected unknown reading}}{\text{aliquot portion}} \times \text{dilution} = \text{ascorbic acid/100 g sample}$$

Analysis of Peas by the Diazotized 4-Methoxy-2-Nitroaniline Method

The diazotized 4-methoxy-2-nitroaniline method described by Schmall, et al. (1953) was used for determining reduced ascorbic acid.

Preparation of Sample Extract. The 100-g samples of peas were cooked in exactly the same manner as for the sodium 2,6-dichlorobenzenoneindophenol method. Peas were blended immediately afterwards in a Waring Blendor with 0.5 percent aqueous oxalic acid and about one ml of butyl stearate. After blending five minutes, the mixture was transferred quantitatively to a 500 ml volumetric flask, made up to volume with 0.5 percent aqueous oxalic acid, thoroughly mixed, and filtered through fluted filter paper. This filtrate was used as the sample extract in the sample solution and the sample blank described below.

Preparation of Sample Solution. Two ml of amino reagent were pipetted into a 200 ml volumetric flask, followed by two ml of nitrite reagent. The solution was swirled until the orange color of the amino reagent disappeared. Then 100 ml of 95 percent ethyl alcohol were added and the contents of the flask mixed. A 40 ml aliquot of the sample extract containing approximately 1.0 mg of ascorbic acid in a 0.5 percent solution of aqueous oxalic acid was added. The solution was mixed and allowed to stand, for development of a yellow color, one minute for each ml of sample extract exceeding five ml. Therefore, in this study a 35 minute standing time was used. After standing, 25 ml of 10

percent sodium hydroxide were added and a bright blue color developed. The solution was swirled, brought up to volume with distilled water, and the contents mixed thoroughly. The solution was filtered to remove particles causing turbidity and/or air bubbles before reading in the colorimeter. Samples were read within 10 minutes to protect from fading, which Schmall, et al. (1953) indicated might occur with certain samples such as fruit juices and chocolate powders.

Preparation of Sample Blank. The sample blank was prepared in exactly the same manner as the sample solution except that the amino reagent was omitted and five ml of 0.5 percent aqueous oxalic acid were added before the sample extract.

Preparation of Reagent Blank. Once daily, the reagent blank was prepared exactly as the sample solution except no sample extract was added and five ml of 0.5 percent aqueous oxalic acid were added, as in the sample blank. No standing time was required.

Preparation of Standard Solutions. Two standard solutions were prepared once daily. Standard A contained one mg of ascorbic acid, whereas, standard B contained 1.4 mg. These solutions were prepared exactly as the sample solution except that no sample extract was added. Also, three ml of aqueous oxalic acid and two ml of ascorbic acid standard were added to standard A, whereas, two ml of aqueous oxalic acid and three ml of ascorbic acid standard were added to standard B. A five minute standing time, determined by preliminary work, was used for development of color in these solutions.

The sequence for adding reagents to solutions prepared for the diazotized 4-methoxy-2-nitroaniline method is given in Table 2.

Table 2. Sequence of addition and amounts of reagents and sample.

Sequence for reagents	Sample soln. (ml)	Sample blank (ml)	Reagent blank (ml)	Standard A (ml)	Standard B (ml)
Amino reagent	2	--	2	2	2
Nitrite reagent	2	2	2	2	2
Alcohol	100	100	100	100	100
Oxalic acid reagent	--	5	5	3	2
Sample	40	40	--	--	--
Standard	--	--	--	2	3
Sodium hydroxide	25	25	25	25	25
Water	To make 200 ml in all flasks				

Measurement of Ascorbic Acid. A Klett-Summerson photoelectric colorimeter containing the same light filter used for the sodium 2,6-dichlorobenzenoneindophenol method (Appendix, p. 43) was adjusted to zero absorbancy with distilled water. At least two readings were taken and recorded within 10 minutes after each solution was prepared and mixed.

