Determination of Fructose in the Presence of a Large Excess of Glucose

Part II. A Modified Anthrone-Sulfuric Acid Method and a Modified Phenol-Sulfuric Acid Method

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Modifications of the anthrone-sulfuric acid method and the phenol-sulfuric acid method are described. By employing the principle of two-point determination of Mokrasch and by modifying the conditions for color development, fructose in the presence of 100-fold excess of glucose can be determined with an error of about 15% by the modified anthrone-sulfuric acid method. The modified phenol-sulfuric acid method also gave the same order of sensitivity and specificity to fructose as the modified anthrone-sulfuric acid method.

The anthrone-sulfuric acid method has been used as a general color reaction of carbohydrates and on this basis has been employed as the method of choice for the determination of total sugars. However, as far as the author is aware, Koehler¹⁾ was the first who developed a method using anthrone-H₂SO₄ reagent for distinguishing fructose from glucose on the basis of different rates of color development and different color intensities of these two sugars. Since then, several workers^{2~5)} developed methods for the determination of fructose in the presence of glucose by controlling the conditions of color development so that fructose developed color while glucose remained inactive or reacted only to a small extent. Bonting⁶⁾ proposed an ultra-micro method by which color was developed in a

total volume of 75 μ l. This method permits the measurement of the smallest amount of fructose among various methods ever proposed, but requires a special micro technique and special apparatuses (a sensitive spectrophotometer and a micro-cell attachment), and is not applicable in ordinary laboratories. Moreover, since this method is based on color development by the heat evolved spontaneously during mixing of the reagents, the conditions (size and wall thickness of test tubes, the rate of mixing the components, *etc.*) should be followed rigorously in order to obtain reproducible results, and the total volume is not to be scaled up without re-examinations of the reaction conditions. Mokrasch⁷⁾ observed that fructose and glucose gave very different patterns of color development in the anthrone-H₂SO₄ method; *i.e.*, fructose gave maximum color (at 620 m μ) in 3 min at 80°C and then the color was decreased, whereas glucose gave the highest color at about 25 min at the same temperature. Therefore, by measuring the color of fructose, glucose and the mixture of

1) L. H. Koehler, *Anal. Chem.*, **24**, 1576 (1952).

2) J. R. Helbert and K. D. Brown, *ibid.*, **27**, 1791 (1955).

3) M. Wenke and J. Labská, *Časopis lékařů českých*, **93**, 23 (1954) [*C. A.*, **48**, 12862e (1954)].

4) C. S. Wise, R. J. Dimler, H. A. Davis and C. E. Rist, *Anal. Chem.*, **27**, 33 (1955).

5) M. A. Jermyn, *Nature*, **177**, 38 (1956).

6) S. L. Bonting, *Arch. Biochem. Biophys.*, **52**, 272 (1954).

7) L. C. Mokrasch, *J. Biol. Chem.*, **208**, 55 (1954).

the two sugars at 3 and 25 min, the contents of fructose and glucose in the mixture can be obtained by calculation. Moreover, this method seemed to be one of the most sensitive methods based on anthrone thus far proposed in the literature. Thus, this method was employed as the basis of a further study. By employing Mokrasch's principle of two-point determination and lowering the reaction temperature to 50°C, fructose in the presence of 100-fold excess of glucose can be determined with an error of about 15%.

The phenol-sulfuric acid method of DuBois *et al.*⁸⁾ has also been widely used as a method for the determination of total sugars. However, this method has never before been used in the preferential determination of fructose in the presence of glucose. In view of the success obtained with the modified anthrone-sulfuric acid method, conditions of this method were re-examined in order to find out a suitable procedure for our purposes (see the preceding paper⁹⁾). Because the results obtained were comparable to but not more promising than the modified anthrone method, this method was not investigated further. However, some data obtained during this investigation are presented here so that some investigators interested in this reaction can utilize these data as a basis for their further studies. One advantage of this method is that the reagent, phenol, is much less expensive than anthrone.

It is reported that fructose also reacts with a mixture of phenol, acetic acid and sulfuric acid to give a green color. Based on this color reaction, Maurmeyer *et al.*¹⁰⁻¹²⁾ developed a

qualitative test as well as a quantitative method for fructose.

