Rapid Colorimetric Methods for Simultaneous Determination of Total Reducing Sugars and Fructose in Citrus Juices

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Ferricyanide is used in a carbonate-phosphate buffer as the oxidizing agent for sugars. The ferrocyanide produced is measured colorimetrically, using the blue solution formed after the addition of Nelson's arsenomolybdate reagent. When heated at 100° C. for 10 minutes, glucose and fructose are oxidized in equal amounts. When heated at 55° C. for 30 minutes, all the fructose, but only one eighth to one ninth of the glucose, is oxidized. The amounts of the two sugars in a mixture can then be readily calculated. Clarification of the juice samples with neutral lead acetate had no effect on sugars as determined with the colorimetric procedure. Comparable results on reducing and total sugar concentration of a number of citrus juice samples were obtained by using this method and the Shaffer-Hartmann volumetric procedure.

MOST METHODS OF DETERMINING SUGAR are based on the oxidation of sugars with an alkaline solution of metal salts, such as copper or iron (ferricyanides). The reduced metals are then determined by gravimetric, titrimetric, or colorimetric means. In recent years the use of ferricyanide for the determination of reducing sugars has materially increased, because this compound is relatively stable in alkaline solution and the reduction product, ferrocyanide, is not reoxidized as readily by dissolved oxygen as the copper salt.

Colorimetric methods for the determination of sugars usually involved the formation of a color compound by the action of the reduced metal ions on some reagents. Folin and Wu (*7*) adopted a phosphomolybdate reagent with cuprous oxide. Nelson (*10, 12*) used an arsenomolybdate solution for the color development in place of the phosphomolybdate in the estimation of the reduced copper. Colorimetric analysis of reducing sugars with the ferricyanide reagent was used by Hoffman (*8*), who measured the decrease in yellow color of the ferricyanide solution after oxidation. Folin (*6*) formed Prussian blue with ferricyanide reagent. The reduction of triphenyltetrazolium chloride has also recently been applied for the colorimetric estimation of reducing sugars by Mattson and Jensen (*9*) and Fairbridge, Willis, and Booth (*5*).

Selective determination of fructose in the presence of glucose generally depends on the choice of alkalizing agents and temperature of reaction bath used in the oxidation. Thus in Nyns' selective method for fructose as modified by Jackson and Matthews (*1*), carbonate and bicarbonate in connection with copper were used. With potassium ferricyanide, Englis and Becker employed a mixture of sodium carbonate and sodium phosphate buffer for the selective oxidation, and determined the reduction product titrimetrically with sodium thiosulfate (*2*) or ceric sulfate (*3*).

A colorimetric method for the determination of fructose in the presence of glucose was described by Englis and Miles (*4*) with the Folin-Denis phosphotungstate phosphomolybdate reagent, and by Mattson and Jensen, who used triphenyltetrazolium chloride.

The method described in the present paper is simple and employs the same reagents as suggested by Becker and Englis (*2*) for the determination of both total reducing sugars and fructose. The extent of reduction of the ferricyanide is determined colorimetrically. The simplicity of the method has an advantage in routine analysis.

Reagents

Alkaline Ferricyanide Solution. Dissolve 160 grams of anhydrous sodium carbonate and 150 grams of disodium phosphate heptahydrate in 850 ml. of distilled water, add 4 grams of potassium ferricyanide, and dilute to 1 liter.

Arsenomolybdate Solution. Dissolve 25 grams of ammonium molybdate tetrahydrate in 450 ml. of distilled water. Add 21 ml. of concentrated sulfuric acid, followed by 3 grams of disodium arsenate in 25 ml. of distilled water. Heat at 55° C. for 30 minutes in a water bath with constant stirring, or in an incubator maintained at 37° C. for 24 to 48 hours.

Sulfuric Acid Solution, 2*N*. Dilute 56 ml. of concentrated sulfuric acid (specific gravity 1.84) to 1 liter.

Sodium hydroxide solutions, 10*N* and 1*N*.

Hydrochloric acid, 1 to 1 by volume.

Procedure

For the determination of sugars, the solution should contain not more than 0.150 gram of reducing sugars per 100 ml. For all citrus juices a 1 to 50 dilution is usually sufficient. Five milliliters of the juice are placed in a 250-ml. volumetric flask and diluted to volume with distilled water.