Calculation of Reduced Ascorbic Acid Values. The following formula was adapted from Schmall, et al. (1953) for use in calculating reduced ascorbic acid.

$$A + \frac{(B - A)\ (E - C)}{D - C} = F.$$

A = mg of ascorbic acid in standard A.
B = mg of ascorbic acid in standard B.
C = corrected reading of standard A (reading of standard A - reading of reagent blank).
D = corrected reading of standard B (reading of standard B - reading of reagent blank).
E = corrected reading of sample [reading of sample solution - (reading of reagent blank + reading of sample blank)].
F = mg of reduced ascorbic acid in sample aliquot.

RESULTS AND DISCUSSION

The investigation was completed within seven weeks after the peas were purchased and stored. During storage the temperature in the freezer varied from -15° to + 20°F. The great amount of fluctuation might be explained, in part, by mechanical difficulty over which there was no control. Boggs, et al. (1960) found that temperature fluctuation alone, however, had no mean effect on ascorbic acid losses of peas, but high storage temperatures caused greater losses of ascorbic acid than low temperatures.

Reduced Ascorbic Acid Content of Peas as Determined by Two Methods

The ascorbic acid values of the peas were subjected to the analysis of variance presented in Table 3 to determine the significance of differences that occurred among the packages of peas and between the two methods of analysis as well as those attributable to the interaction of packages x methods. When appropriate, Fisher's (Fisher, 1949) least significant difference was calculated.

Table 3. Analysis of variance of reduced ascorbic acid in peas as determined by two methods.

Source of variation	D/F	Ms and Significance
Packages	5	0.2704
Methods	1	221.2 ***
Packages x methods	5	0.9921 *
Remainder	36	0.1886
Total	47	

*** - Significant at the 0.1% level.
* - Significant at the 5.0% level.

Differences among packages were non significant, whereas, differences attributable to method of analysis and the interaction of packages x methods were significant at the 0.1 percent and 5.0 percent level, respectively. The significant differences attributable to the interaction of packages x method probably occurred in package V (Tables 3 and 4). The mean value for package V analyzed by Method b was significantly higher than that for any other package analyzed by Method b, whereas, the mean value for package V analyzed by Method a was significantly lower than that for either package III or IV. The lowest mean value for all packages analyzed by Method a was obtained in package V.

Variations from Package to Package Within One Grade

All packages contained peas that varied widely in size from large mature peas to smaller, less mature ones. Ascorbic acid values for peas, that occurred within and among packages, analyzed

Table 4. Mean ascorbic acid values of methods[1] with the interaction of packages x methods as the source of variation.

Packages	Methods a (mg/100 g peas)	Methods b (mg/100 g peas)
I	16.49	11.93
II	16.76	12.34
III	16.96	12.11
IV	17.09	12.21
V	16.17	13.18
VI	16.36	12.29

lsd* = 0.62

[1] a = sodium 2,6-dichlorobenzenoneindophenol method
b = diazotized 4-methoxy-2-nitroaniline method

* The means at the ends of the brackets are significantly different.

lsd* = least significant difference at the 5.0% level.

by a given method are illustrated in Figures 1 and 2. According to the Bartlett test (Snedecor, 1956), the sampling variances within the same treatment and package were homogeneous. For individual samples analyzed by the sodium 2,6-dichlorobenzenoneindophenol method (Method a) the ascorbic acid values ranged from 15.77 mg/100 g peas to 17.35 mg/100 g. A range of 11.25 mg/100 g to 13.75 mg/100 g was found with the diazotized 4-methoxy-2-nitroaniline method (Method b). These values are fairly close to the average values reported by Watt, et al. (1950, p. 38) for cooked peas (15 mg/100 g) and frozen peas (18 mg/100 g). Variations in mean ascorbic acid values for packages of U.S. Grade A

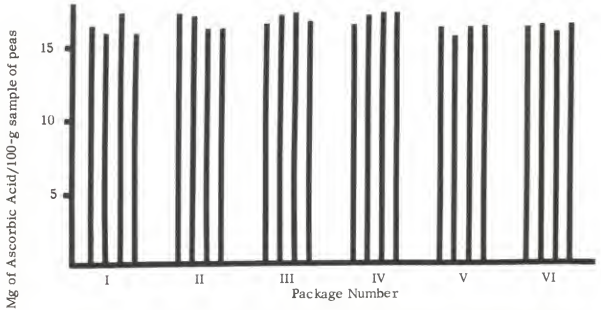


Figure 1. Ascorbic acid values of 100-g samples within a package analyzed by Method a (sodium 2, 6-dichlorobenzenoneindophenol method).