After this work was completed, Handel¹³⁾ used anthrone-H₂SO₄ reagent at room temperature to determine successfully fructose in the presence of 10-fold excess of glucose by this procedure.

EXPERIMENTAL

Reagents. Anthrone reagent is prepared by dissolving 100 mg of anthrone (Tokyo Kasei Co.) in cold diluted H₂SO₄ which has been prepared by adding 100 ml of conc. H₂SO₄ to 29 ml of distilled water under cooling in running tap water. This reagent is usable for more than one month when stored in a dark bottle in a refrigerator. Phenol reagent is prepared by mixing 80 g of redistilled phenol with 20 g of distilled water. This reagent is stable for a long time in a refrigerator, and on color change to pink, is discarded and prepared fresh.

Other details are described in the preceding paper.⁹⁾

RESULTS AND DISCUSSION

A. Anthrone-sulfuric acid method.

Principle of the method. Small test tubes (14 mm *i.d.* × 100 mm) containing 3.6 ml of the anthrone reagent are cooled in ice water and to each tube is added, slowly and without mixing, 0.6 ml of a sugar solution. After the tubes are incubated for a further few minutes in ice water, the tubes are shaken and then transferred to a water bath kept at a constant temperature. After the incubation for various time intervals, the tubes are cooled in running tap water and the absorbances at 625 mμ are read in a spectrophotometer.

Figure 1 shows the time course of color development when 30 μg each of fructose and/or glucose are used as the sugar and the incubations are at 80 ± 1°C (as specified by Mokrasch⁷⁾). It is evident from the figure that fructose gives maximum color at 2~5 min and then the color decreases slowly with time. On the other hand, glucose color is maximum at 20~30 min. A mixture of fructose and glucose gives a progress curve which

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11) R. K. Maurmeyer, E. M. Livingston, R. Michaels, *Mikrochim. Acta*, **1956**, 1600 [*C. A.*, **51**, 2475h (1957)].

12) E. M. Livingston, R. K. Maurmeyer and A. Worthman, *Microchem. J.*, **1**, 261 (1957) [*C. A.*, **52**, 2664d (1958)].

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FIG. 1. Time Course of Color Development in the Modified Anthrone-Sulfuric Acid Method.

The reaction mixture contained 3.6 ml of the anthrone-sulfuric acid reagent and 0.6 ml of a sugar solution containing fructose (F) and/or glucose (G) as indicated in the figure. The components were mixed in ice-water and then heated in a water bath at $80 \pm 1^\circ\text{C}$ for various times.

is almost identical to the sum of fructose and glucose curves.

In the method of Mokrasch, t_1 and t_2 were 3 and 25 min, respectively. Because D_{t_1} and D_{t_2} may equal to the sum of colors of fructose and glucose at respective heating times, then we obtain

$$D_{t_1} = C_F \cdot E_F + C_G \cdot E_G \cdot k_G$$

and

$$D_{t_2} = C_F \cdot E_F \cdot k_F + C_G \cdot E_G$$

Solving these equations, we obtain

$$C_F = (D_{t_1} - k_G \cdot D_{t_2}) / E_F (1 - k_F \cdot k_G) \quad (1)$$

and

$$C_G = (D_{t_2} - k_F \cdot D_{t_1}) / E_G (1 - k_F \cdot k_G) \quad (2)$$

where

E_F : Absorbance at $625 \text{ m}\mu$ obtained from $1 \mu\text{mole}$ of fructose at time t_1

E_G : Absorbance at $625 \text{ m}\mu$ obtained from $1 \mu\text{mole}$ of glucose at time t_2

k_F : Ratio of absorbances at times t_2 and t_1 for fructose

k_G : Ratio of absorbances at times t_1 and t_2 for glucose

D_{t_1} and D_{t_2} : Absorbances of the sample at times t_1 and t_2 , respectively

