The determination of ascorbic acid (Vitamin C) in highly colored plant tissues.

THE DETERMINATION OF ASCORBIC ACID (VITAMIN C) IN HIGHLY COLORED PLANT TISSUES

POLAND, Jr. - 1938

THE DETERMINATION OF ASCORBIC ACID (VITAMIN C) IN HIGHLY COLORED PLANT TISSUES

Edwin F. Poland, Jr.

Thesis submitted for
the degree of
Master of Science

MASSACHUSETTS STATE COLLEGE, AMHERST

May 20, 1938

Table of Contents

Introduction

Since 1932, the Tillmans's titration method for determining ascorbic acid has been modified several times, so that ascorbic acid in non-pigmented plant tissues can now be determined with considerable accuracy. However, a number of attempts to apply it to colored fruits and vegetables have been made without much success.

In the past, it has been possible to determine the ascorbic acid content of colored plant tissues only by means of the bioassay. This procedure, which requires 90 days, is subject to a variety of experimental errors, especially when the ascorbic acid content is low or when the material is not particularly palatable to the animals, as is the case with many of the colored plant tissues. Another disadvantage is that a constant supply of fresh material cannot be maintained throughout the 90 day bioassay period, because many of these colored tissues are the small pigmented fruits which are available only during short seasons.

Because the animal method takes a long time and is subject to error, and because colored plant tissues are widely used in the diet, a quick, more or less simple method has been sought.

This work was undertaken, therefore, to find, if possible,

either by adapting the regular modified Tillmans's procedure, or by developing an entirely new method for the determination of ascorbic acid in colored plant tissues.

Review of Literature

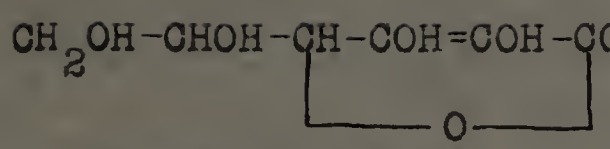
A review of the literature on the chemical determination of ascorbic acid reveals that, while there are several methods that have been worked out, there are no methods available for the chemical determination of ascorbic acid in colored plant tissues.

King (1936), in his report on ascorbic acid gives a very complete review of the methods more commonly used. The most widely used chemical method is the one which the dye 2-6 dichlorophenol indophenol oxidizes quantitatively the ascorbic acid, molecule for molecule. The results of this method have been shown to correlate closely the results of the bioassay in work done by Tillmans, Hirsch and Hirsch (1932), Harris et al (1933), and Bessey and King (1933). Another method that has gained favor, because of the stability of the color and the simplicity of preparing reagents, is the ferricyanide titration of Tauber and Kleiner (1935). Bezssonoff's (1934) color reagent ($MoO_3P_2O_5(WO_3)_{17}(H_2O)_{14}$) has been used but it has been found that this reagent is not specific in all cases. A method by Roe (1934) wherein the

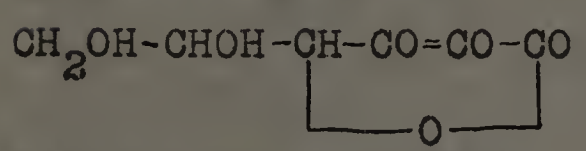
ascorbic acid is decomposed to furfural and a color developed with aniline, has possibilities, but as yet has not been extensively used. King's laboratory found that titrating directly with the oxazine dye, prune, as described by Melville and Richardson (1934) was not practical. Bachstez and Cavallini (1934) developed a color tecnic using ($UO_2(OAc)_2$). Szent-Gyorgyi (1934) developed a color reaction with $FeSO_4$ exposed to air. A report by Martini and Bonsignore (1934) stated that methylene blue was more specific than the indophenol, but King did not confirm this. Micro methods have been developed by Birsch, Harris and Ray (1933) and Glick (1935).