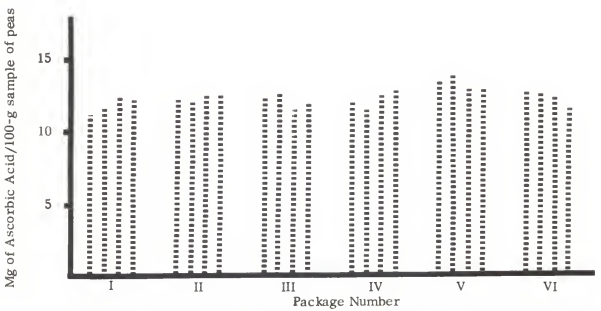


Figure 2. Ascorbic acid values of 100-g samples within a package analyzed by Method b (diazotized 4-methoxy-2-nitroaniline method).

Fancy peas analyzed by each method are shown in Table 4.

Although the differences in reduced ascorbic acid among individual samples of peas seemed small (Figures 1 and 2), some of the mean values (Table 4) were significantly ($P < .05$) different from each other. With Method a the mean ascorbic acid value for package IV was significantly ($P < .05$) greater than that for packages V and VI. Package III also had significantly ($P < .05$) more ascorbic acid than package V. All other differences between packages analyzed by Method a were non significant. When analyzed by Method b package V contained significantly ($P < .05$) more ascorbic acid than any of the other packages.

Variations Resulting from Time of Analysis

Since some of the packages were shown to be different, when the interaction of packages x methods was considered (Table 4) and since packages and method were somewhat confounded with the time of analysis, t-tests were made to determine if there were significant variations in ascorbic acid values. When each method was considered separately there was no difference that could be attributed to the time of analysis. When Method a was compared with Method b, and when the analyses done in the morning and the afternoon were considered separately, it was shown that Method a gave decidedly higher numerical results than Method b at each time of day.

Comparison of the Two Methods

Under the conditions of the study reported here Method b, the diazotized 4-methoxy-2-nitroaniline method, was observed to require: (1) more time for analysis, (2) a greater number of reagents, (3) more steps in the procedure, and (4) as great or greater skill for ascorbic acid analysis when compared with Method a, the sodium 2,6-dichlorobenzenoneindophenol method. The increased number of reagents and steps in Method b allowed greater chance for error. For these reasons Method a was considered more suitable than Method b for routine ascorbic acid analyses. Previous work has shown that the ascorbic acid values obtained with Method a seemed to give a good indication of quality when compared with palatability scores.

Since the diazotized 4-methoxy-2-nitroaniline method has been found to be highly specific for ascorbic acid (Schmall, et al., 1953), it would be beneficial to determine the reasons why it gave lower values than the sodium 2,6-dichlorobenzenoneindophenol method. One might investigate whether or not substances other than ascorbic acid interfere in the sodium 2,6-dichlorobenzenoneindophenol method. A modification of the indophenol-xylene extraction method for determination of interference described by Robinson and Stotz (1945) might be applied to the sodium 2,6-dichlorobenzenoneindophenol method. Formaldehyde was used in one of the modifications to prevent the reducing action of ascorbic acid. Therefore, the addition of formaldehyde to a portion of the sample solution would show the reducing power of substances

other than ascorbic acid that might be in the solution, providing that the formaldehyde did not react in any other way with the sample solution or the dye.

Another problem that should be investigated is the determination of both the reduced and dehydroascorbic acid. Recent knowledge of human utilization of dehydroascorbic acid (Linkswiler, 1958) makes this type of information important for determining the true nutritive value of foods. Davey, et al. (1956) found that peas contained 19.0 to 21.6 mg of total ascorbic acid per 100 g and that 4.7 to 6.3 mg of this was in the dehydro form. Six vegetables, including peas, were analyzed for reduced ascorbic acid and dehydroascorbic acid by Noble and Hanig (1948). When peas were cooked in a tightly covered pan they contained 16.3 to 18.7 mg reduced ascorbic acid and 0.9 to 2.7 mg of dehydroascorbic acid per 100 g. Wilson, et al. (1959) suggested there is a need for revision of food tables to include both reduced and dehydroascorbic acid.