C_F : μmoles of fructose in the 0.6 ml of the sample

C_G : μmoles of glucose in the 0.6 ml of the sample

Time course of color development at various temperatures. A survey of the literature shows that there is some variations in the concentrations of reagents among many methods based on the anthrone- H_2SO_4 color reaction; especially, the temperature and time of heating. For example, the conditions used and millimolar absorbance values ($\epsilon \times 10^{-3}$)* (given in parenthesis) obtained by various investigators are: Koehler,¹¹ 1~2 min at 98°C (1.83); Helbert and Brown,²¹ 7 min at 60°C (13.0); Wenke and Labská,³¹ 8 min at $50 \sim 51^\circ\text{C}$; Wise *et al.*,⁴¹ 20 ± 0.1 min at $50 \pm 0.5^\circ\text{C}$ (15.7); Jermyn,⁵¹ 20~60 min at room temperature (17°C) (0.94 at 10 min and 2.26 at 60 min); Bonting,⁶¹ 10~50 min at room temperature (8.37); and Mokrasch,⁷¹ 3 min at 80°C (21.7). Thus, these workers searched for the condition under which fructose color is nearer to the maximum and, at the same time, glucose color is nearer to the minimum. The two-point determination of Mokrasch presumes that these two requirements will never be satisfied simultaneously. Therefore, in principle, the condition of color development is not important, provided there is no error in the determinations. But, in practice, small errors in the determinations are inevitable, and they influence seriously on subsequent calculations. Therefore, in order to find out

* The millimolar absorbance values given here were calculated by the author from the data given in the literature.

FIG. 2. Time Course of Color Development in the Modified Anthrone-Sulfuric Acid Reaction at Various Temperatures.

Experimental details are the same as given in the legend to Fig. 1, except the reaction mixture was heated in a water bath maintained at various temperatures as indicated in the figure. The figures in parentheses on each fructose curve shows the color intensity ratio of fructose to glucose at the time of maximum fructose color at each temperature.

a condition under which errors in the determinations will have minimum influence on subsequent calculations, it seems necessary to compare the time course of color development by both fructose and glucose at various temperatures.

Figure 2 shows the time course of color development by fructose and glucose at various temperatures between 40 and 80°C. When the temperature is varied, the time course differs to a remarkable extent, especially with glucose. The time course of the color intensity ratio of fructose to glucose, as calculated from the data of Fig. 2, is shown in Fig. 3. Since there is a remarkable difference between the rates of color development of both sugars at various temperatures, the color intensity ratio at the time of the maximum fructose color varies with the temperature; the larger is the ratio, the lower the temperature. Because the maximum color of fructose is practically constant between 50–80°C, a lower temperature is to be preferred in order to more satisfy

FIG. 3. Time Course of the Color Intensity Ratio of Fructose to Glucose at Various Temperatures.

Calculated from the data of Fig. 2. The point on each curve surrounded by a circle indicates the time when the fructose color is at the maximum.

the two requirements of sensitivity and specificity. At 40°C, the rate of color development is too slow.

When the method of Mokrasch is examined with the above results in mind, apparently glucose may develop an appreciable color even after heating for 3 min at 80°C. Therefore, when a sample solution contains a large excess of glucose over fructose, glucose color will represent a greater portion of the sample color even at 3 min. Mokrasch selected the 3 min and 25 min heating times at 80°C because his aim was to determine both fructose and glucose simultaneously. In order to determine fructose content preferentially and as accurately as possible, it would be better to develop color at 50°C and select two times: one at the maximum fructose color, and the other an appropriate time for the convenience

TABLE I. DETERMINATION OF FRUCTOSE IN THE PRESENCE OF 100-FOLD EXCESS OF GLUCOSE BY THE MODIFIED ANTHRONE-SULFURIC ACID METHOD

Experiment No.	E_F	E_G	k_F	k_G	Calculated		F.***
					F.	G.	
1	3.42	0.150	0.977	0.387	8.30	920	25.3
2	3.31	0.148	0.940	0.388	8.70	1010	26.2
3	3.34	0.151	1.03	0.381	9.80	966	26.4
4	3.50	0.153	1.07	0.393	8.85	1027	25.6
5	3.44	0.153	1.04	0.379	9.43	976	26.0
6	3.71	0.157	0.965	0.392	8.55	990	24.9
7	3.52	0.149	0.995	0.400	10.1	947	26.2
8	3.68	0.162	1.03	0.410	10.0	945	26.9
9*	3.51	0.255	1.05	0.284	11.7	865	29.6
10**	3.74	0.186	1.02	0.356	9.97	913	26.0

The reaction mixture contained 3.6 ml of the anthrone reagent and 0.6 ml of a sugar solution containing either 10 μ g of fructose or 1.0 mg of glucose, or a mixture of 10 μ g of fructose and 1.0 mg of glucose. The components were mixed in ice water and heated in a water bath at $50 \pm 0.1^\circ\text{C}$ for 15 and 40 min. Absorbance was read against a reagent blank prepared with distilled water in place of a sugar solution. See the text for symbols.