To overcome rapid oxidation of the ascorbic acid during extraction, several modifications of the extracting material have been suggested. Fujita and Iwatake (1935) found that the use of a 2 percent metaphosphoric acid instead of trichloroacetic was helpful. A combination of 2 percent metaphosphoric and 3 percent trichloroacetic acid was found by King and Musulin (1936) to be very helpful. Mack and Tressler (1937) found that the use of a strong acid such as 10 percent sulfuric delayed the catalytic action of such metals as copper and iron.

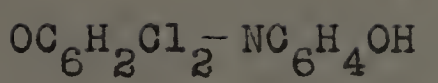
In conclusion King (1936) gives the probable reaction of the ascorbic acid and the dye as follows:



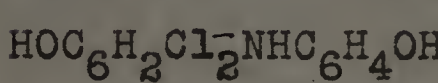
l-ascorbic acid



dehydro ascorbic acid



2-6 dichlorophenol indophenol



phenolic leuco base
(colorless)

This leads to the conclusion that theoretically other oxidizing agents should be more specific. However, since the dye reaction is satisfactory, very few others have been widely used.

Experimental Using the Fellers-Maclinn Modified Technic with Colored Plant Tissues

Reagents

Iodine solution

Dissolve 1.3 grams of iodine and 25 grams of potassium iodide and make up to 1000 cubic centimeters with distilled water. Standardize against sodium thiosulfate. Store in a cool place.

Thiosulfate solution

Dissolve 2.5 grams of sodium thiosulfate in distilled water and make up to 1000 cubic centimeters with distilled water. Standardize against .01N iodine.

Acid mixture

Dissolve 30 grams of trichloroacetic acid and 20 grams of metaphosphoric acid in distilled water and make up to 1000 cubic centimeters with distilled water.

Dye solution

Dissolve .1 gram of 2-6 dichlorophenol indophenol in small portions of hot distilled water and make up to 400 cubic centimeters. Store in cold place. Do not use after 5 days.

Starch solution

Dissolve .5 gram of soluble starch in 100 cubic centimeters of cold distilled water. Store in cold place.

Ascorbic acid solution

Dissolve .1 gram of pure ascorbic acid crystals in acid mixture and make up to 100 cubic centimeters with acid mixture. Store in cold place. Standardize against dye as used.

Standardization of the Dye (Buck and Ritchie 1938)

Dissolve 4 grams of KI in 15 cubic centimeters of distilled water in a 300 cubic centimeter Erlenmeyer flask. Add 10 cubic centimeters of dye. Add 10 cubic centimeters of dilute hydrochloric acid and let stand for 2 minutes. Titrate with standard thiosulfate solution, approximately .01N.

Calculation:

1 cubic centimeter of .01N thiosulfate = 1 cubic centimeter of .01N iodine = .88 milligrams of ascorbic acid.

Extraction of Ascorbic Acid

Weigh out a 10 or 20 gram sample and place in a mortar. Add small amount of washed sand and approximately 10 to 15 cubic centimeters of acid mixture. Triturate thoroughly. Transfer the material to a centrifuge tube, washing the mortar out with acid mixture. Centrifuge at 2000 revolutions per minute for 15 minutes. Decant into a 100 cubic centimeter flask and make up to 100 cubic centimeters with acid mixture.

Modifications

Carbon decolorization

At first, attempts were made to remove the color from colored solutions by using carbon in varying amounts at various pH values. The color was removed quite easily but very little of the ascorbic acid was left in a titratable form. According to Roe (1934), ascorbic acid is oxidized by activated carbon to dehydroascorbic acid which does not react with the dye.

