# THE OSAZONE METHOD FOR DETERMINING ASCORBIC ACID IN FRUITS AND VEGETABLES

Thesis for the Degree of M. S.
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This is to certify that the

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F. W. Fabian

Major professor

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# THE OSAZONE METHOD FOR DETERMINING ASCORBIC ACID IN FRUITS AND VEGETABLES

By

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A THESIS

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## Introduction

For three hundred years medical science has been aware of a substance in foodstuffs which protects against scurvy. It was in 1747 that Lind found that the feeding of oranges and lemons to British sailors would prevent or cure the disease. Progress in the study of this vitamin was slow until its isolation from lemon juice by King in 1932.

Chemically, ascorbic acid is a six-carbon compound closely related in structure to the simple sugars. Since it is a potent reducing substance, early chemical methods of measuring it were based on this property, such as the one by Tillmans in 1927 using dichloro-phenol-indophenol dye. At the present time there are a great many methods and modifications for determining ascorbic acid using various dyes and newer equipment such as photelometers based on the reducing ability of ascorbic acid.

Further study of this vitamin, however, revealed two forms besides the active one, both with antiscorbutic properties (9) yet no reducing ability. One was called dehydro ascorbic acid, an oxixided form,; the other ascorbigen, a combined form of ascorbic acid with a protein. Only in recent years have chemical methods of measurement been made available for these two forms. Roe and his collaborators (29,30) have now developed a highly specific chemical method for determining the active and oxidized forms of vitamin C by means of a osazone reaction. This method was originally worked out for body fluids but was recently modified (31) so as to be applicable to plant tissues.

The present work, as originally planned, was to be a study of the applicability of the first Roe method for determining the active and oxidized forms of vitamin C in fruits and vegetables. For comparative

purposes the ascorbic acid content of the same substances was determined by dye titration and by the photelometer methods. Upon publication of the modified method by Roe et al for plant tissues, it was also included.

## LITERATURE REVIEW

The occurrence of fully developed cases of scurvy is, fortunately, relatively rare in civilized countries at the present time. Common even in the days of the Greeks, the plague took a heavy toll of human lives for hundreds of years. Even Vasco de Gama lost a hundred men from his original crew of a hundred and sixty from the disease. No tonic or drug seemed to prevent the palpitations, the swelling and tenderness of the legs, the bleeding gums, the vomiting, diarrhea, - all the common symptoms of frank scurvy. Finally in 1720 Kramer (11) found that citrus fruits both prevented and cured the disease and thirty years later Lind, a British naval surgeon, recommended that all British sailors be required by law to have fresh citrus fruits. Progress was slow, however, because of the lack of quantitative methods of determining the antiscorbutic properties in various food. Impetus to the systematic study of the occurrence of the vitamin in food material and its stability was given by the tremendous problem of civilian and army rationing during the first World War. Thus the beginning of our modern knowledge of the quantitative determinations are found in the work of Holst and Frolich, (14) in 1907. Using the guinea pig method they made comparisons of the antiscorbutic value of foods and gave specific directions as to the preparation. Check and Hume (5), two Englishmen, continued the studies of Holst and Frolich.

The real progress in the systematic study of vitamin C began with its isolation from lemon juice by King (18) in 1932. Then its structure as a six carbon compound related to the simple sugars and having a empirical formula of $C_6H_8O_6$ was established. From the knowledge of the chemical properties of ascorbic acid, chemical methods of determination were devised

which today have almost entirely replaced the biological guinea pig method of assay. Tillmans (37) relying on the reducing ability of vitamin C developed the first chemical method and the one still most widely used today. Using a dye solution of 2.6 dichlorophenol indophenol, only the reduced form of ascorbic acid was measured. Later workers introduced modifications when sulfhydryl compounds (6), ferrous and ferric compounds (1) reductones (25) were found to interfere. Other dye solutions were used also such as Tauber's method (36) with Prussian blue and Lund's (23) method using methylene blue. When photelometers were introduced, other methods of determinations were developed making use of them such as the methods of Mindlin and Butler (24), Bessey (2), Evelyn (8), and Woessner (38).

All the dye titration methods, however, measured just the reduced form of vitamin C. Dehydro ascorbic acid could be determined by reduction with hydrogen sulfide to the active form and then titrated with the dye solution. The procedures (2,7) which have been published are contradictory and not adapted to routine procedure. Therefore, the dehydro ascorbic acid content materials has been fairly consistently disregarded as too unstable to be measured (4). In 1944 a new chemical method was published by Roe and Oesterling (31) which was specific for dehydro and which opened this field for investigation.

Not satisfied with the dye methods of measurement as being specific enough, Roe, a biochemist at George Washington University, became interested in developing a method based on a principle other than oxidation-reduction. He found that by using 2, 4 dinitrophenyl hydrazine, an osazone derivative of dehydroascorbic acid would be formed and accordingly published his first method in 1939 (29). Later modifications of the original method were developed for blood and urine in 1943 (30) and for plant tissue in 1944 (31).

The osazone method is suitable for routine work and is widely used in laboratories at the present time.

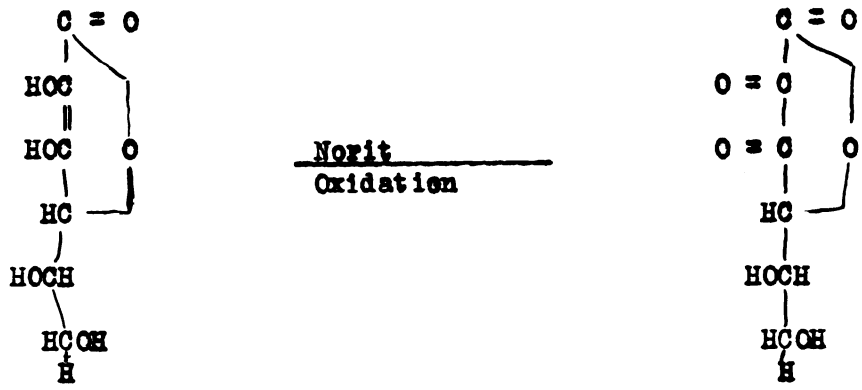
With the advance in methods, the comparative value of foods has become common knowledge. Mustard greens, peppers, cauliflower, cabbage, parsley, and strawberries were found to have high ascorbic values and nutritional requirements for humans were also determined. The unfavorable effect of heavy metals especially copper (12), of high temperature (19), of exposure to air (16,17,32), of dissolved oxygen (19) of enzymes (37) and many other conditions has been determined. Favorable methods (16,14,4) of retaining the vitamin have also been worked out. Previous work would fill several volumes and even though frank scurvy itself has almost disappeared, surveys have shown that subclinical vitamin C is still widely prevalent. The work of the past must be continued in the future until proper nutrition has eradicated vitamin C deficiency entirely.