Determination of Total Reducing Sugars. One milliliter of the dilute juice is transferred to a 100-ml. volumetric flask and 5 ml. of the ferricyanide reagent are added. Fifteen to 30 flasks can be placed on a single rack and immersed in a boiling water bath for 10 minutes. After heating, the flasks are quickly cooled in running water and the contents are partially neutralized with 10 ml. of 2*N* sulfuric acid solution. The contents of the flask are thoroughly mixed until no more gas is evolved. Four milliliters of the arsenomolybdate are then added. The contents of the flask are again mixed and diluted to volume. The absorbance of the solution is obtained from a photometer using a green filter (515 mμ). The zero setting on the photometer is made with a reagent blank run like the sample.

Determination of Fructose. In heating a mixture of glucose and fructose at a certain temperature—e.g., 55° C.—with the alkaline ferricyanide reagent, the rate of oxidation of glucose is considerably less than that of fructose. The result obtained is termed apparent fructose. This is slightly higher than the true fructose content in the sample, which can be calculated by using appropriate formulas. The procedure for the determination of this apparent fructose is the same as that for the total reducing sugars, except that the temperature and

duration of heating are changed to 55° C. for 30 minutes.

Determination of Total Sugars and Sucrose. The sucrose in the sample is inverted with hydrochloric acid. Fifty milliliters of the diluted juice are placed in a 150-ml. beaker and 10 ml. of hydrochloric acid (1 to 1) are added. After the beaker has stood at room temperature for 18 hours, 5 ml. of 10*N* sodium hydroxide solution are added and the contents are adjusted to a pH between 5 and 7 with 1*N* sodium hydroxide solution, using a pH meter. They are then transferred to a 100-ml. volumetric flask and diluted to volume. A 1-ml. aliquot of this solution is pipetted into a 100-ml. volumetric flask, and the procedure described for the determination of reducing sugars is followed. The amount of sucrose is calculated from the difference between the reducing sugar content before and after inversion, multiplied by the factor of 0.95.

Calibration

Spectral Characteristics and Standard Curves. The color of the solution after the addition of arsenomolybdate was studied by the means of a Beckman Model B spectrophotometer between 400 and 700 mμ, using a 1-cm. cell. The results are shown in Figure 1. The absorbance increased with increasing wave length, but did not reach a maximum within the range of wave length studied. By selecting a filter in the 500-mμ area, the range of sugar concentration determined can be increased. For this reason a 515-mμ filter was used with a Lumetron colorimeter Model 402E, using 1-cm. cells.

To construct the standard curves, known glucose and fructose solutions containing 0.020, 0.040, 0.060, 0.080, 0.100, 0.120, and 0.140 gram of each sugar per 100 ml. were used. Standard

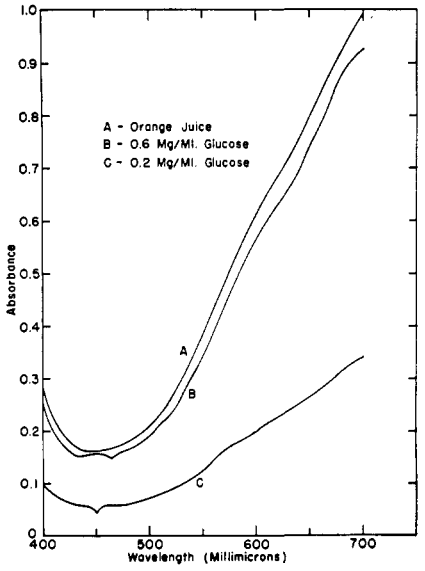


Figure 1. Absorption curves of color produced by reducing sugars with ferricyanide and arsenomolybdate reagents

cruves for glucose and fructose were made at both 100° and 55° C. (Figures 2 and 3). At 100° C. glucose and fructose have similar rates of oxidation, and the curves are identical. At 55° C. the values for fructose were about eight times higher than those for glucose of same concentrations. All curves were found to obey the Beer's law and to pass through the origin.

