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THE COLORIMETRIC DETERMINATION OF CARBOHYDRATES IN PLANTS BY THE PICRIC ACID REDUCTION METHOD I. THE ESTIMATION OF REDUCING SUGARS AND SUCROSE[1]

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A study of the estimation of sugars in the complex mixtures in plants was commenced by Brown and Morris,[2] followed by the work of Parker[3] and the more complete studies of the Rothamsted group.[4] These workers have tested many points of procedure of the analytical details in the cupric reduction methods and it is apparent that the difficulties have been clearly recognized. Even such skilled and careful workers as Brown and Morris considered the degree of accuracy as being equivalent to 1 mg. of the cupric oxide weighed, corresponding to 0.5 mg. of glucose.

One of the chief objections to copper reduction methods is the difficulty in securing absolute uniformity in the conditions of reduction, which is so essential, since Nef[5] has shown that a number of products, variable in nature and quantity with the concentration of the sugar solution and alkali are formed when the sugar molecule is acted upon by alkaline Fehling solution. The main sources of error due to (a) lack of temperature control and (b) auto-oxidation have been largely overcome by Quisumbing and Thomas;[6] notwithstanding which, considerable difficulty was experienced in our Laboratories in applying it to the determination of small quantities of sugar in leaves and spurs of apple trees, in connection with biochemical studies relating to the effect of fertilizers on the physiological functions of the apple.[7]

A combination of the gravimetric and volumetric methods (Bertrand) is quite extensively used at the present time in biochemical laboratories. The procedure introduces the errors of both; moreover, it is not generally recognized that the standard permanganate solution must be standardized

[1] Read at the Milwaukee meeting of the American Chemical Society, September, 1923.

The cost of this investigation has largely been defrayed by a grant from the Adams Fund for Agricultural Research.

[2] Brown and Morris, *J. Chem. Soc.*, **63**, 604 (1893).

[3] Parker, *Biochem. J.*, **6**, 1 (1912).

[4] Davis, *J. Agr. Sci.*, **5**, 434 (1912–13). Davis and Daish, *ibid.*, **6**, 152 (1914). Daish, *ibid.*, **6**, 255 (1914). Davis and Sawyer, *ibid.*, **6**, 406 (1914). Davis, Daish and Sawyer, *ibid.*, **7**, 255 (1915–16). Davis and Sawyer, *ibid.*, **7**, 352 (1915–16). Davis, *ibid.*, **7**, 737 (1915–16).

[5] Nef, *Ann.*, **357**, 214 (1907); **376**, 1 (1910); **403**, 204 (1914).

[6] Quisumbing and Thomas, THIS JOURNAL, **43**, 1503 (1921).

[7] Thomas, *Soil Sci.*, **15**, 1 (1923).

against the cuprous oxide produced by a known quantity of pure glucose and not against oxalic acid. Results obtained in our Laboratories indicate that the values are from 8 to 20% too low when oxalic acid is used.

Inasmuch as colorimetric methods for the determination of sugars have been used with a high degree of accuracy in the sphere of animal physiological chemistry, the present paper is the result of an investigation in the application of Benedict and Osterberg's method[8] to the plants used in the investigation to which reference has been made, due consideration being given to the limitations set by them and to the findings of Falk and Noyes.[9]

General Considerations as to the Applicability of the Method

Of the sugars occurring in plants uncombined, glucose, fructose, sucrose and possibly the pentoses, arabinose and xylose, are the only ones which are important from the standpoint of the plant biochemist. With the exception of sucrose, these have a reducing action on Fehling's copper solution and are accordingly classed as "reducing sugars." These same sugars also have a reducing action on picric acid in alkaline solution, reducing it to a colored substance, presumably picramic acid.

From the experimental data to be presented, it has been found that sucrose also (up to 0.1 *M* concentration) has no reducing action on Benedict and Osterberg's picrate-picric solution. Experiments were also carried out on the quantitative relation between the cupric and picric reducing values of each of these sugars. Inasmuch as these values do not differ very greatly for the respective sugars, we should expect the values obtained by the cupric and picric acid reduction methods to agree with one another within the limits of experimental error.

