0273-2289/95/5303—0207$05.60

A Modification of the Phenol/Sulfuric Acid Assay for Total Carbohydrates Giving More Comparable Absorbances

KIMBERLEY A. C. C. TAYLOR

Department of Animal and Poultry Science, University of Guelph, Guelph Ontario, Canada N1G 2W1

Received December 7, 1993; Accepted August 22, 1994

ABSTRACT

The conditions and acid strength of the phenol/sulfuric acid assay were investigated to improve agreement between absorbances obtained from different sugars. It was found that by increasing acid strength and by cooling the tubes in water after a short reaction time, the values obtained for several sugars, including fructose and xylose, agreed, on an equimolar value, with that for glucose.

Index Entries: Sugar analysis; carbohydrate; colorimetric assay; phenol-sulfuric acid.

INTRODUCTION

Perhaps the easiest and most well-known colorimetric assay for sugars is that devised by Du Bois et al. (*1*) utilizing phenol and sulfuric acid. The assay involves mixing sugar, water, and phenol, then adding sulfuric acid, and allowing the heat of reaction to drive formation of the absorbing compound. The assay is simple and universally accepted, requiring only chemicals, tubes, and a spectrophotometer. In this laboratory, the assay has been used to determine sugar monomers and complex soluble carbohydrates. With care in the suspension, the assay can even be used to quantitate a crystalline cellulose, such as Avicel.

One drawback of the assay is the variable absorbance response to different sugars. This can sometimes make determining true sugar content difficult in mixtures or in biological samples.

Rao and Pattabiraman (2) have described a modification that reduces the variability of the assay toward different sugars, while giving an increase in sensitivity. Their method relies on avoiding sulfonation of the phenol by adding it after the sugars have been converted to furfurals and lowering the heat of reaction (2,3). When this modification was used in this laboratory, the reaction of xylose was still found to be almost double that of glucose. Since we wished to use this assay to determine sugar contents in animal feeds that contain hemicelluloses, pectins, and cellulose, we wished to obtain a xylose absorbance closer to that for glucose to avoid having to estimate the sugar compositions of the samples. An investigation of the assay conditions was undertaken to determine if this variation could be further reduced.

MATERIALS AND METHODS

Chemicals Used

Concentrated H_2SO_4 (96%) and liquid phenol were from Fisher Scientific. All other chemicals were from Sigma.

Standard Method

The Rao and Pattabiraman (2) method (R-P) is as follows. One milliliter of water with the sugar to be assayed is placed in a 16 × 150 mm glass test tube. Three milliliters of concentrated sulfuric acid (resulting mix defined as 75% acid) are added, and the solution cooled to room temperature. Fifty microliters of 90% phenol are added, and the tubes allowed to stand at room temperature for 30 min. They are then read at 480 nm on a spectrophotometer.

The method above was modified as follows. Water (0.8 mL) and sugar are added to a 16 × 150 mm glass test tube; 3.2 mL of concentrated sulfuric acid (defined as 80% acid) are added and the tubes mixed quickly. The tubes are allowed to reach the maximum reaction temperature, for at least 1 min. They are then cooled to room temperature using a water bath to reduce the temperature quickly. Fifty microliters of 90% phenol are then added, and the tubes allowed to stand at room temperature for 30 min. The absorbance is read at 480 nm.

RESULTS AND DISCUSSION

Using the R-P method to determine the standard curves of glucose, fructose, and xylose gave the results shown in Fig. 1. Glucose and fructose