## PRELIMINARY EXPERIMENTAL WORK

### The Osazone Method

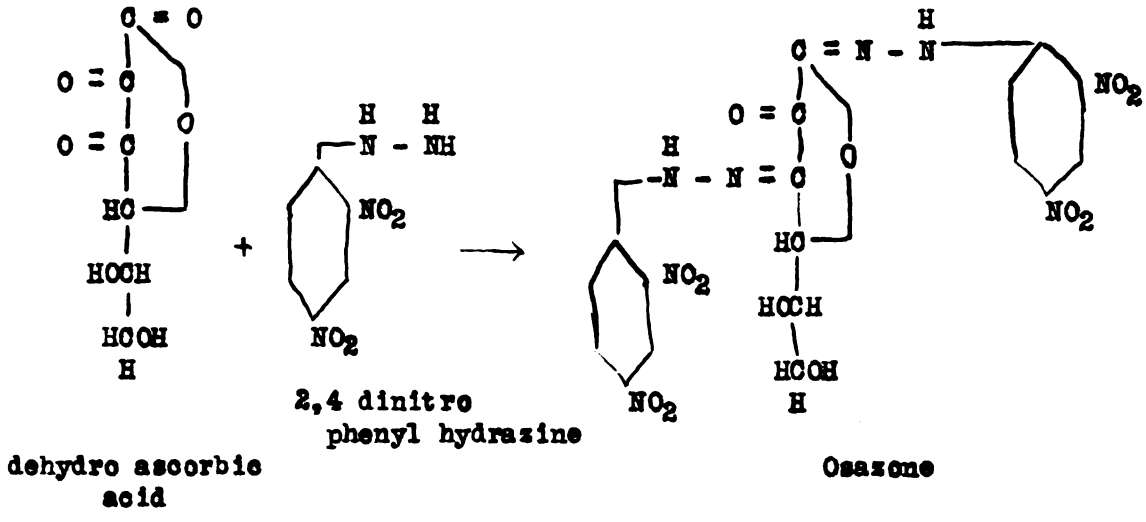
The color reaction as published by Roe and Kuether (30) determines active and dehydro ascorbic acid in 4 percent trichloroacetic by oxidation with norit. The activated charcoal, norit must be freed from traces of iron and other interfering metals by repeated washings with 10 percent hydrochloric acid and distilled water. An aliquot of the norit treated filtrate was stablized from further oxidation by the addition of one drop of a 10 percent alcoholic solution of thiourea. 2, 4 dinitrophenyl hydrazine reagent then formed the red osazone crystals when held at 37° C. for three hours. At the end of this incubation period, the solutions are iced and 85 percent sulfuric acid is slowly added to dissolve the osazones. The intensity of the color produced is the measure of the ascorbic acid content as read in a photelometer with a suitable filter. All photelometer work was done on the Cenco, Sanford, and Sheard type using a green filter of 525 wave length.

The chemistry of the method is as follows:




1 - ascorbic acid

dehydro ascorbic acid




The following modifications of the original procedure were investigated:

1. Effect of temperature

Because of the desirability of decreasing the time of the determination, temperature variations were made. Pure ascorbic acid solutions in four percent trichloroacetic prepared at the same time were placed at 37° C. for three hours and 55° C. for various time periods. Repeated experiments with standard solutions incubated in a water bath at 55° C. for one hour show the same results as 37° C. for three hours as seen in table 1.

Since the results appear to be consistant, a one-hour incubation period in a 55° C. water bath was adopted rather than the original three hour incubation period at 37° C.

2. Effect of oxidizing agents

Since norit is of uncertain origin and composition, the extent of its oxidation would be difficult to prove. Aerosol, iodine, copper chloride, and potassium permanganate decolorized with hydrogen peroxide were tried in an attempt to oxidize the active ascorbic acid present further than the

dehydro form. Paget and Berger (26) found that permanganate oxidized ascorbic acid to oxalic acid. If any oxidation products beyond dehydro-ascorbic acid such as diketogulonic acid, l-threonic acid, or oxalic acid formed osazones, the method would not measure true physiological activity. The results obtained are shown in table 2.

It is evident then that other oxidizing agents allow osazone formation to the same extent or more than norit itself. Since ascorbic acid has been found to be easily oxidized and thereby destroyed (12), the osazone formation is difficult to explain when copper chloride or permanganate are used. However, the only breakdown product available in pure form is oxalic acid, solutions of which gave no osazones when tested.

### 3. Effect of various amounts of norit

When standard runs were made weighing the norit analytically to see if any appreciable amount of ascorbic acid was absorbed, tests show that absorption did not occur unless the quantity was increased to three times the amount called for.

### 4. Effect of acidity

Norit oxidizes only in acid solutions having a pH of two or less. A reagent such as acetic or trichloroacetic is necessary, metaphosphoric acid standard solutions forming no osazones. Acetic acid is absorbed by the norit to give active oxygen needed for rapid oxidation of ascorbic acid.

### 5. Effect of sugar

Since sugars form osazones, dextrose was added to standard ascorbic acid solutions and the osazone procedure followed through to determine if increased photelometer readings would be obtained. Pure dextrose and cane

sugar solutions of two and ten percent do form osazones, which can be read as ascorbic acid on the photelometer. However, when a five percent sugar solution was combined with ascorbic acid in trichloroacetic, no increase was obtained until the standard reached 24 gamma per milliliter. After this level was reached, the increase was unmistakebably large showing that sugar in high concentrations would interfere.

## Pure ascorbic acid solutions

Previous to any sample determinations it was necessary to know how long the ascorbic acid content would be measureable by the osazone method. To obtain this information the following effects were studied.

### 1. Effect of temperature

#### 1. 25° C.

The results are shown in table three.