It was from a consideration of these facts that the application of the colorimetric method was first investigated through the use of lead acetate with the view of simplifying the technique. However, as will be shown, the use of this reagent was not a success.

It will be noticed that maltose has not been mentioned here as being among the sugars occurring in plants, for it is improbable that it occurs except as a laboratory product in the degradation of starch. It is true that Brown and Morris[2] found it in leaves of Tropoelum, but Davis and Sawyer[10] have attributed its presence to the failure of Brown and Morris to inactivate the diastase present, owing to the procedure adopted. The leaves were dried at 50°. Under such conditions considerable quantities of glucose and maltose would be formed and the latter would persist, owing to the destruction of the maltase at the temperature used.

[8] Benedict and Osterberg, *J. Biol. Chem.*, **34**, 195 (1918).

[9] Falk and Noyes, *ibid.*, **52**, 109 (1920).

[10] Davis and Sawyer, *J. Agr. Sci.*, **5**, 454 (1912–13).

Following the method of Mulliken[11] and also that of Sherman and Williams[12] no maltose was found in any of the extracts concerned in the present investigation.

Experimental Part

For the convenience of workers who may desire to use this colorimetric method the preparation of the reagents—(a) the picrate-picric solution as developed by Benedict and Osterberg[13] and (b) the mercuric nitrate solution used by them is given here.

Preparation of the Picrate-Picric Solution

Thirty-six g. of picric acid (dried at 60° and purified if necessary)[14] is added to 500 cc. of a 1% solution of sodium hydroxide in a liter flask. This is followed by 400 cc. of hot water. The mixture is shaken occasionally until the picric acid is dissolved, and afterwards cooled and diluted to one liter.

Preparation of the Mercuric Nitrate Solution Used as a Clarifying Agent

A more dilute solution of the mercuric nitrate than that used by Benedict and Osterberg was employed. A solution of 110 g. of mercuric oxide added gradually to 80 cc. of concd. nitric acid is stirred, then heated to boiling, and cooled, and to it is added 30 cc. of a 5% solution of sodium hydroxide. This is then made up to one liter and stored in the dark.

The Standard Glucose Solution

Benedict and Osterberg describe the preparation of a permanent standard.[15] However, thus far, no attempt has been made in the course of this investigation to prepare a permanent color standard. In all our experiments the color of the standard and unknown were developed under exactly the same conditions. Although for low concentrations of sugars a standard containing 0.016% of glucose, which was used in the preliminary experiments, is satisfactory; for general work a standard containing 0.025% of glucose is recommended.

The Picrate-Picric Reducing Power of Fructose, Arabinose and Xylose

The following data are given as indicative of the purity of the sugars employed.

Glucose.—Bureau of Standards product; $[\alpha]_D^{20}$, + 52.76; ash, 0.003%; moisture, 0.03%.

Sucrose.—Bureau of Standards product; $[\alpha]_{589.25}^{200}$, 66.529; ash, 0.003%; moisture, 0.003%.

Fructose.—Pfanstiehl brand; $[\alpha]_D^{20}$, —91.66; ash, 0.095%; moisture (after drying under reduced pressure), 0.005%.

l-Arabinose.—Pfanstiehl brand; $[\alpha]_D^{20}$, +104.5; moisture, 0.10%.

l-Xylose.—Pfanstiehl brand; $[\alpha]_D^{20}$, +18.5; moisture, 0.10%.

The picrate-picric reducing power, by which is meant the ratio between the amounts of glucose and the respective sugar, required to reduce equal

[11] Mulliken, "Identification of Pure Organic Compounds," John Wiley and Sons, **1908**, 1st ed., vol. I, p. 32.

[12] Sherman and Williams, THIS JOURNAL, **28**, 629 (1906).

[13] Ref. 8, p. 198.

[14] Folin and Doisy, *J. Biol. Chem.*, **28**, 349 (1910).

[15] Ref. 8, p. 200.

amounts of picric to picramic acid, was determined by comparing, in a a series of experiments, the color values developed under the same conditions, of equal concentrations of the standard glucose and the sugar, the reducing power of which is to be obtained. Table I gives the comparison of the cupric and picrate-picric reducing powers.