SUMMARY

The purpose of this investigation was: (1) to compare values for reduced ascorbic acid in individually frozen peas as determined by the sodium 2,6-dichlorobenzenoneindophenol and the diazotized 4-methoxy-2-nitroaniline methods and (2) to determine any variations that existed in the reduced ascorbic acid content within one grade of frozen peas from different packages, analyzed either in the morning or afternoon.

The study was set up using a randomized block design with 12 replications for each of two methods of ascorbic acid analysis. Two-pound packages from one lot of U. S. Grade A Fancy, individually frozen peas were purchased and divided into 100-g samples. Four of the 100-g samples from each package were cooked and analyzed for reduced ascorbic acid content by each method. Data were analyzed by analysis of variance, Fisher's test for least significant difference, the t-test, and the Bartlett test.

Significantly higher ascorbic acid values always were obtained with Method a (the sodium 2,6-dichlorobenzenoneindophenol method) than with Method b (the diazotized 4-methoxy-2-nitroaniline method). Significant differences attributable to the interaction of packages x methods, with particular reference to package V analyzed by Method b, could not be explained completely. According to the Bartlett test, the sampling variances within the same treatment and package were homogeneous. T-tests were made to determine if the time of analysis caused variations in ascorbic acid values. When each method was considered separately, there was no difference that could be attributed to the time of analysis. When Method a was compared with Method b and when the analyses done in the morning and the afternoon were considered separately, decidedly higher numerical results were obtained with Method a than with Method b at each time of day.

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APPENDIX

SOLUTIONS

All solutions containing water were prepared using water which had been distilled and further purified in a demineralizer (Crystalab Deeminizer, Model C1-5, Crystal Research Laboratories, Inc., Hartford, Conn.). Solutions were mixed thoroughly by inverting the flask 10 times when made up to volume at 25°C.

Solutions for Method a (the Sodium 2,6 Dichlorobenzenoneindophenol Method)

Metaphosphoric Acid. A stock solution of 10 percent metaphosphoric acid was prepared by dissolving 200 g of metaphosphoric acid in distilled water in a two liter volumetric flask. This solution was made up to volume, mixed, and refrigerated when not in use. A one percent solution was prepared daily by adding 100 ml of the 10 percent metaphosphoric acid to 900 ml of water in a 1000 ml stoppered graduated cylinder and mixing.

Sodium 2,6-Dichlorobenzenoneindophenol Dye. The dye solution was prepared by weighing 40 mg of dye on a Mettler Gramatic balance and dissolving the dye in hot water as it was filtered into a two liter volumetric flask. The solution was cooled, made up to volume, and standardized. It was refrigerated when not in use and a reading of dye plus acid (blank reading) was taken daily to determine if any changes had occurred in the dye. When changes occurred the dye was restandardized.

Ascorbic Acid. Accuracy is essential in preparing this solution used for standardizing the dye. The dye is sensitive

to slight variation in ascorbic acid concentration and the readings obtained from dye standardization are used in calculating the ascorbic acid values of the unknowns.

The solution was prepared by weighing exactly 25 mg of pure ascorbic acid crystals (General Biochemicals, Inc.) on a Mettler Gram-atic balance. Immediately after weighing the crystals were brushed through a dry funnel into a 250 ml volumetric flask containing a small amount of freshly prepared one percent metaphosphoric acid. The funnel was rinsed with more acid and the solution made up to volume.

Solutions for Method b (the Diazotized 4-Methoxy-2-Nitroaniline Method)

Amino Reagent. Five hundred mg of pure 4-methoxy-2-nitroaniline were weighed on a Mettler Gram-atic balance. Using a dry funnel this reagent was put into a 250 ml volumetric flask and dissolved in 125 ml of glacial acetic acid. The mixture was diluted to volume with 10 percent sulfuric acid and mixed thoroughtly. Sulfuric acid (10 percent wt/vol) was prepared by cautiously adding 15 ml concentrated sulfuric acid to 225 ml water, and mixing. The amino reagent, stable at room temperature for at least two months (Schmall, et al., 1953) was dated immediately.