Dilution

Following this, the dye titration was modified by dilution. Work with colored solutions such as cranberry, grape, blueberry and currant juices to which had been added known amounts of ascorbic acid, gave results, shown in Table 1. These results showed that the added ascorbic acid could be determined with considerable accuracy. Of course, great care as to details was necessary to obtain the correct endpoint. These data seemed to indicate that this procedure could be applied to unknown pigmented tissues. Where considerable quantities of ascorbic acid were present the titration of pigmented foods was much easier and more accurate than where only small amounts of ascorbic acid were present. Several of these pigmented foods were tried and the results are given in Table 2.

The procedure used is as follows: Extract the materials as described in the previous section. Take two, 10 cubic centimeter portions, place in tall glass cylinders, approximately $1\frac{1}{4}$ inches by $5\frac{1}{2}$ inches, and dilute with distilled water to within one inch of the top. Titrate one preparation directly with the dye using a glass stirring rod to mix the solution. Use the second preparation as a standard to compare the titrated sample with. The endpoint in this titration is observed by

Table 1

Titration, by the Dilution Procedure, of Colored Juices Containing Known Amounts of Ascorbic Acid

Colored juice	Added ascorbic acid	Titrated ascorbic acid
	mg. per 10 cc.	mg. per 10 cc.
Grape	.50	.51
Blueberry	.50	.49
Currant	.40	.41
Cranberry	.49	.50

Table 2

Titration of Various Colored Fruits
by the Dilution Procedure

Fruit	Variety	Ascorbic acid
		mg. per gram
Currants (frozen)	Perfection	.13
Red raspberries (frozen)	Cuthbert	.09
Black raspberries (frozen)	Plum Farmer	.08
Blueberries (frozen)		.07
Blueberries (frozen)	Jersey	.08
Strawberries (fresh)	Local A & P	.55
Strawberries (frozen)	Local A & P	.49
Cherries (canned)		.06
Cherries (frozen)		.06

looking down through the liquid in the two cylinders, which are held side by side and comparing colors. In as much as all of these colored tissues give considerable color even to dilute solutions, the endpoint can be noted only by an increase in the intensity of the color or by a difference in shade as compared with the standard. To make it easier to observe the first color change, a white based burette standard and a strong light were used.

Since this method of determining the endpoint, even after much practice, is difficult to judge accurately, its use would probably be subject to a personal factor. Therefore, the colorimeter was used in an attempt to standardize the endpoint. A small portion of the standard was placed in the left-hand cup of a Klett colorimeter and a small portion of the solution being titrated in the right-hand cup. The standard was set at a definite point determined more or less by the intensity of the color. The right-hand cup was moved up and down until the two halves of the color matched. A reading was then taken and the difference between the standard and the unknown was noted. It was found that the addition of one drop of dye would increase the difference from 0.1 to 0.2. Since this might be easily missed in balancing the colors, a difference of from 0.2 to 0.4 was arbitrarily set as the endpoint. In these titrations an endpoint correction factor was subtracted from

the results in order to give a more accurate value. This factor was arrived at by observing the amount of dye required to produce a definite pink color in a volume of water and acid mixture equal to the volume being titrated.

This procedure, while not extremely accurate, could be used with certain of the lighter colored tissues, such as strawberries, apples and others.

Sample calculation

1. Sample 10 grams of strawberries
2. Extracted material diluted to 100 cubic centimeters.
3. 10 cubic centimeters of dilute material contains ascorbic acid from 1 gram of sample.
4. 10 cubic centimeters diluted to 100 cubic centimeters and titrated directly.
5. Dye equals .35 milligrams of ascorbic acid.
6. Dye used = 1.50 cubic centimeters

   Sample used = 1 gram

   1.50 cc. x .35 mg. = ascorbic acid per gram of strawberrics.

The new petroleum ether-ethyl ether method

Although the results obtained by the previous method were reasonably satisfactory, they showed that there was still much to be desired. First, they were generally slightly higher when checked with known amounts of ascorbic

acid. Second, the endpoint was too difficult to observe accurately each time. Therefore, it seemed advisable to try to find a simpler and more accurate method. The first attempt made was to remove the color with various solvents and combinations of solvents. These trials were without success. Out of this failure grew the idea that perhaps a chemical could be found to fill three requirements.