* Heating times (t_1 and t_2) were 15 and 60 min, respectively.

** Heating times (t_1 and t_2) were 14 and 40 min, respectively.

*** Calculated by the usual one-point determination principle (absorbance was measured after heating for 15 min).

of the experiment and not necessarily the time at the maximum glucose color.

From the results of Figs. 2 and 3 and other similar experiments, it seemed appropriate to select 15 and 40 min heating times at 50°C . Table I shows the results of 10 such experiments in which a mixture of 10 μ g of fructose and 1.0 mg of glucose per 0.6 ml of the sample solution was assayed by the above procedure. Although the time course of color development as well as the absolute color intensity of fructose or glucose varies more or less from experiment to experiment, fructose in the presence of 100-fold excess of glucose may be determined with an error of about 15%. The last column of Table I shows the results obtained when the fructose content was determined by the principle of one-point determination; *i. e.*, the fructose content was obtained from the absorption value of the sample tube at 15 min heating by consulting with a calibration curve of fructose. The advantage of the two-point determination is obvious when the results obtained by the two methods are

compared with each other.

Recommended procedure. Eight small test tubes containing 3.6 ml of the anthrone reagent are incubated in ice-water and to two tubes each are added 0.6 ml of distilled water, a standard fructose solution, a standard glucose solution, and the sample solution, respectively. The tubes are incubated in ice-water for a further few min, shaken to mix the components, and then transferred to a water bath at $50 \pm 0.1^\circ\text{C}$. After 15 and 40 min heating, 4 tubes each from the different series are removed and transferred back to the ice-water bath. Absorbances of the sugar tubes at 625 $m\mu$ are read against the reagent blank tube prepared with distilled water. Fructose and glucose contents are calculated from equations (1) and (2) given in the text, in which t_1 and t_2 represent 15 and 40 min, respectively.

B. Phenol-sulfuric acid method.

Time course of color development with fructose and glucose at various temperatures. Small test tubes containing 3.0 ml of conc. H_2SO_4 are incubated in an ice-water bath for at least

5 min. To each tube are added, slowly and without mixing, 1.2 ml of a sugar solution and 0.03 ml of 80% phenol reagent. The tubes are incubated in ice-water for a further few min, shaken well to mix the components, and then left at room temperature (27~28°C). During mixing of the components, there was some evolution of heat, although the components had been sufficiently cooled in ice-water. There was also a slightly yellow color appearing in the mixed solution, but this color faded after a few min. The absorption spectra for fructose and glucose both gave absorption maxima at 490~494 m μ . Figure 4 shows the time course of color development as observed at 490 m μ . The color development with fructose was rather rapid during the first hour, then slowed down and remained practically constant after the second hour, while the glucose color increased continuously for at least 4 hr. The time course of color develop-

FIG. 4. Time Course of Color Development in the Phenol-Sulfuric Acid Reaction.

The reaction mixture contained 3.0 ml of conc. H_2SO_4 , 1.2 ml of a sugar solution containing fructose (F) and/or glucose (G) as indicated in the figure, and 0.03 ml of 80% aq. phenol. The components were mixed in ice-water, and then left at room temperature (27~28°C) for various times as indicated in the figure.