Nitrite Reagent. The sodium nitrite was dried in a muffle furnace at 80°C for one hour, cooled and stored in a desiccator, and a 0.2 percent solution prepared daily. Two hundred mg of sodium nitrite were weighed on a Mettler Gram-atic balance and

poured through a dry funnel into a 100 ml volumetric flask. The watch glass was rinsed and the sodium nitrite dissolved in water and mixed. Scudi and Ratish (1938) questioned the stability of a nitrite solution.

Alcohol. The alcohol used in this experiment was 95 percent ethyl.

Sodium Hydroxide. A solution of sodium hydroxide was made up daily because silica from the flask dissolves and contaminates the solution if allowed to stand (McDowell, 1960). Twenty-five mg of sodium hydroxide were weighed on an Ohaus balance and dissolved in water in a 250 ml volumetric flask. After cooling to 25°C it was made to volume and mixed.

Oxalic Acid Reagent. A 0.5 percent oxalic acid solution was prepared daily by adding 100 ml of a five percent stock solution of oxalic acid to 900 ml of water in a 1000 ml graduated cylinder and mixing. The five percent stock solution of oxalic acid was prepared by weighing on a Mettler Gram-atic balance 50 g of anhydrous oxalic acid, which had been dried in a muffle furnace at 80°C for one hour, cooled, and stored in a disiccator. Stoppered glass weighing bottles were used for weighing. Using a dry funnel this reagent was put into a one liter volumetric flask, dissolved in water, made to volume, and mixed.

Ascorbic Acid Standard. (0.5 mg per ml of 0.5 percent aqueous oxalic acid) This solution was prepared by weighing exactly 25 mg of ascorbic acid on a Mettler Gram-atic balance. The ascorbic acid crystals were weighed and brushed through a

dry funnel into a 50 ml volumetric flask, containing a small amount of 0.5 percent aqueous oxalic acid within a five minute period to avoid oxidation. The solution was made up to volume and thoroughly mixed.

STANDARDIZATION OF DYE FOR METHOD "a"

The dye was standardized by using three solutions of different known concentrations of pure ascorbic acid in one percent metaphosphoric acid. Solutions containing three, flour, and five μg per ml were prepared by using a five ml graduated pipette and pipetting three, four, and five ml of the ascorbic acid solution described above into three 100 ml volumetric flasks and making these up to volume with one percent metaphosphoric acid. A Klett-Summerson photoelectric colorimeter containing a light filter with a spectral range of approximately 500 to 570 mμ was set at zero absorbancy with distilled water. Five ml of dye were pipetted into matching test tubes with an automatic pipette. To obtain the reading of the dye plus acid, five ml of one percent metaphosphoric acid were added to a test tube containing dye using a volumetric pipette. The tube was inverted quickly three times and placed in the colorimeter and the reading taken within 15 seconds after the beginning of the addition of the acid. This was repeated until two readings were in agreement.

Five ml aliquots of each concentration of ascorbic acid were pipetted into the dye and read in the same manner as the acid.

The following formula was used for calculating the ascorbic

acid factor. This factor was used later in calculating the ascorbic acid concentration of the unknown solution.

$$\text{Ascorbic acid factor} = \frac{\text{concentration of ascorbic acid in dilution}}{\text{blank reading - ascorbic acid reading}}$$

Table 5. Ascorbic acid values for each sample and package. Method a.

Package	Day	AM Sample number	AM Ascorbic acid value (mg/100 g)	PM Sample number	PM Ascorbic acid value (mg/100 g)	Mean ascorbic acid value (mg/100 g)
I	1	--	--	3	16.56	
		--	--	7	16.03	
	2	--	--	5	17.35	
		--	--	1	16.03	
						16.49
II	3	--	--	4	17.35	
		--	--	6	17.09	
	4	2	16.30	--	--	
		8	16.30	--	--	
						16.76
III	5	--	--	8	16.56	
		--	--	2	17.09	
	6	6	17.35	--	--	
		4	16.82	--	--	
						16.96
IV	7	--	--	2	16.56	
		--	--	4	17.09	
	8	--	--	8	17.35	
		--	--	6	17.35	
						17.09
V	9	4	16.30	--	--	
		2	15.77	--	--	
	10	8	16.30	--	--	
		6	16.30	--	--	
						16.17
VI	11	--	--	4	16.30	
		--	--	8	16.56	
	12	--	--	6	16.03	
		--	--	2	16.56	
						16.36

Table 6. Ascorbic acid values for each sample and package. Method b.