TABLE I

COMPARISON OF THE PICRATE-PICRIC AND CUPRIC REDUCING POWERS OF THE PRINCIPAL SUGARS OF PLANTS

Material	Glucose	Fructose	*l*-Arabinose	*l*-Xylose	Sucrose
Picrate-picric	1.00	0.99	1.01	1.08	0.00
Cupric	1.00	.91	1.00	1.01	.00

Glucose, fructose and sucrose are the sugars that occur in largest amounts in plants. Although the ratio of the picrate-picric reducing power to the cupric reducing power is 99:91, it is improbable that the ratio of glucose to fructose in the plant will be less than unity, for from the work of Davis[16] it seems justifiable to assume that glucose and fructose exist in the leaves and stalks of plants as invert sugar and that they travel to the root in nearly equal proportions (depending upon the consumption in respiratory processes as functions of growth and new tissue formation). For this reason we would expect the results obtained by the two methods to agree closely.

The Failure of Lead Acetate as a Clarifying Agent

The use of this reagent for the removal of proteins, gums, etc., resulted in higher values for reducing sugars by the colorimetric than by the gravimetric method. The disparity, which was far greater than would be accounted for by any differences in the cupric and picric reducing values of the sugars present, was definitely established as being due to the presence of acid-hydrolyzable material (not of pentosan nature),[17] which the lead acetate had not precipitated. An attempt was made to determine the nature of this material by treating 200 g. of leaves according to the method of Schulze and Winterstein.[18] After the decomposition of the mercuric nitrate precipitate, filtration and evaporation of the filtrate under reduced pressure, a thick, dark brown, viscous material was obtained, which thus far has resisted all attempts at crystallization. It showed a marked difference in the cupric and picrate-picric reducing values, the latter being considerably greater than the former. Resort was then made to Benedict and Osterberg's mercuric nitrate solution to precipitate interfering materials, with very satisfactory results.

[16] Davis, *J. Agr. Sci.*, **7**, 327 (1916).

[17] A determination of pentosans gave only 0.0026 g. in 50 cc. of the original alcoholic solution, equivalent to 0.08 mg. in 10 cc. of the deleaded solution used in the colorimetric determination.

[18] Schulze and Winterstein, Abderhalden, "Handbuch der Biochem. Arbeitsmethoden," **1910**, vol. 2, pp. 510–521.

Preparation of the Plant Extracts

A representative sample (about 50 g.) of the plant parts is plunged into 95% boiling alcohol, previously redistilled over potassium hydroxide to remove acids and aldehydes, to which has been added 0.2 g. of calcium carbonate to prevent any hydrolyzing action by native acids. Allowing for the moisture present in the plant parts, the alcohol should be brought to a concentration of 70 to 75% by the addition of water, if necessary. The material can remain under alcohol for any length of time that may be convenient to the investigator, when, after being filtered and washed with 75% alcohol, it is dried in an oven at 70°, and then pulverized in a suitable mill[19] to pass an 80-mesh sieve.

The complete removal of the sugars is effected by extracting the powdered plant in a Soxhlet apparatus by using the alcoholic filtrate until the percolate is colorless.[20] The extract is then transferred to a 250cc. or 500cc. volumetric flask, according to the quantity of sugars present. The concentration of sugars in this extract should preferably be from 0.1 to 1.0%. The starch and other polysaccharides remain in the residue.

The Estimation of Reducing Sugars

A 20cc. to 100cc. aliquot portion containing from 0.025 to 0.150 g. of sugars is evaporated at a low temperature to remove the alcohol, the residue is dissolved with water and washed into a 400cc. beaker with 100 cc. of water. A slight excess of mercuric nitrate solution, of which 10 cc. was sufficient in our experiments, is added to the solution. Then, as directed by Benedict and Osterberg, small quantities of solid sodium bicarbonate are added gradually and the liquid stirred after each addition, until all frothing ceases. At this stage the bicarbonate is added carefully until the solution is alkaline to litmus. It is important to avoid any very large excess of sodium bicarbonate because of the subsequent treatment. This solution is then rapidly filtered into a 250cc. flask, the precipitate washed with a little 5% sodium bicarbonate solution, the solution and wash liquid are made up to the mark with water and shaken.