Calculation

Total Reducing Sugars. From the standard curve in Figure 2, the k value for the total reducing sugars is calculated by the formula:

$$k = c/a \qquad (1)$$

where k = factor for unit absorbance, or slope of curve
c = concentration in gram reducing sugars per 100 ml.
a = absorbance of solution at that concentration

The k values at different sugar concentrations are averaged and designated as K. The total reducing sugar content, S, of the sample is calculated from the formula:

$$S = K.\ A.\ D. \qquad (2)$$

where S = total reducing sugar concentration of sample, grams per 100 ml. of juice
K = average slope of curve
A = absorbance of sample
D = dilution factor

Fructose. The K_f and K_g values for fructose and glucose, respectively, oxidized at 55° C. are obtained from the standard curves of these two sugars in Figure 3, in the manner described for K. The ratio K_g to K_f or Q is used in the calculation of true glucose and fructose of the sample with the following simultaneous equations:

$$G + F = S \qquad (3)$$

$$G/Q + F = L \qquad (4)$$

where G = percentage glucose in sample
F = percentage fructose in sample
S = percentage total reducing sugar (sum of G and F)
L = percentage apparent fructose
Q = ratio between K_g and K_f (K_g/K_f)

In a mixture of glucose and fructose both sugars present are assumed to be fructose. The apparent fructose, L, is calculated from Formula 2, substituting L for S and K_f for K. Solving Equations 3 and 4 simultaneously, one obtains Formula 5 for percentage of glucose in the sample.

$$G = (S - L) \times \frac{Q}{Q - 1} \qquad (5)$$

Figure 2. Relationship of concentration of sugars and absorbance of color formed with reagents

Absorbance measured at 515 mμ after heating at 100° C. for 10 minutes

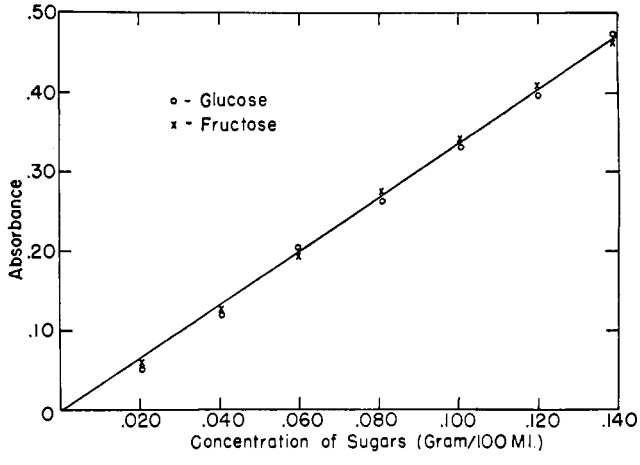
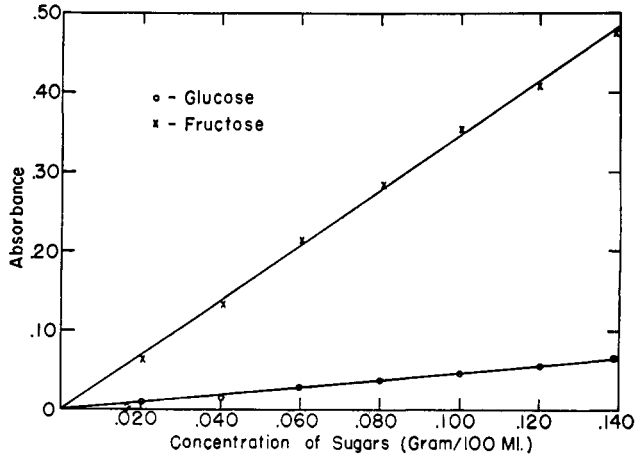


Figure 3. Relationship of concentration of sugars and absorbance of color formed with reagents

Absorbance measured at 515 mμ after heating at 55° C. for 30 minutes



The quotient, $Q/(Q-1)$, is used as a constant in calculations of true glucose value. True fructose value can be obtained by difference.

Experimental

Time of Heating. In order to determine the optimum time of heating for the oxidation of total reducing sugars by this procedure, 1 ml. of a 0.1% glucose solution and 5 ml. of the ferricyanide reagent were placed in each of twenty 100-ml. volumetric flasks. In each of another ten 100-ml. flasks were placed 1 ml. of distilled water and 5 ml. of the ferricyanide reagent to serve as blanks. The flasks were placed in a boiling water bath. At 2-minute intervals, duplicate samples and a blank were removed from the water bath. Similar runs were made using fructose. The results are shown in Figure 4. For complete oxidation of glucose, 8 minutes at 100° C. are required. Complete oxidation of fructose at that temperature occurred at about 6 minutes.