While the dye titration showed complete disappearence of vitamin C in distilled water in two hours, the osazone method showed no change and even increased on standing. Evidently the ascorbic acid forms a stable compound as measured by the osazone method.

#### 2. 55° C.

Since ascorbic acid is readily destroyed by heat (16,21,22), standard solutions in water were incubated at 55° C. to see if the osazone method would bring this out. Results of unusual stability are shown in table 4.

There is then considerable destruction of pure ascorbic acid in water solution when held at 55° C. for 24 hours especially in the higher concentrations. Little or no further decrease is found when the same samples remain at room temperature for six days. If ascorbic acid itself in water solutions is stable for this length of time, samples would be suitable for testing if

held in acid solutions at five° C. overnight.

3. Effect of aeration on standard ascorbic acid solutions.

In 1941, Strohecker and others (34) found that the stability of ascorbic acid was more affected by the oxygen of the air than by temperature. A series of aeration experiments using standard ascorbic acid solutions was undertaken using the osazone method of measurement. The results appear in table five.

Since the stability was greater than would be expected, aeration of pure ascorbic acid in water was repeated many times using different samples of vitamin C, different distilled water, and even vacuum to suck in room air. Results revealing unbelievable stability by the osazone method were also verified by the dye titration method.

In contradiction to Keys work also (17), the reason for pure ascorbic acid in water to disappear by dye titration measurement in two hours but remain 70 percent after two days aeration is unknown.

4. Effect of hydrogen sulfide and nitrogen treatment on ascorbic acid oxidized by norit

Only reversibly oxidized solutions of ascorbic acid are anticorbutic acid and therefore physiologically valuable. Further oxidation products beyond dehydroascorbic acid are neither reversible by reduction nor active in disease prevention. In using the osazone method it was extremely important to know if the resulting norit treated solutions were reversibly oxidized. Positive results given in table seven are one of the strongest facts in favor of the specificity of the osazone method as a true measure of active and dehydro ascorbic acid.

The procedore for reducing dehydro ascorbic acid to active ascorbic

acid by Bessey (2) was used. Pure ascorbic acid solutions were oxidized to the dehydro form and buffered to a pH of 3.5 with a citrate buffer. Hydrogen sulfide was bubbled through the buffered solution for 30 minutes and after 24 hours nitrogen was bubbled through the same solutions for two hours. The hydrogen sulfide reduces the dehydro ascorbic acid and the nitrogen drives off the hydrogen sulfide. All the ascorbic acid is then present in the reduced form and is measured by dye titration. Iodine is the oxidizing agent in common use for converting ascorbic acid; hence it served as a comparison to norit.

**Table 1.** Photelometer readings of standard solutions of ascorbic acid by the original osazone method.

| Gamma per ml. ascorbic acid | 37° C. | | 55° C. | |
|---|---|---|---|---|
| | 3 hr. | 1 hr. | 2 hr. | 3 hr. |
| 0.0 | 99 | 99 | 99 | 99 |
| 4.8 | 93 | 91 | 92 | 92 |
| 8.0 | 90 | 87.5 | 87.5 | 86.5 |
| 11.2 | 85 | 83 | 82.5 | 82 |
| 16.0 | 79.5 | 78 | 75 | 75 |
| 24.0 | 72 | 70 | 67 | 66 |
| 32.0 | 64 | 64 | 59 | 58 |
| 40.0 | 58 | 58.5 | 55 | 53 |

**Table 2.** The effect of oxidizing agents on standard solutions of ascorbic acid as shown by photelometer readings with the osazone method.

| Gamma per ml. ascorbic acid | Oxidizing agents | | | | | |
|---|---|---|---|---|---|---|
| | Norit | Nothing | $CuCl_2$ | Aerosol | $KMnO_4$ | $H_2O_2$ |
| 0.0 | 99 | 98 | 99 | 99 | 99 | |
| 8.0 | 88 | 89 | 88 | 88 | 88 | |
| 16.0 | 77.5 | 80.0 | 66.5 | 78.5 | 80 | |
| 24.0 | 69.5 | 74.5 | 58.5 | 71 | 71 | |

**Table 3.** Comparison of standard ascorbic acid solutions of five milligrams per 100 milliliters by dye titration and osazone method.

| Time in hours of standing at 25° C. | Osazone method ascorbic acid in distilled water | Dye titration in milliliters | |
|---|---|---|---|
| | | Ascorbic acid in water | Ascorbic acid in 4% trichloroacetic |
| 0 | 88 | 16.9 | 17.2 |
| 0.25 | | 12.9 | |
| 0.50 | | 9.0 | |
| 0.75 | | 5.8 | |
| 1.0 | 88 | 3.6 | 15.1 |
| 2.0 | 88 | 0.7 | |
| 3.0 | 88 | | 9.2 |
| 4.0 | 89 | | 6.9 |
| 5.0 | | | 4.1 |
| 24 | 85 | | |
| 48 | 81 | | |
| 72 | 81 | | |

**Table 4.** The stability of ascorbic acid in water by the osazone method.

| Milligrams per 100 ml. assayed at the beginning | pH of beginning solutions of water | Percentage loss | |
|---|---|---|---|
| | | 55° C. for 24 hours | 55° C. for 24 hours and 6 days standing at 25° C. |
| 0.55 | 5.08 | 92 | 92 |
| 2.2 | 4.31 | 60 | 59 |
| 3.2 | 4.01 | 66 | 66 |
| 6.4 | 3.60 | 67 | 58 |
| 10.0 | 3.40 | 53 | 47 |

Table 5. The stability of ascorbic acid in water after aeration as measured by the osazone method.

| Milligrams per 100 ml. of ascorbic acid originally | pH | Percentage loss | |
|---|---|---|---|
| | | 24 hours aeration | 24 hours aeration plus six days at 25° C. |
| 0.55 | 5.39 | 25 | 38 |
| 2.13 | 4.29 | 5 | 17 |
| 3.22 | 3.92 | 7 | 20 |
| 6.40 | 3.62 | 20 | 30 |
| 10.00 | 3.38 | 10 | 12.5 |

Table 6. Aeration results of pure ascorbic acid in water using the dye titration method of measurement.