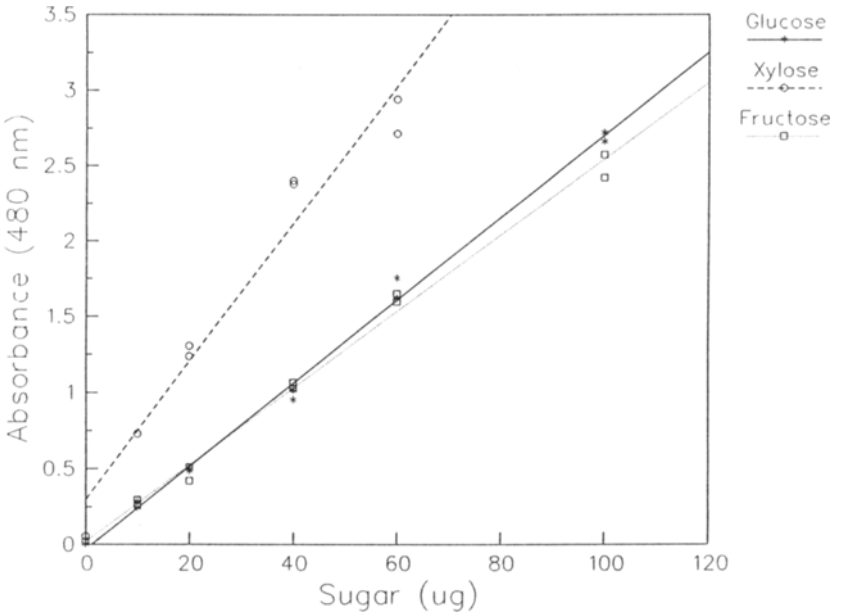

Fig. 1. Standard curves for glucose (solid line), fructose (dotted line), and xylose (dashed line) using the R-P method. In this case, the tubes were cooled in water after acid was added to the water and sugar, and the reaction was allowed to proceed for 10 min. The absorbance was read after the phenol was added and the tubes were held at room temperature for 2 h.

Table 1
Conversion of Sugars to Furfurals at Various Temperatures

	Sugar, A_{480nm}					
	Glucose		Xylose		Fructose	
Temperature	10 min	20	10 min	20	10 min	20
Room temp.	0.125	0.144	0.285	0.296	0.625	0.660
40°C	0.132	0.196	1.064	1.458	0.949	0.975
60°C	0.311	0.465	1.758	1.653	0.974	0.867
95°C	0.633	0.482	1.595	1.524	0.746	0.491
STD R-P[a]	0.744		1.587		0.715	

[a]Rao and Pattabiraman (2) method.

were close in their absorbances, but xylose showed a much higher absorbance. At 20 μg of each sugar, fructose was 94%, and xylose was 259% of the glucose absorbance. The chromagen was quite stable, showing 97, 95, and 87% of full absorbance for 20 μg glucose, xylose, and fructose, after standing at room temperature overnight.

Table 1 shows the results of incubating the sugar and acid at different temperatures. Acid and water (75% acid) were mixed and cooled to room

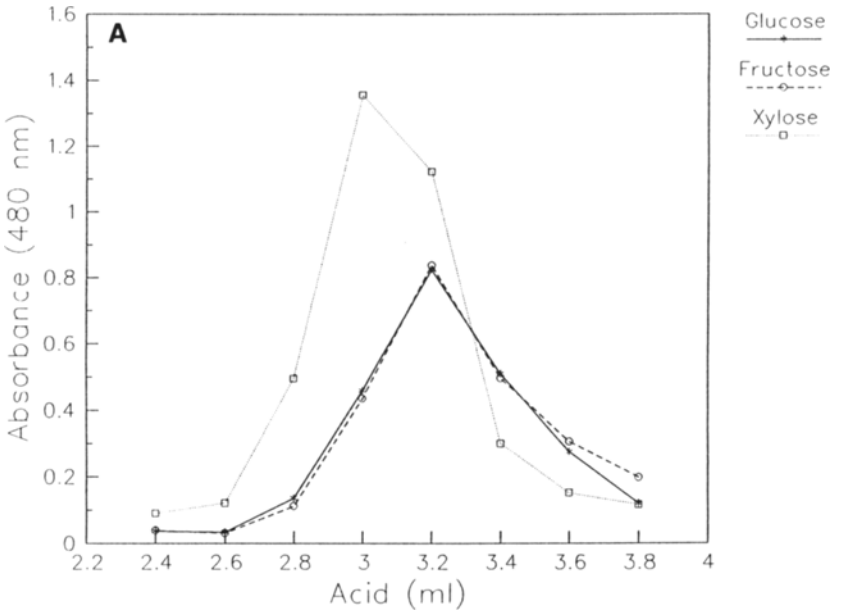

Fig. 2 (*caption on following page*).