Table I. Recovery of Glucose and Fructose in Mixture

Sugars Added, Mg./Ml.		Sugars Found[a], Mg./Ml.	
Glucose	Fructose	Glucose	Fructose
0.10	0.90	0.11	0.91
0.20	0.80	0.20	0.81
0.30	0.70	0.32	0.69
0.40	0.60	0.42	0.59
0.50	0.50	0.52	0.49

[a] Average of duplicate determinations.

For determining the optimum heating time in the selective oxidation of fructose in the presence of glucose, temperatures of 65° and 55° C. with various time intervals were tried. At 65° C. the difference in the amounts of fructose and glucose oxidized narrowed as heating time was increased. The ratio of fructose oxidized to glucose oxidized changed from 3 to 1 after 30 minutes' heating to 2 to 1 after 90 minutes' heating. When heated for 120 minutes, the amounts of the two sugars oxidized were nearly equal. When the heating temperature was reduced to 55° C., fructose was completely oxidized in 30 to 40 minutes, while only $^1/_9$ to $^1/_8$ of the glucose had been oxidized. The amount of glucose oxidized increased with heating time, while that of fructose remained relatively unchanged or decreased slightly (Figure 5).

Determining Glucose and Fructose in Mixture. A series of sugar solutions containing glucose and fructose in various proportions to provide 0.100 gram of total reducing sugars per 100 ml. was prepared. Total reducing sugars and apparent fructose content were determined according to prescribed methods and the glucose and fructose were calculated. The results are shown in Table I.

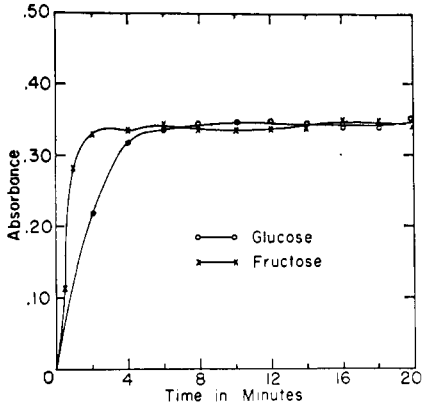


Figure 4. Effect of duration of heating at 100° C. on color development by glucose and fructose with reagents

Absorbance measured at 515 mμ

Comparison of Methods. In order to compare the proposed colorimetric procedure with one of the present methods for sugar determination the Shaffer-Hartmann volumetric method (*11*) was selected. The results of 12 duplicate reducing and total-sugar determinations on orange juices by the two methods are shown in Table II. A correlation coefficient of nearly 1 was obtained when all 48 pairs of analyses were used in the calculation. As seen in Table II, the results obtained with the colorimetric method were consistently higher than with the Shaffer-Hartmann procedure on sample before inversion, but differences in the results obtained by the two methods were not so significant after the samples had been inverted with hydrochloric acid.

Effect of Clarification with Neutral Lead Acetate. Lead acetate is generally used in the clarification of sugar solutions before analyses are made. It was advisable to ascertain whether the lead clarification process in any manner interferes with this colorimetric method of sugar determination. Orange juice samples were prepared in the manner described by diluting 5 ml. of juice to 250 ml. with distilled water. Five replicate samples were made from a single juice. In another test of five replicate samples 2 ml. of saturated neutral lead acetate solution were added before making to volume. The solutions were filtered, and excess lead in the filtrate was removed by incorporating it with an amount of dry sodium oxalate crystals, calculated to precipitate the lead. The lead oxalate was filtered off, and the solutions were used in the determinations. The results of the clarified and nonclarified samples are shown in Table III. It is evident that the clarification of juice with neutral lead acetate does not interfere with the determination of sugar by the colorimetric procedure.