| Milligrams per 100 ml. in standard. | Beginning dye titration in ml. | Aeration | | | |
|---|---|---|---|---|---|
| | | One day | | Two days | |
| | | Dye titration in ml. | % loss from original | Dye titration in ml. | % loss |
| 2.0 | 13.3 | 11.7 | 12 | 9.1 | 30 |
| 4.0 | 31.5 | 28.4 | 10 | 23.2 | 28 |
| 6.0 | 48.5 | 36.7 | 25 | 32.7 | 30 |

Table 7. The reversibility of oxidized solutions of ascorbic acid when treated with hydrogen sulfide and nitrogen.

| Oxidizing Agents | | Milligrams per 100 ml. ascorbic acid in 4% trichloracetic | | |
|---|---|---|---|---|
| | | 0.0 | 2.0 | 4.0 |
| Control | Dye titration | 1.0 | 19.1 | 36.3 |
| | | 0.0 | 18.1 | 35.3 |
| | Percent recovery | | 100 | 100 |
| Iodine | Dye titration | 2.2 | 16.6 | 30.7 |
| | | 0.0 | 14.4 | 28.4 |
| | Percent recovery | | 81.0 | 80 |
| Norit | Dye titration | 0.9 | 17.0 | 29.7 |
| | | 0.0 | 16.1 | 28.8 |
| | Percent recovery | | 96 | 81 |

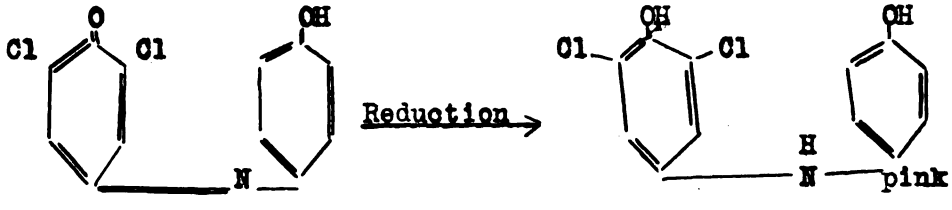
## EXPERIMENTAL

### Preparation of sample

Ten grams of vegetable solids were extracted with a strongly acidic solution by grinding in a mortar and pestle with sand. The mixture was then centrifuged and the extraction repeated. After three extractions the volume of the extracted material was made up to 100 ml. and assayed. Fruit and vegetable juices were assayed as such by simply preparing a one to ten dilution. Samples were made in duplicates and assayed by each method. Two acid extracting solutions were necessary, eight percent trichloroacetic for the beginning work and five percent metaphosphoric acid for the later work. Extraction of vitamin C was found to be equally effecient with either solution.

### The dye titration method

The determination of vitamin C by titration with 2, 6 dichlorophenol indophenol originally suggested by Tillmans (37) was used throughout these experiments. Utilizing the reducing ability of ascorbic acid, the dye is changed from a blue to a pink color.




2, 6 dichloro indophenol

Dye solutions were made by dissolving specially prepared tablets in distilled water at 80° C. Because of the instability of the dye solution, standardization with pure ascorbic acid previous to sample determinations was necessary. The faintest pink color lasting for 15 seconds was taken

as the endpoint in all titrations. An example of dye standardization and the calculation of ascorbic acid content is a sample is given in table 8.

Although the dye titration method is a rapid means of measuring the ascorbic acid content, it determines only the active or reduced form. In addition the method lacks specificity since any reducing compound such as glutathione, cysteine, and phenolic compounds present may produce the same effect as ascorbic acid on the dye. Highly colored solutions obscure the endpoint and cause erroneous results. Photelometers have therefore been adopted to make the dye determinations more accurate.

## The dye photelometer method

Bessey's modification (2) of the method of Mindlin and Butler (24) followed in this assay work has many decided advantages. Eliminating the personal factor of endpoint titrations, the assay of highly colored, turbid solutions becomes possible. Differing slightly chemically from the dye titration, ascorbic acid changes the dichloro indophenol to the colorless leucobase.

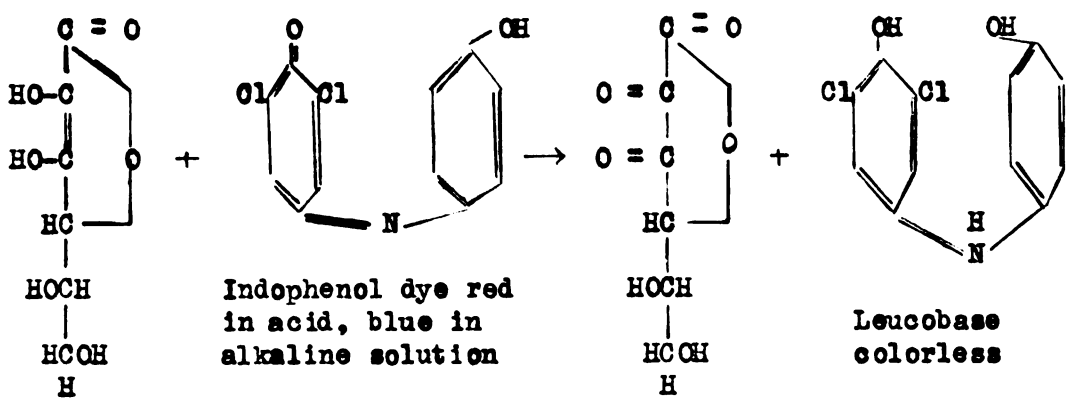


**Table 8.** The standardization and calculations of ascorbic acid content by the visual dye titration method.

Dye solution - ten tablets
Standard ascorbic acid - 10 gamma/ milliliter

| ML. standard used | Final volume | Dye titration in mls. | | Average dye titration | Titration minus blank | Ml. dye / ml. standard |
|---|---|---|---|---|---|---|
| 0 | 25 | 1.3 | 1.2 | 1.2 | 0.0 | |
| 5 | 25 | 4.7 | 5.3 | 5.0 | 3.8 | .76 |
| 10 | 25 | 9.5 | 8.9 | 9.2 | 8.0 | .80 |
| 15 | 25 | 13.8 | 13.3 | 13.5 | 12.3 | .82 |
| 20 | 25 | 18.8 | 17.5 | 17.5 | 16.3 | .815 |

Milligrams/100 ml. in standard = milligrams/ml dye • 100

For example: $\frac{1.0}{.81 \cdot 100} = .0123$

On milliliter dye solution equals .0123 milligrams per 100 vitamin C.