temperature, sugar was added, and then the mixture was incubated for 10 or 20 min at various temperatures. The assay was compared to the standard R-P method in which the only heat was from the reaction of acid with water. After heating, all tubes were cooled in water, and absorbances were taken 30 min after addition of 50 μL 90% phenol. The best agreement of sugar absorbances was found using the standard R-P method. Xylose and especially fructose seemed to be more reactive than glucose, their absorbances peaking at 60°C and then declining at higher temperatures, presumably because of the destruction of the sugar. A similar lowering of absorbance was often seen when comparing 10- to 20-min assays at the same (higher) temperatures. In contrast, glucose absorbances rose with both increasing incubation temperatures and in most cases, times, indicating that this sugar is more resistant to conversion by acid and/or more stable once in its furfural forms. It was theorized that a short high-temperature treatment might be the most appropriate reaction condition, at least for glucose. A change in the acid content of the assay might also be useful.

Figure 2 (A–C) shows the effect of changing acid concentration on the final absorbance for three sugars. For the data in Fig. 2A (mixing), the sugar was present when the acid was added to the water and the heat generated allowed to drive the reaction. For the assays in Fig. 2B (boiling), the water and acid were premixed and cooled to room temperature before adding the sugar and incubating in a boiling water bath for 10 min. The effect of a prolonged, high incubation temperature was investigated and is reported in Fig. 2C (combined heating). Acid was added to the sugar and water, the tubes allowed to reach maximum temperature, then placed