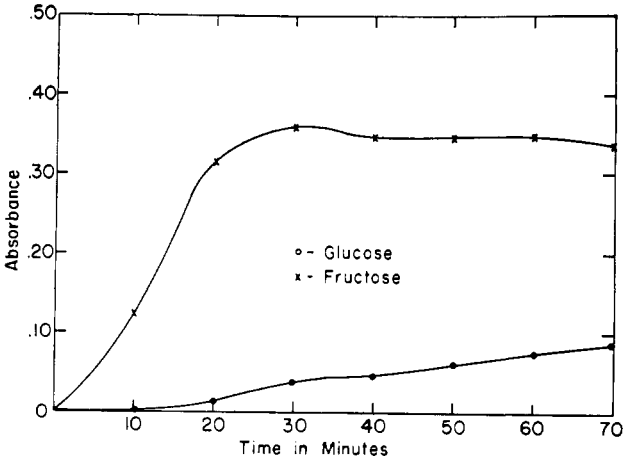
Discussion

The use of ferricyanide for the oxidation of sugars has an advantage over copper solution, as the ferrous ions are not easily oxidized by the atmospheric oxygen. Arsenomolybdate reagent forms a blue compound with the ferrous ions. This color is stable and may be determined in a colorimeter within 30 minutes after the reaction is completed, or may be left at room temperature over night; the changes in absorbance upon standing for 18 hours are about 2% (Table IV). The procedure may also be interrupted after the completion of oxidation with the ferricyanide reagent but before the addition of sulfuric acid, provided the reagent mixture is stored at 0° C.

The colorimetric determination of fructose in presence of glucose can be used advantageously in differentiating these two sugars in plant extracts, where they are the only two reducing sugars present in large quantities.

Figure 5. Effect of duration of heating at 55° C. on color development by glucose and fructose with reagents

Absorbance measured at 515 mμ



Because of the simplicity of this method, it lends itself readily to routine determination of sugar compositions in citrus juices. The analyst can adapt this method easily for the determination of sugars in the juice of other fruits or in other plant extracts.

The only known reducing substance in the citrus juice which would inter-

Table II. Determination of Sugar in 24 Samples of Orange Juice

Sample	Sugar Concentration, Grams/100 Ml.			
	Before Inversion		After Inversion	
	Colorimetric method	Shaffer-Hartmann method[a]	Colorimetric method	Shaffer-Hartmann method[a]
1	3.97	3.72	8.79	8.70
	4.00	3.75	8.79	8.74
2	4.17	3.97	9.11	9.26
	4.20	4.00	9.24	9.26
3	4.37	4.09	9.38	9.54
	4.36	4.09	9.32	9.54
4	4.57	4.30	9.54	9.68
	4.53	4.24	9.51	9.61
5	4.54	4.29	9.53	9.70
	4.53	4.33	9.53	9.72
6	4.54	4.28	9.53	9.78
	4.49	4.19	9.59	9.82
7	4.41	4.12	9.38	9.19
	4.34	4.01	9.40	9.05
8	4.63	4.46	9.80	9.25
	4.62	4.39	9.64	9.57
9	4.76	4.54	10.07	9.80
	4.74	4.60	9.91	9.74
10	4.78	4.60	10.15	10.07
	4.79	4.59	9.99	10.03
11	4.89	4.79	10.23	10.23
	4.89	4.66	10.12	10.25
12	4.93	4.89	10.18	10.19
	4.92	4.77	10.18	10.20
Correlation coefficients				
Before inversion	0.9818[b]			
After inversion	0.8944[b]			
Combined	0.9985[b]			

[a] As % invert sugars.
[b] Significant at 1% level.

Table III. Effect of Neutral Lead Acetate Clarification of Orange Juice on Colorimetric Method

Replicates	Total Reducing Sugars		Apparent Fructose	
	Clarified	Not clarified	Clarified	Not clarified
	Absorbance			
1	0.410	0.412	0.280	0.283
2	0.412	0.407	0.276	0.276
3	0.415	0.415	0.276	0.280
4	0.424	0.415	0.276	0.276
5	0.416	0.420	0.274	0.276

fere with this colorimetric procedure is ascorbic acid, which when present in amounts of 100 mg. per 100 ml. will give sufficient color to interfere. However, the ascorbic acid contents of various citrus juices vary between 25 to 60 mg. per 100 ml. The diluted juice used in present method contains such a small amount that its interference is negligible. The following substances known to occur in citrus fruits were tried with the reagents, but no color was formed: naringin, hesperidin, citric acid, arginine, asparagine, glutamic acid, aspartic acid, and aerine.

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Table IV. Absorbance at 515 Mμ at Intervals after Color Development

Time after Color Development, Min.	Absorbance (Duplicate Samples)	
0	0.370	0.370
10	0.370	0.372
30	0.377	0.375
60	0.380	0.380
18 hours	0.384	0.384

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