Sample calculations

| Juice tested | Original dilution | ml. titrated | dye titration | dye titration minus blank | Calculation | Results in ml. |
|---|---|---|---|---|---|---|
| Orange | 1 - 10 | 5 | 24.5 | 23.3 | 4.66 • .0123 • 10 • 100 = | 58.5 |
| Tomato | 1 - 10 | 10 | 20.8 | 20.8 | 1.96 • .0123 • 10 • 100 = | 24.1 |

When a solution of five percent meta phosphoric acid buffered to a pH of 3.5 is added to the dye, an intense pink color is formed. The presence of ascorbic acid decreases the intensity of the pink color as measured by the photelometer. Pure ascorbic acid in five percent meta phosphoric acid served to standardize the dye and unknown samples are assayed for vitamin C content by referring to the standard curves. Turbidity and interfering colors are eliminated by taking an initial photelometer reading and a final photelometer reading in which all the dye has been completely reduced by a crystal of ascorbic acid, only residual color remaining. The method of deriving a standard curve is given in table nine.

The logarithm of the final reading minus the initial reading is used to plot the standard curve. Typical curves are seen in the following chart. (Figure 1)

To calculate the ascorbic acid content of any sample, the logarithm $\frac{\text{average standard reading}}{\text{initial sample reading}}$ - logarithm $\frac{\text{average standing reading}}{\text{final sample reading}}$ was obtained. This number corresponds to the milligrams per 100 milliliters ascorbic acid as read from the standard curve. By correcting for the dilution factor the final result was obtained. To follow through the calculations, a sample of tomato juice was diluted one to ten with five percent metaphosphoric acid. Five milliliters of this dilution were taken for assay, diluted with metaphosphoric to 25 milliliters and buffered to a pH of 3.5. The buffered sample was made to 100 milliliters. Using four milliliters of dye and four milliliters of buffered sample, the initial photelometer reading was taken. A small amount of ascorbic acid crystals was added and a final photelometer reading taken of the residual color. To calculate the amount of ascorbic acid in the original material, the formula given above was

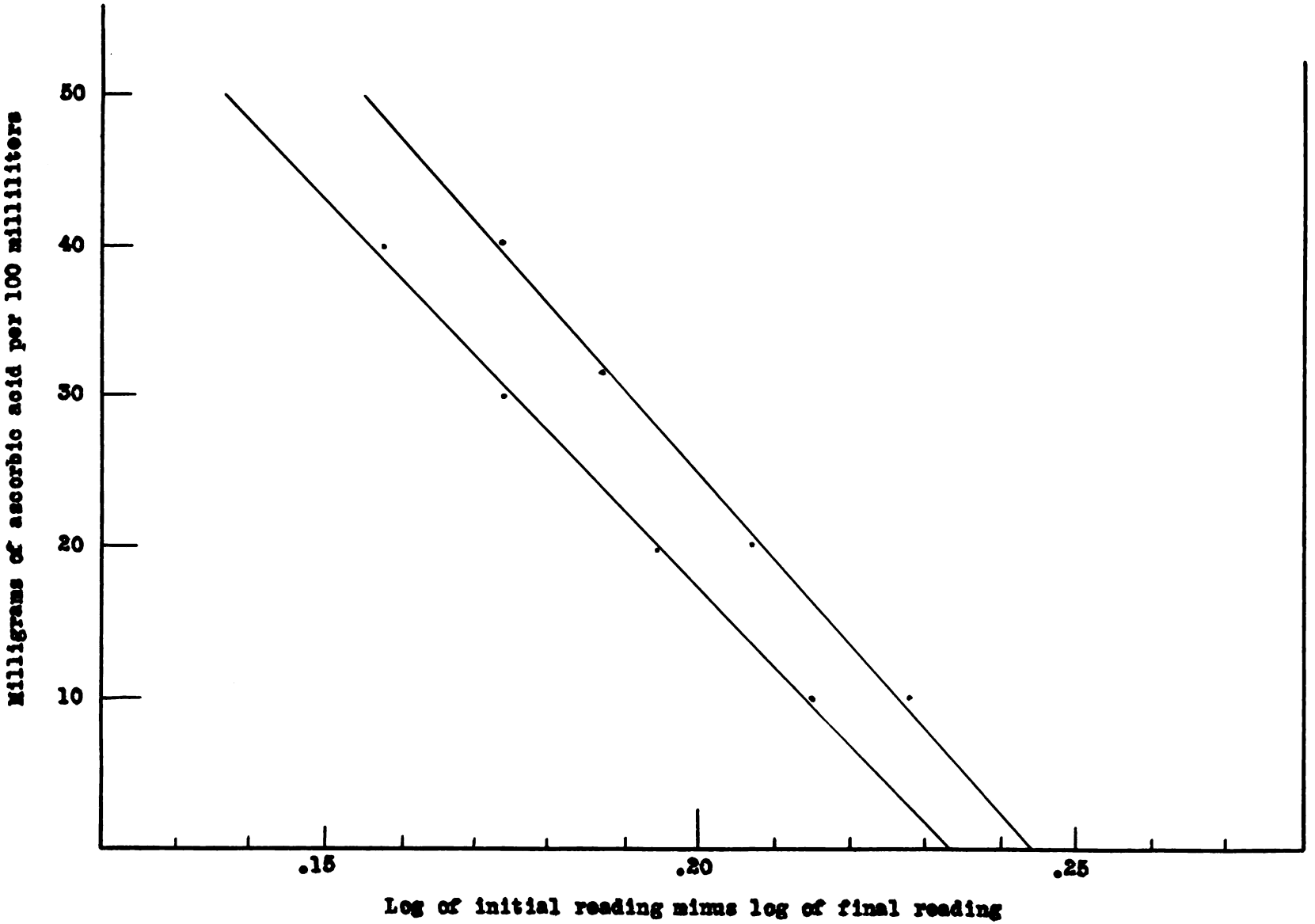



Fig. (1) Typical standard curves for the determination of ascorbic acid by the dye photelometer method