(1). That it form a colorless layer above the colored solution.

(2). That it would allow the dye to pass unchanged to the colored solution.

(3). That it would indicate, by the development of a pink color, the excess of dye left when the ascorbic acid in the colored solution was completely oxidized.

Several solvents such as chloroform, toluene, hexane, ether, and petroleum ether were tried both alone and in combinations, however, none was satisfactory. All except petroleum ether, either absorbed the dye or decolorized it.

Petroleum ether fulfills two of the above requirements for a desirable reagent. Because of its lower specific gravity, it will form a layer above the colored solution, and, due to the insolubility of the dye in the petroleum ether, the dye will pass through this layer unchanged. For these reasons petroleum ether was used as a base. How-

ever, since it will not show an excess of dye by the development of a pink color, it is essential to add some chemical to overcome this defect.

Ethyl ether also satifies two of the requirements. It will form a layer if used in sufficiently large quantities and it will indicate an excess of dye by the formation of a pink color. However, because of the solubility of the dye in ethyl ether the dye will not pass through unchanged.

While ethyl ether is soluble in water up to about ten percent, it is many times more soluble in petroleum ether and apparently the petroleum ether shows a preferential solubility for the ethyl ether. It was possible, therefore, to mix the two ethers in various proportions in an attempt to obtain the ideal mixture. Many of these mixtures were tried and the proportion was found finally which satisfied most completely the three specifications; one part of ethyl ether to twenty parts of petroleum ether. In working with this proportion on colorless solutions of ascorbic acid, it was found that the ethyl ether not only dissolved the dye enough to show a pink color, but also showed a preferential solubility for the dye. On shaking, all of the excess color could be concentrated in the ether layer, leaving a clear, colorless layer below.

Using the above mixture, a procedure was worked out which gave consistent results on ascorbic acid solutions. Also it has given satisfactory results for the colored

tissues on which it has been tried. The detailed procedure is as follows: Weigh out a 10 gram sample, place it in a mortar, add a small amount of sand, 10 to 15 cubic centimeters of acid mixture and triturate thoroughly. Transfer the material to a centrifuge tube washing the mortar with acid mixture. Centrifuge at 2000 revolutions per minute for 15 minutes. Decant the liquid into a 100 cubic centimeter volumetric flask and dilute to the mark with distilled water. Ten cubic centimeters of this dilute extraction are then placed in a 50 cubic centimeter test tube and covered with a layer of approximately 10 cubic centimeters of ether mixture. A test tube is used because a wider band is formed by the ether mixture thereby partially solving the problem of reflected color. The colored solution is then titrated directly with the dye, stoppered with a rubber stopper and shaken after each addition. As some of the dye will be taken up by the ether layer, mixing of some sort is necessary to bring the dye in contact with the ascorbic acid contained in the colored solution. In some cases shaking is undesirable because it tends to cause the formation of a rather stable emulsion. This is particularly true where large quantities of pectin are present. Therefore, in these cases, it seems advisable to stir the dye into the colored solution. In making this titration the dye should be added slowly. If the dye is added rapidly, or, too much at a

time, it collects in a heavy layer on top of the colored solution, and more of the dye goes into the ether layer. This necessitates extra shaking to bring the dye into contact with the ascorbic acid. If an emulsion is formed, which does not readily break down, placing the test tube in a beaker of hot water or using a stirring rod to break the bubbles will help remedy this condition.

In all of these titrations an endpoint correction factor was subtracted from the titration figure to give more accurate results. This factor was obtained by titrating the dye against 10 cubic centimeters of acid mixture plus 10 cubic centimeters of the ether mixture. The amount of dye necessary to produce a definite pink color is the factor. In general, this amounts to about two drops of dye.

The endpoint is best observed by looking through the ether band at a white background and comparing the color of the unknown with the color of a standard obtained as described above.