Package	Day	AM Sample number	AM Ascorbic acid value (mg/100 g)	PM Sample number	PM Ascorbic acid value (mg/100 g)	Mean ascorbic acid value (mg/100 g)
I	1	6	11.25	--	--	
		8	11.78	--	--	
	2	2	12.40	--	--	
		4	12.29	--	--	
						11.93
II	3	7	12.29	--	--	
		3	12.09	--	--	
	4	--	--	1	12.50	
		--	--	5	12.50	
						12.34
III	5	7	12.29	--	--	
		1	12.60	--	--	
	6	--	--	3	11.66	
		--	--	5	11.88	
						12.11
IV	7	5	11.98	--	--	
		1	11.56	--	--	
	8	7	12.50	--	--	
		3	12.81	--	--	
						12.21
V	9	--	--	5	13.34	
		--	--	3	13.75	
	10	--	--	7	12.81	
		--	--	1	12.81	
						13.18
VI	11	7	12.60	--	--	
		1	12.50	--	--	
	12	5	12.40	--	--	
		3	11.66	--	--	
						12.29

A COMPARISON OF THE SODIUM 2,6-DICHLOROBENZENONEINDOPHENOL AND THE DIAZOTIZED 4-METHOXY-2-NITROANILINE COLORIMETRIC METHODS FOR DETERMINING REDUCED ASCORBIC ACID CONTENT OF FROZEN PEAS

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The amount of reduced ascorbic acid in fruits and vegetables has been used as one method of indicating quality. A number of methods have been used for determining reduced ascorbic acid. However, since there appeared to be no one entirely satisfactory method for measuring ascorbic acid content, it seemed desirable to compare two methods considered applicable to a large number of routine laboratory analyses for reduced ascorbic acid.

The purpose of the investigation was: (1) to compare values for reduced ascorbic acid in individually frozen peas as determined by the sodium 2,6-dichlorobenzenoneindophenol and the diazotized 4-methoxy-2-nitroaniline methods and (2) to determine any variations that existed in the reduced ascorbic acid content within one grade of frozen peas from different packages, analyzed either in the morning or afternoon.

The study was set up using a randomized block design with 12 replications for each of the two methods of reduced ascorbic acid analysis. Two-pound packages from one lot of U. S. Grade A Fancy, individually frozen peas were purchased and divided into 100-g samples. Four of the 100-g samples from each package were cooked and analyzed for reduced ascorbic acid content by each method. Data were analyzed by analysis of variance, Fisher's test for least significant difference, the t-test, and the Bartlett test.

Significantly higher ascorbic acid values always were obtained with Method a (the sodium 2,6-dichlorobenzenoneindophenol method) than with Method b (the diazotized 4-methoxy-2-nitroaniline method). Significant differences attributable to the interaction

of packages x methods, with particular reference to package V analyzed by Method b could not be explained completely. According to the Bartlett test, the sampling variances within the same treatment and package were homogeneous. T-tests were made to determine if the time of analysis caused variations in ascorbic acid values. When each method was considered separately, there was no difference that could be attributed to the time of analysis. When Method a was compared with Method b, and when the analyses done in the morning and the afternoon were considered separately, Method a gave decidedly higher numerical results than Method b at each time of day.

Since higher values for reduced ascorbic acid were obtained with Method a (the sodium 2,6-dichlorobenzenoneindophenol method) than with Method b (the diazotized 4-methoxy-2-nitroaniline method), a modification of Method a using formaldehyde was suggested. Such a modification might determine if there are substances other than ascorbic acid which interfere and cause higher values to be obtained with Method a than with Method b.

Recent research has shown ascorbic acid and dehydroascorbic acid are utilized equally well by humans. Hence, it would be desirable to determine both forms of ascorbic acid.