**Table 9.** Standarization of dye and calculation of samples in the photelometer method.

Number of dye tablets in aqueous solution = 10
Number of milligrams per 100 ml. ascorbic acid in five percent metaphosphoric acid = 8

| ml. standard solution | Total acid vol. in ml. | Final buffered volume. | pH | milligrams per 100 ml. ascorbic | Initial reading | Final reading | Log. of final reading minus original reading |
|---|---|---|---|---|---|---|---|
| 0 | 25 | 100 | 3.5 | 0.0 | 58 | 98 | .2337 |
| 5 | 25 | 100 | 3.5 | 10 | 60 | 99 | .2147 |
| 10 | 25 | 100 | 3.5 | 20 | 63.5 | 98 | .1941 |
| 15 | 25 | 100 | 3.5 | 30 | 65.5 | 98.5 | .1739 |
| 20 | 25 | 100 | 3.5 | 40 | 68.5 | 98 | .1577 |
| 25 | 25 | 100 | 3.5 | 50 | 7.2 | 99 | .1361 |

applied. Thus:

$$\log \frac{98.5}{56} - \log \frac{98.5}{99} = .2249$$

.2249 on the standard chart represents five milligrams per hundred milliliters. Multiplying by the dilution factor, the original sample contains $\frac{5 \cdot 100}{10 \cdot 5}$ or 10 milligrams vitamin C per hundred milliliters. In this way, all samples assayed by the dye photelometer method were calculated.

## The osazone method

The procedure followed for the osazone method has already been given. Originally eight percent trichloroacetic acid was used. Standard curves were made with pure ascorbic acid solutions such as the ones shown in Fig. 2. Calculations of samples were made by reading the milligrams per 100 ml. from the chart and multiplying by the dilution factor. An example would be as follows:

Orange juice

| | |
|---|---|
| Original dilution with trichloracetic | = 1 - 10 |
| Mls of original assayed | = 5 |
| Final dilution in mls | = 25 |
| Milliliters of oxidized portion assayed | = 4 |
| Photelometer reading | = 69 |
| Milligrams per hundred from chart | = 2.55 |

$\frac{2.55 \cdot 10 \cdot 25}{5 \cdot 4}$ = 53.1 milligrams per hundred ascorbic acid

The modified osazone method (31) used five percent metaphosphoric acid, one percent thiourea and 10 percent acetic acid as the extracting solution. Standard curves were prepared as previously described and this was used for sample calculations. The chief advantage of the modification was the introduction of a new chemical assay for dehydroascorbic acid alone. Using metaphosphoric acid, an aliquot of four milliliters was taken before norit

oxidation. The same procedure as for total ascorbic acid was then followed, that is, the addition of dinitrophenyl hydrazine, the incubation at 55° C. for one hour, and the photelometer readings. A separate chart was prepared for dehydro ascorbic acid by oxidizing a pure ascorbic acid solution with bromines. The curve is seen in Fig. 2, and the samples were calculated the same way as shown in the previous example.

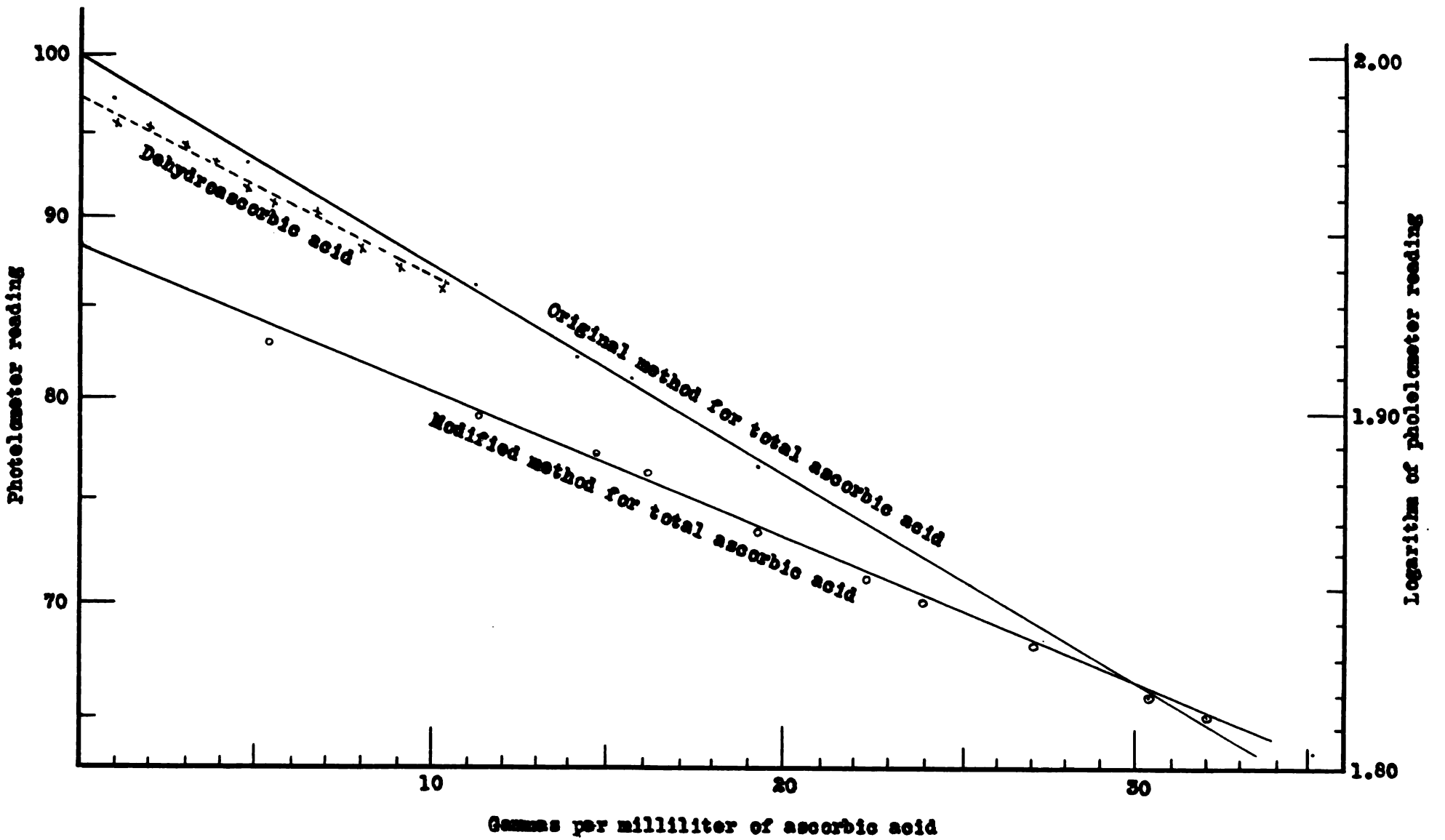



Fig. (2) Standard curves for the determination of ascorbic acid by the osazone method

# RESULTS

## Comparative studies with dye titration and the original osazone method

Both methods of assay were run in duplicates on the same sample extracted on eight percent trichloroacetic. The dye titration was carried out on the first day after extraction and the osazone method on the following day, the extracted sample being held at 5° C.