Using the method as described, the ascorbic content of several colored fruits and fruit preparations was determined. Table 3 gives the ascorbic acid value of three colored fruit juices. To check the accuracy of the method, a known amount of ascorbic acid was added to an equal quantity of each of the juices. The titration values obtained were equivalent to the sum of the unknown amount of ascorbic

Table 3

Titration of Colored Juices Using the Ether Mixture

Colored material	Unknown ascorbic acid	Added ascorbic acid	Total ascorbic acid
	mg. per cc.	mgs.	mgs.
Strawberry juice	.56	.72	1.28
Raspberry juice	.21	.72	.92
Cherry juice	.11	.72	.81

acid, as previously determined, plus the known amount added. These results indicate that both large and small amounts of ascorbic acid can be determined with considerable accuracy by means of this method. Table 4 gives the ascorbic acid content of various cranberry products as determined by three procedures, (1) dilution using dye, (2) dilution using iodine, and (3) direct titration using the ether mixture. The first three products were commercial preparations obtained from Cranberry Canners Incorporated. The last four products were made in the laboratory from frozen cranberries that had been in storage for six months. These products were prepared according to the procedure outlined in the booklet entitled, "Fascinating Cranberries and How to Serve Them," distributed by the American Cranberry Exchange. In view of the fact that most of these products contained very little ascorbic acid the data presented are of little significance, because, in many instances the results approach the range of experimental error. Table 5 gives the ascorbic acid content of a number of colored berries as determined by the dilution and ether mixture procedures. These data show that while the two methods check each other closely, the latter method gives generally lower and more accurate results. This method, if properly used, is capable of giving consistently good results.

Sample calculation

1. Sample 10 grams of strawberries

Table 4

Titration of Cranberry Products by the Dilution Procedure Using Dye and Iodine and by the Ether Procedure

Product	Dye	Iodine	Ether
	mg. per gram	mg. per gram	mg. per gram
Ocean Spray cranberry juice cocktail	.025	.03	
Ocean Spray cranberry sauce (strained)	.021	.028	
Ocean Spray cranberry sauce (whole)	.025	.026	
Frozen cranberries (6 months storage)	.038	.046	.030
Cranberry cocktail (frozen berries)	.00	.00	.00
Cranberry jelly (frozen berries)	.02	.02	.00
Cranberry sauce (frozen berries)	.02		.01
Cranberry relish (frozen berries)	.08	.06	.05

Table 5

Comparison of Results of Dye Titration by the Two Procedures, Dilution and Ether Mixture

Fresh	Variety	Dilution	Ether mixture
		mg. per gram	mg. per gram
Currants (frozen)	Perfection	.13	.11
Red raspberries (frozen)	Cuthbert	.09	.05
Black raspberries (frozen)	Plum Farmer	.08	.07
Blackberries (frozen)		.07	.06
Blueberries (frozen)	Jersey	.08	.05
Strawberries (frozen)	Local A & P	.49	.46
Strawberries (fresh)	Local A & P	.55	
Cherries (canned)		.06	
Cherries (frozen)			.06

2. Extracted material diluted to 100 cubic centimeters
3. 10 cubic centimeters of dilute material contains ascorbic acid from 1 gram of sample
4. Dye equals .35 milligrams of ascorbic acid.
5. Dye used = 1.50 cubic centimeters

   Sample used = 1 gram

   1.50 cc. x .35 mg. = .53 mg. ascorbic acid per gram of strawberries.