TABLE II. TIME COURSE OF COLOR DEVELOPMENT IN THE PHENOL-SULFURIC ACID REACTION AT VARIOUS TEMPERATURES

		Time in minutes													
		2	4	6	8	10	15	20	25	30	40	60	120	180	240
F.	80°	20.8	23.6	22.6											
	70°	20.7	23.3	24.4	24.4	23.4	22.2								
	60°		21.0		22.4		23.6	23.1		22.7					
	50°					22.2		23.0	24.3	24.2	24.1	24.1			
	40°					19.1		21.1		24.1	24.2	24.3	26.2		
	Room temp. (27~28°C)					12.6		16.6		19.1	20.7	22.9	25.5	26.1	26.7
G.	80°	4.05	10.9	11.9											
	70°	3.24	8.50	10.3	10.4	10.8	11.3								
	60°		3.46		6.74		8.84	9.11		9.53					
	50°					1.78		3.19	3.43	4.61	4.99	5.92			
	40°					1.30		1.03		1.48	2.36	1.89	2.81		
	Room temp. (27~28°C)					0.503		0.647		0.785	0.861	1.07	1.53	1.83	2.08
Ratio, F./G.	80°	5.14	2.16	1.90											
	70°	6.39	2.74	2.37	2.34	2.17	1.97								
	60°		6.07		3.33		2.67	2.54		2.39					
	50°					12.5		7.22	7.00	5.25	4.83	4.07			
	40°					14.7		20.5		16.3	10.2	12.8	9.34		
	Room temp. (27~28°C)					25.0		25.7		24.4	24.0	21.4	16.7	14.3	12.8

The reaction mixture contained 3.0 ml of conc. H_2SO_4 , 1.2 ml of a sugar solution containing an appropriate amount of either fructose or glucose (depending on the temperature used), and 0.03 ml of 80% aq. phenol. The components were mixed in ice-water and transferred to a water bath maintained at the specified temperature for various times. The absorbance was expressed in terms of millimolar absorbance ($\epsilon \times 10^{-3}$) units.

TABLE III. COLOR DEVELOPMENT IN A MODIFIED PHENOL-SULFURIC ACID REACTION

See the text for a general procedure.

Expt. No.	Sugar soln. ml	80% Phenol ml	75 vol % H ₂ SO ₄ ml	Temp. of mixing	Color development	$\epsilon \times 10^{-3}$		Ratio F./G.
						F.	G.	
1	1.2	0.03	3.0	Room temp.	100°C, 2.5 min	3.70	0.10	37.0
2	1.2	0.03	3.0	Ice-water	60°C, 10 min	2.79	0.017	169
					80°C, 7 min	5.46	0.132	41.4
3	0.6	0.03	3.0	Ice-water	60°C, 5 min	5.99	0.055	109
					8 min	9.15	0.086	106
					10 min	10.4	0.137	75.5
					15 min	12.45	0.224	55.7
4	0.6	0.03	3.0	Ice-water	80°C, 3 min	12.5		
					4 min		0.535	
					6 min	14.3	0.990	14.5
					9 min	13.6	1.83	7.45
					12 min	12.4	2.36	5.26
					15 min	11.1	3.10	3.59

ment with fructose and glucose and the color intensity ratio of these two sugars at various temperatures are shown in Table II. As is evident from the table, the sensitivity of this modified procedure for fructose is about twice as high as the original method ($\epsilon \times 10^{-3} = 11.3$). However, this method also gives a higher color intensity to glucose, thus leading to a lower color intensity ratio (*i.e.*, a lower specificity for fructose) when compared to similar results obtained by the modified anthrone-H₂SO₄ method.

A modified procedure using 75 % sulfuric acid. In order to eliminate the heat evolved spontaneously during dilution of the conc. H₂SO₄ reagent referred to in the preceding section, the concentration of the sulfuric acid reagent was reduced to 75%, because it was probable that the heat was responsible to the high

glucose color obtained. Table III shows the results of some experiments in which fructose and glucose were treated under various conditions. When the volume ratio of 75% H₂SO₄ and the sugar solution was kept the same as in the original method, the color intensity obtained with fructose was very low, owing to the reduced concentration of sulfuric acid in the final reaction mixture. When the volume of the sugar solution was reduced to 1/2 in order to keep the final sulfuric acid concentration as high as possible, the sensitivity and specificity to fructose obtained was comparable to those in the modified anthrone-sulfuric acid method. But, because this method did not seem to be more promising than the modified anthrone-sulfuric acid method, it was not investigated further.