As shown in table 10 the two methods gave about the same ascorbic acid content in the canned vegetables assayed. Theoretically the dye titration which measures only the reduced form, would give slightly lower values than the osazone method which measures both active and dehydroascorbic acid. This was not found to be true, however, since the osazone values were sometimes higher and sometimes lower. In table 11 showing comparisons with canned tomato juice samples, the osazone results showed some decrease after five days holding.

## Comparative studies with dye titration, dye photelometer, and modified osazone method

Table 12 shows the results obtained by three methods of assay using the same sample extracted in five percent metaphosphoric. The dye determinations were run on only the metaphosphoric solutions, acetic acid and thiourea being added for the osazone determination. The inaccuracy of the dye titration method using a visual endpoint in contrast to the dye photelometer method was not observed. In general, both methods gave comparable results with the same samples. Apparently the solutions were not highly colored enough at the dilution used to interfere with the pink endpoint of the dye titration. The modified osazone method in general gave comparable

results with the dye method, green snap beans being an exception. Even in the modified method, however, the results were not consistantly higher due to the small amount of dehydro ascorbic acid present, nor were the results obtained for total ascorbic acid any noticeable improvement over the original method. When the same samples were held at 5° C. and retested after seven days, the dye titration showed a decrease in all cases while the osazone results were inconsistant. In some samples, a slight decrease was observed while other samples remained the same or showed an apparent increase in vitamin C.

## Dehydroascorbic acid determinations

With the modified osazone method it was possible to determine the amount of dehydroascorbic acid present in the foods tested. Seeking an answer for the unusual stability of the osazone samples, dehydro determinations also were repeated after seven days. Decided and consistant increase in dehydo is shown in table 13. Evidently ascorbic acid in plant tissue is oxidized to dehydro on standing and remains stable in this form longer than would be expected from previous work (4,9,28).

Table 10. Comparative results of dye titration and original osazone method for determining ascorbic acid in canned vegetables - average of two determinations.

| Food Tested | Milligrams per hundred grams | | | |
|---|---|---|---|---|
| | Solids | | Juices | |
| | Dye Titration | Original Osazone | Dye Titration | Original osazone |
| Peas, sweet | 11.2 | 13.5 | 16.8 | 12.2 |
| Peas, sweet | 15.8 | 16.0 | 16.8 | 17.7 |
| Peas, Alaskan | 11.0 | 14.0 | 17.0 | 12.0 |
| Peas, Alaskan | 6.5 | 8.5 | 17.5 | 13.5 |
| Green snap beans | 8.3 | 12.5 | 14.0 | 8.3 |
| Green snap beans | 6.0 | 9.0 | 8.3 | 11.0 |
| Green snap beans | 2.4 | 1.7 | 1.6 | 8.0 |
| Spinach | 10.8 | 15.0 | 15.0 | 3.8 |
| Spinach | 3.6 | 6.0 | 5.8 | 6.0 |
| Corn - whole kernel | 3.3 | 9.0 | 7.7 | 9.1 |
| Corn- whole kernel | 0.0 | 3.8 | 0 | 4.1 |
| Beets | --- | 10.0 | 0.0 | 3.0 |
| Carrots | 6.2 | 4.4 | 8.8 | 7.3 |

**Table 11.** Comparative results of dye titration and original osazone method for determining ascorbic acid in canned tomato juice - average of duplicate determinations.

| Number | Pounds vacuum | Milligrams per hundred milliliters | | |
|---|---|---|---|---|
| | | Dye titration | Original osazone | |
| | | | Fresh sample | Sample in 5 da. at 5°C. |
| 1 | 0 | 6.4 | 5.0 | 4.3 |
| 2 | 8 | 10.3 | 9.0 | 6.8 |
| 3 | 0 | 6.1 | 3.7 | 3.7 |
| 4 | 4 | 9.4 | 8.5 | 6.6 |
| 5 | 3 | 9.1 | 7.5 | 6.9 |
| 6 | 7 | 4.8 | 4.5 | 4.9 |
| 7 | 8 | 11.2 | 10.9 | 8.2 |
| 8 | 7 | 12.5 | 10.9 | 9.2 |
| 9 | 2 | 7.9 | 11.0 | 5.9 |

Table 12. Comparative results of dye titration, dye photelometer, and modified osazone method for determining ascorbic acid in canned vegetables - average of two determinations.

| Food Tested | | Milligrams per hundred grams | | | | |
|---|---|---|---|---|---|---|
| | | Freshly opened samples | | | Samples after holding seven days at 5° C. | |
| | | Dye titration | Dye photelometer | modified osazone | Dye titration | Modified osazone |
| | solids | 6.6 | 3.5 | 3.6 | 3.9 | 4.0 |
| Peas | juices | 7.6 | 10.0 | 10.5 | 6.4 | 9.4 |
| | solids | 6.0 | 6.7 | 4.2 | 3.9 | 5.3 |
| Peas | juices | 7.6 | 6.7 | 10.3 | 6.7 | 11.3 |
| | solids | 8.9 | 10.0 | 16.5 | 5.6 | 8.2 |
| Peas | juices | 11.3 | 11.5 | 9.4 | 7.4 | 10.0 |
| Green snap | solids | 2.5 | 5.2 | 0.5 | 1.3 | 1.0 |
| beans | juices | 2.5 | 3.0 | 0.5 | 2.2 | 0.0 |
| Green snap | solids | 2.8 | 3.2 | 1.5 | 0.0 | 0.0 |
| beans | juices | 2.8 | 2.3 | 1.2 | 0.0 | 0.0 |
| Spinach | solids | 31.4 | 27.0 | 30.0 | 25.3 | 30.0 |
| | juices | 39 | 36 | 34 | 33.0 | 34.0 |
| Spinach | solids | 19.5 | 15.0 | 9.4 | 11.0 | 17.5 |
| | juices | 22.0 | 19.7 | 12.5 | 17.9 | 20.0 |
| Carrots | solids | 2.4 | 0.0 | 0.0 | 0.7 | 0.0 |
| | juices | 2.5 | 0.0 | 0.0 | 0.6 | 0.0 |
| Carrots | solids | 2.1 | 0.0 | 0.0 | 0.0 | 0.0 |
| | juices | 2.1 | 0.0 | 0.0 | 0.0 | 0.0 |
| Tomatos | solids | 16.6 | 17.0 | 14.3 | 14.5 | 14.0 |
| | juices | 16.5 | 20.0 | 16.0 | 15.0 | 15.5 |
| Kidney | solids | 10.4 | 3.2 | 0.0 | 0.0 | 0.0 |
| beans | juices | 9.8 | 10.0 | 3.5 | 0.0 | 1.6 |
| Beets | solids | none | 10.5 | 0.0 | none | 0.0 |
| | juices | possible | 13.0 | 0.0 | possible | 0.0 |