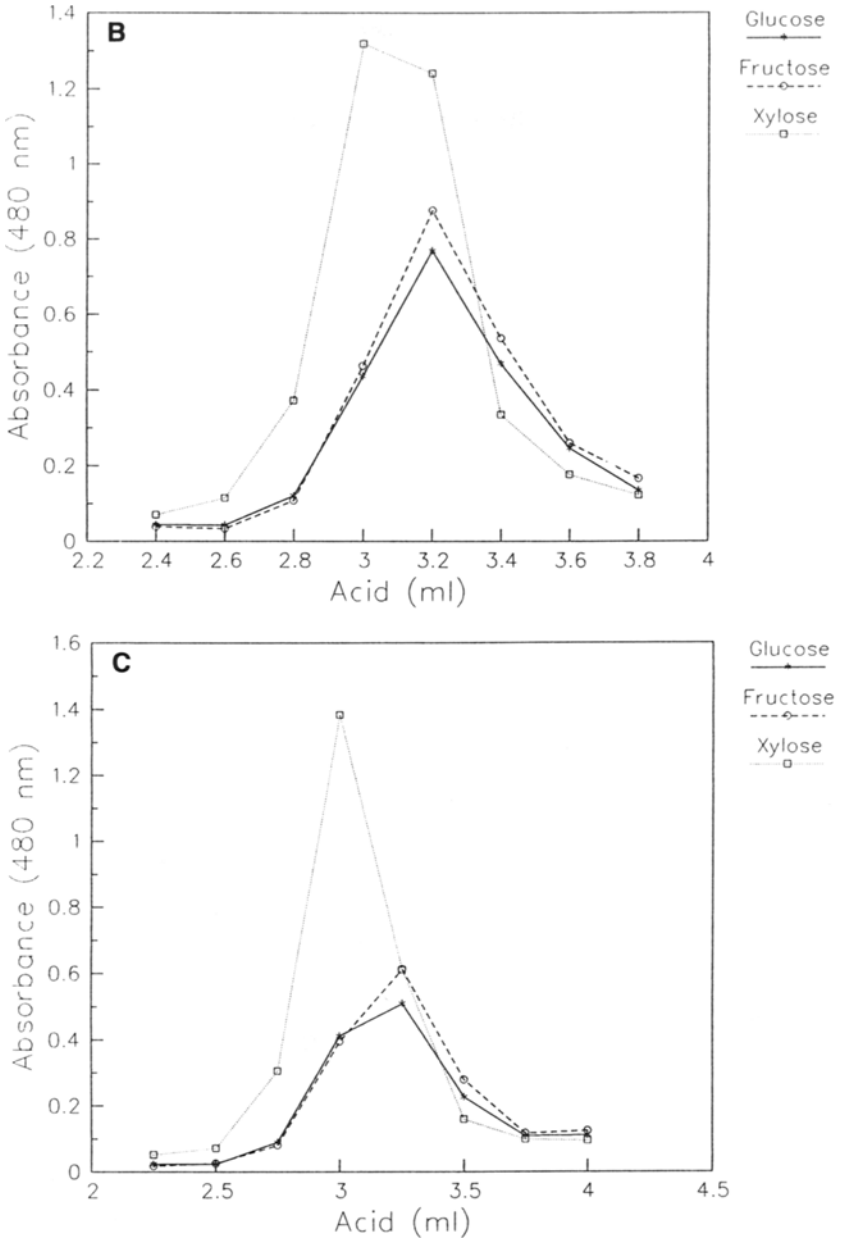

Fig. 2. (**A**) The effect of varying acid concentration on absorbance for 20 μg of glucose (solid line), fructose (dotted line), and xylose (dashed line). Acid was added to appropriate levels of sugar and water to make up 4-mL vol. The tubes were allowed to reach their maximum temperature, cooled in a water bath to room temperature, and then left at room temperature for 30 mins after phenol (50 μL of 90%) addition. The absorbance at 480 nm was recorded. (**B**) The assay conditions were the same as for Fig. 2A with the exception that acid and water were premixed and cooled, the sugars added at room temperature, and the tubes then boiled for 10 min. (**C**) The assay was performed as per Fig. 2A with the exception that the tubes were boiled for 10 min after the acid was added to the sugar and water, and allowed to go to maximum temperature.

Table 2
Effect of Initial Temperature and Method of Cooling on Conversion of Sugars to Furfurals

Preheat[a]	Cool	Sugar, A_{480nm}		
		Glucose	Xylose	Fructose
For 75% Acid				
Y	Air	0.542	1.517	0.400
Y	Water	0.161	1.528	0.592
N	Air	0.521	1.447	0.513
N	Water	0.597	1.375	0.575
For 80% Acid				
Y	Air	0.690	0.683	0.640
Y	Water	0.694	0.783	0.697
N	Air	0.722	0.711	0.608
N	Water	0.736	0.870	0.738

[a]Preheated in boiling water bath prior to addition of acid; Y, yes; N, no.

in the boiling water bath for a further 10 min before being cooled in water to room temperature and the phenol added. The mixing system, used in Fig. 2A, in addition to being the simplest method, showed the closest agreement at all acid levels between glucose and fructose absorbances. Possible reasons for this have already been discussed.

In Fig. 2A, the xylose, glucose, and fructose absorbances were equal at 3.32 mL (83%) acid. This equivalence point was for equal weights of the sugars. When the sugars were compared on an equal molar basis, the xylose, being 120% (by moles) compared to the hexose sugars, showed 120% of the glucose absorbance at 3.2 mL (80%) acid. This acid concentration also allowed maximum sensitivity to the hexose sugars. As a result, an acid concentration of 80% was deemed most suitable for the assay, giving maximum sensitivity and most equal absorbance for equimolar sugar concentrations.

The effect of an initial high-temperature infusion and the method of cooling the tubes after converting the sugars to their furfural forms were investigated and are reported in Table 2. For the elevated initial temperature infusion, tubes containing sugar and water were preheated in a boiling water bath before acid was added. After all tubes had reached their maximum temperatures (at least 1 min after acid addition), some were cooled rapidly in water, and some set aside for 30 min to air-cool. After all tubes reached room temperature, 50 μL 90% phenol were added and the tubes left standing for 30 min before reading the absorbance at 480 nm.

Rao and Pattabiraman (2) state that the assay tubes may be cooled in a water bath or in the air before adding phenol. In this experiment, the effects of air or water cooling were variable in the 75% acid (R-P) assay.