The concentration of this solution with respect to the sugars present should be within the limits 0.01 to 0.07%. From 30 cc. to 50 cc. of this solution is transferred to a 75cc. test-tube and 0.3 to 0.5 g. of zinc dust added. One drop of concd. hydrochloric acid is then added to reduce the small amount of mercury present in the solution, resulting from the solubility of the basic carbonate formed. Care must be taken in this treatment that sufficient sodium bicarbonate is present to prevent the solution becoming acid after the addition of the hydrochloric acid, as otherwise zinc carbonate will be precipitated in the subsequent treatment. The tube is then stoppered loosely and shaken, after which it is allowed to stand for 15 minutes and the contents finally filtered through a hardened filter paper. From 5 cc. to 10 cc. of the filtrate, tested with ammonium sulfide to ascertain whether all traces of mercury have been removed, is pipetted into a 50cc. Pyrex test-tube and 10 cc. of the picrate-picric solution is then added, followed by 2 cc. of a 25% solution of sodium carbonate. When less than 10 cc. of the sugar solution has been used, water equal to the deficiency is added, making the total volume of the solution 22 cc. A standard color solution is simultaneously prepared, using 10 cc. of standard glucose solution, 10 cc. of the picrate-picric solution and 2 cc. of 25% sodium carbonate solution. The tubes are then plugged with cotton and immersed for 20 minutes in a water-bath maintained at 95°; at the expiration of this time the tubes are removed and cooled to room temperature. The standard is diluted to 35 cc., or 70 cc., according to the quantity of sugar in the unknown, which is also suitably diluted,

[19] In our Laboratories the plants are first ground in a Seck mill and finally in a horizontal plate mill made by H. Dreefs, Halle, Germany.

[20] In our experiments no trace of any sugars was detected after the percolates became colorless.

and the color comparison made in any of the standard colorimeters according to the individual operator's preference.

As Benedict and Osterberg point out, it is absolutely essential to remove all traces of mercury, for apparently mercuric salts possess a reducing action on sugar solutions.[21]

A comparison of results obtained on apple tissues by the method outlined above with those of the cupric reduction method of Quisumbing and Thomas[6] from reducing values determined on smaller quantities of glucose (1 to 10 mg.) than given in the reducing tables of these investigators, is shown in Table II. It will be seen that more uniform and consistent results can be obtained and in much less time by this colorimetric method.

TABLE II

EXPERIMENTS SHOWING THE COMPARISON OF RESULTS OBTAINED ON PLANT TISSUES BY THE GRAVIMETRIC AND COLORIMETRIC METHODS

Material	Date March	Gravimetric Mg. of glucose in 50 cc. of solution	Colorimetric Mg. of glucose in 50 cc. of solution
Spurs	8	31.4	27.7
	10	28.9	28.0
	13	30.4	28.2
	15	29.0	27.9
	15	28.1	27.6
Leaves	15	20.2	20.6
	15	20.0	20.6
	18	19.0	20.7
	18	19.6	20.7

The method was further tested by adding various known amounts of glucose and fructose to plant extracts of known sugar content.

Table III shows the recoveries obtained.

TABLE III

EXPERIMENTS, USING THE COLORIMETRIC METHOD, ON THE RECOVERY OF GLUCOSE A ND FRUCTOSE ADDED TO PLANT MATERIALS

Material	Mg. of glucose added	Mg. of fructose added	Mg. of reducing sugars present in 50 cc. of solution	Total found Mg.	Recovered Mg.	Calcd. Mg.	%
Spurs	10	none	31.8	41.7	9.9	10	99.00
	none	10	31.8	41.8	10.0	10	100.00
	20	none	31.8	51.9	20.1	20	100.50
	none	20	31.8	51.6	19.8	20	99.00
	10	10	31.8	51.8	20.0	20	100.00
Leaves	15	none	20.5	35.6	15.2	15	101.33
	none	15	20.5	35.4	14.9	15	99.33
	10	10	20.5	55.5	19.8	20	99.00

[21] Knapp, *Z. anal. Chem.*, 9, 395 (1870).