Table 13. Comparative results of dye titration, dye photelometer and modified osazone method for testing ascorbic acid in canned fruit juices.

| Juices Tested | Milligrams per hundred grams | | | | |
|---|---|---|---|---|---|
| | Freshly opened samples | | | Samples after holding seven days at 5° C. | |
| | Dye titration | Dye photelometer | Modified osazone | Dye titration | Modified osazone |
| Tomato juice | 23.8 | 23.0 | 23.3 | 19.5 | 20.6 |
| Tomato juice | 22.8 | 20.2 | 22.8 | 20.3 | 26.2 |
| Orange juice | 61.8 | 66.0 | 56.7 | 50.2 | 53.0 |
| Orange juice | 54.0 | 52.0 | 50.0 | 49.3 | 53.1 |
| Orange juice | 53.8 | 49.0 | 53.0 | 49.3 | 55.0 |
| Pineapple juice | 14.3 | 13.0 | 12.5 | 11.6 | 8.6 |
| Pineapple juice | 9.7 | 7.1 | 11.0 | 8.5 | 9.1 |
| Grapefruit juice | 32.8 | 29.0 | 34.0 | 31.4 | 33.8 |
| Grapefruit juice | 33.5 | 32.0 | 28.0 | 31.4 | 33.8 |

Table 14. Dehydroascorbic acid results on canned vegetables, vegetable and fruit juices - average of two determinations.

| Food tested | | Milligrams per hundred grams: Freshly opened sample | Milligrams per hundred grams: Sample after holding seven days at 5° C. |
|---|---|---|---|
| | solids | 1.3 | 4.0 |
| Peas | juices | 0.0 | 0.0 |
| | solids | 1.3 | 4.5 |
| Peas | juices | 0.3 | 2.6 |
| | solids | 0.0 | 4.8 |
| Peas | juices | 0.0 | 3.8 |
| Green | solids | 0.0 | 1.6 |
| snap beans | juices | 0.0 | 1.0 |
| Green | solids | 1.0 | 2.6 |
| snap beans | juices | 0.0 | 1.2 |
| | solids | 2.6 | 8.0 |
| Spinach | juices | 0.0 | 6.3 |
| | solids | 4.3 | 8.9 |
| Spinach | juices | 1.9 | 7.1 |
| | solids | 0.0 | 1.6 |
| Carrots | juices | 0.0 | 0.0 |
| | solids | 0.0 | 1.0 |
| Carrots | juices | 0.0 | 0.0 |
| | solids | 0.0 | 4.0 |
| Tomatoes | juices | 0.5 | 3.5 |
| | solids | 0.0 | 2.0 |
| Kidney beans | juices | 2.5 | 4.5 |
| | solids | 0.0 | 0.0 |
| Beets | juices | 0.0 | 0.0 |
| Tomato juice | | 1.3 | 9.3 |
| Tomato juice | | 0.6 | 7.8 |
| Orange juice | | 2.3 | 9.3 |
| Orange juice | | 2.1 | 7.5 |
| Orange juice | | 4.2 | 7.5 |
| Pineapple juice | | 0.0 | 4.5 |
| Pineapple juice | | 2.2 | 3.5 |
| Grapefruit juice | | 1.3 | 5.9 |
| Grapefruit juice | | 1.3 | 5.9 |

## GENERAL DISCUSSION

It is evident from the results obtained that further verification of the osazone method is necessary. In general, the results are comparable to the dye methods, but they are not consistantly higher to the amound of dehydro present as they should be. For total ascorbic acid content, the original (30) and modified (31) methods are equally comparable to the dye determinations. The real advantage in the modified method (31) is that it is now possible to determine dehydro chemically.

Since the oxidized form, dehydro, is no more stable than the reduced form, the results obtained from holding the samples are difficult to explain. Apparently under these testing conditions, reduced ascorbic acid forms a more stable compound. Dehydro results on the freshly prepared samples verify the fact that plant tissues contain little or no dehydro. The very consistant and decided increase of dehydro ascorbic acid on standing, however, needs facts for explanation which are not available at present.

## SUMMARY

Results obtained from the comparative studies on fruits and vegetables of reduced and oxidized ascorbic acid show that

1. A modification of the osazone method reduced the time of incubation from three hours at 37° C. to one hour at 55° C. with comparable results.

2. The original osazone method gave the same general values for the ascorbic acid content of foods as the dye titration method.

3. The modified osazone method was no improvement over the original osazone method as a measure of active and reduced ascorbic acid.

4. The dye titration method gave values which approximately equalled the dye photelometer method. Evidently the vegetables when diluted were not highly colored enough to interfere with the pink endpoint of the dye titration.

5. The osazone method gave approximately the same value for ascorbic acid as the dye methods.

6. On storage, the osazone method showed less decrease in ascorbic acid content than the other methods studied. Under the conditions of the osazone test, ascorbic acid reamined in a stable form much longer than it did under the conditions for the other tests. If storage is necessary, the osazone method of measurement would be invaluable.

7. Dehydroascorbic acid as measured by the osazone method was present only in small amounts in the original plant tissue.

8. On storage dehydroascorbic acid was found to greatly increase, indicating that reduced ascorbic acid on storage changes to the dehydro form.

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