Summary and Conclusions

1. The review of literature discusses the more commonly used methods of determining ascorbic acid chemically, some of the modifications that have been suggested, and the probable reaction between the dye and the ascorbic acid.
2. A list of the reagents used in this work and a description of their preparation is given.
3. The use of activated carbon to remove the color was tried and found unsatisfactory.
4. The modified titration tecnic, as adapted by Fellers and Maclinn (1938) in this laboratory, was further modified to adapt it to colored plant tissues. The modification is to dilute the extracted material approximately one to ten and, using tall cylinders, to compare the unknown with a standard.
5. A new method is described using a colorless layer of a mixture of petroleum ether and ethyl ether to indicate by the development of a pink color, when an excess of dye is present, the complete oxidation of the ascorbic acid in the colored solution. The optimum ratio of ethyl ether to petroleum ether is 1 to 20 by volume.

Of the two methods used, the one using the ether mixture gives the most accurate results and is the easier to manipulate. This method should be of considerable value in simplifying vitamin C studies.

Bibliography

Bessey, O. A. and King, C. G., 1933. Distribution of vitamin C in plant and animal tissues and its determination. J. Biol. Chem. 103, 687.

Bezssonoff, N., 1934. Identification of vitamin C. Bull. Soc. Chim. biol. 16, 1107, 1160. C. A. 29, 201.

Birsch, T. W., Harris, L. J. and Ray, S. N., 1933. A microchemical method for determining the hexuronic acid (vitamin C) content of foodstuffs, etc. Biochem J. 27, 590.

Bachstez, M. and Cavallini, G., 1934. A color reaction of isoascorbic acid (isovitamin C). Ztschr. physiol. chem. 228, 25. C. A. 29, 199.

Buck, R. E. and Ritchie, W. S., 1938. New method for the standardization of dye used for determination of cevitamic acid (vitamin C). Ind. Eng. Chem. Anal. Ed. 10, 26.

Fellers, C. R. and Maclinn, W, A., 1938. Unpublished data.

Fujita, A. and Iwatake, D., 1935. Determination of vitamin C with 2-6 dichlorophenol indophenol. Biochem. Ztschr. 277, 284, 296.

Glick, D., 1935. The chemical determination of minute quantities of vitamin C. J. Biol. Chem. 109, 433.

Harris, L. J., et al., 1933. Chemical test for vitamin C and the reducing substances present in tumor and other tissues. Nature 132, 27, 28.

King, C. G., 1936. Vitmin C, ascorbic acid. Physiol. Rev. 16, 238.

King, C. G. and Musmlin, R. R., 1936. Metaphosphoric acid in the extraction and titration of vitamin C. J. Biol. Chem. 116, 409.

Mack, G. L. and Tressler, D. K., 1937. A critical investigation of the Tillmans's method for the determination of ascorbic acid. J. Biol. Chem. 118, 3.

Martini, E. and Bonsignore, A., 1934. A new method for the chemical determination of vitamin C. Bull. Soc. ital. biol. sper. 9, 388. C. A. 28, 6460.

Melville, J. and Richardson, G. M., 1934. Prune as an oxidation-reduction indicator. Its stability for titration of ascorbic acid. Biochem. J. 28, 1565.

Roe, J. H., 1934. Detection of ascorbic acid as furfural. Science 80, 561.

Szent-Gyorgyi, A., 1934. A color reaction of ascorbic acid. Ztschr. physiol. chem. 225, 168. C. A. 28, 6460.

Tauber, H. and Kleiner, T. S., 1935. An enzymic method for the estimation of true vitamin C. J. Biol. Chem. 108, 563.

Tillmans, J., Hirsch, P and Hirsch, W., 1932. Reduction capicity of plant foodstuffs and its relation to vitamin C. Ztschr. Untersuch. Lebensm. 63, 1.

Acknowledgments

I wish to take this opportunity to offer my sincere thanks to Dr. C. R. Fellers for his guidance and suggestions in carrying out this work, to Dr. Helen S. Mitchell and Dr. W. A. Maclinn for their assistance in the writing of this paper, and to Mr. Defelice and other graduate students for their patient assistance and suggestions.

Approved by:

Carl R. Ellers

Helen S. Mitchell

Graduate Committee

Date: May 31, 1938