The Estimation of Sucrose

It has been pointed out (p. 1663) that sucrose up to 0.1 *M* concentration was found to have no picrate-picric color value.

The experiments were conducted by (1) a direct comparison of sucrose solutions of various concentrations with the standard, the colors of which were developed under the same conditions, and (2) a comparison of the colors developed, likewise under the same conditions, of known amounts of sucrose added to the standard solution with a standard solution of the same concentration with respect to glucose. The results of a series of experiments indicated that the sucrose gave no color value up to 0.1 *M* concentration.

Inasmuch, therefore, as the products of hydrolysis of sucrose are equivalent amounts of glucose and fructose, it should be possible to determine accurately the quantity of sucrose present in solution not exceeding 0.1 *M* concentration by determining the increase in color obtained after inversion.

In order to test this, experiments were carried out by hydrolyzing pure sucrose solutions under the Herzfeld[22] condition of inversion,[23] which consists in adding slowly 5 cc. of hydrochloric acid (d., 1.888), in a 50cc. sugar flask containing a thermometer, in a water-bath heated to about 72–73°. The heating is adjusted so that the solution in the flask reaches 69° in three minutes. This temperature is then maintained for seven minutes longer. The solution is then removed and cooled to room temperature and neutralized with sodium hydroxide solution.

Table IV gives the results obtained.

TABLE IV

RECOVERY OF SUCROSE SOLUTION BY THE COLORIMETRIC METHOD

Calcd., 33.7 mg. in 50 cc. of solution

Found, mg. in 50 cc. of soln.....	33.0	33.1	33.4	33.7	33.6	33.7
Recovery, %.....	97.9	98.2	99.1	100.0	99.7	100.0

It is of interest to note that the results obtained are not due to any compensating factors, such as, for instance, the sodium chloride formed by the neutralization of the hydrochloric acid, since in parallel blank experiments using the same quantities of hydrochloric acid and sodium hydroxide no color value due to the presence of sodium chloride formed was found in these experiments. This point is emphasized here because of the apparently contradictory results obtained by different workers. Thus, though Okey[24] found a retardation in the rate of production of the colored substance, there appears to be no indication that there was any error in the color value as finally determined, due to the presence of sodium chloride.

[22] Herzfeld, *Z. Ver. deut. Zuckerind*, **38**, 699 (1888).

[23] Rose, *J. Biol. Chem.*, **46**, 529 (1921), has described a method of inversion by picric acid.

[24] Okey, *ibid.*, **38**, 33 (1919).

Denise and Rose[25] found an error of 17% for solutions containing molar concentrations of sodium chloride, but whether they found an increase or decrease in the color value has not been made clear.

There is, however, no destruction of glucose by the hydrochloric acid under the conditions of inversion adopted. One of us (W. T.) will show, in the following paper, that some glucose can be destroyed under certain conditions, such as when a solution containing glucose is boiled with even a low concentration of hydrochloric acid, as in the secondary hydrolysis of starch conversion products.

On the basis of the diluted solutions finally taken for the determination the concentration of the sucrose in a plant containing even as high a content of sucrose as 10% would only be about 0.025 *M*.

Summary

The method of Benedict and Osterberg for the determination of sugars in urine has been adapted, with modifications, to the determination of reducing sugars and sucrose in plant extracts. The details of the determination are given.

This colorimetric method is superior to the gravimetric, volumetric and, of course, optical methods for the determination of small amounts of sugars.

The use of mercuric nitrate as a precipitating agent for the removal of proteins, tannins, amino acids and other interfering substances should give truer values for reducing sugars than lead acetate, since the former removes substances having a slight reducing action on Fehling and a much greater reducing action on the picrate-picric solution. The nature of this material has not as yet been determined.

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[25] Denise and Rose, *Proc. Assoc. Exp. Biol. Med.*, **20**, 77 (1922).