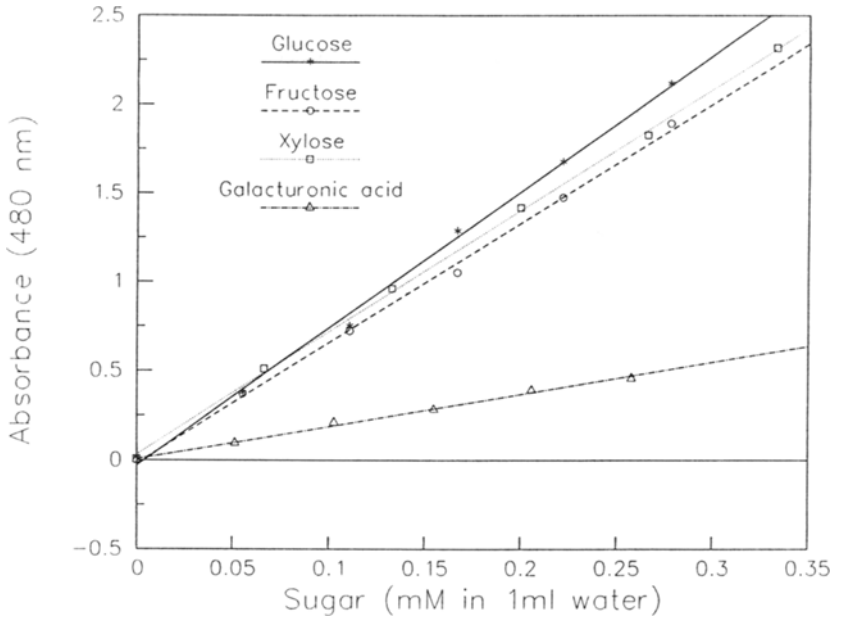

Fig. 3. Standard curves for glucose (solid line), fructose (dashed line), xylose (dotted line), and galacturonic acid (dots and dashes). Eighty percent acid assay performed as described in Materials and Methods.

When the 80% acid assay tubes were rapidly cooled (in water), the absorbances tended to be higher for xylose and fructose, but stayed the same for glucose.

Preheating the tubes gave inconsistent results, quite likely owing in part to the violent reaction that occurred on addition of acid. Quantities of the reacting substances can be lost as reagents sometimes boil out of the tubes. Even when this is controlled, the higher temperatures could destroy or volatilize the furfural, causing unpredictable losses.

Using 80% acid and water cooling, the xylose values match those for glucose on an equimolar basis, whereas for 75% acid (R-P), the xylose is two to three times as high as glucose for the same quantities of sugar. Overall, in the 80% acid assay, glucose and fructose showed absorbances 1.29-fold stronger than those of the 75% assay, whereas xylose showed a relative value of 59%.

The R-P assay was thus modified by increasing the acid content and using stricter temperature control. The assay is still simple, and requires minimal equipment and time.

Figure 3 shows the standard curves obtained for glucose, xylose, fructose, and galacturonic acid using the new method. All sugars show good linearity, with the hexoses and pentoses showing equal absorbance for equimolar concentrations. The uronic acid sugar shows reduced reactivity in the assay.

Table 3
Reactivity of Other Carbohydrates Using the 80% Acid Assay

Sample	A_{480nm}	Relative to glucose = 100
D-Glucose	0.745	100
D-Fructose	0.655	89
D-Xylose	0.979	131
D-Galacturonic acid	0.161	22
Myoinositol	0.0	0
L-Fucose	0.0	0
L-Rhamnose	0.005	1
D-Cellobiose	0.730	98
Soluble starch	0.681	91
Carboxymethyl cellulose	0.515	69
Oat spelt Xylan	0.870	117
Citrus pectin	0.216	29

Several carbohydrates (equal weights) were tested using the new assay, and the results are reported in Table 3. Three sugars showed no reaction, myoinositol, a cyclitol, L-fucose (6-deoxy-L-galactose), and L-Rhamnose (6-deoxy-L-mannose). The modified glucose residues contained in carboxymethylcellulose may have acted to reduce the absorbance (69%) of this compound. As expected, xylose and xylan both show higher absorbances than glucose. Pectin and galacturonic acid showed similar, low absorbances.

Sodium tetraborate added to the assay at 10 mg/mL was also examined in an effort to increase the reactivity of uronic acids. Most sugars showed no effect of borate ions, whereas the uronic acid sugars showed only a slight increase in absorbance.

REFERENCES

1. Du Bois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956), *Anal. Chem.* **28,** 350–356.
2. Rao, P. and Pattabiraman, T. N. (1989), *Anal. Biochem.* **181,** 18–22.
3. Rao, P. and Pattabiraman, T. N. (1990), *Anal. Biochem.* **189,** 178–181.