A Manual Method for Reducing Sugar Determinations with 2,2′-Bicinchoninate Reagent

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A method was developed to analyze reducing sugars manually by use of a 2,2′-bicinchoninate reagent. Potassium phosphate was used to buffer the reagent. Ethylene glycol increased the sensitivity of the assay for several sugars. Sugar determinations can be performed in the presence of borate ion. In comparative assays of a number of monosaccharides and disaccharides, the bicinchoninate reagent was about as sensitive as the Nelson reagent but more convenient to use. The effects of ethylene glycol concentration, reagent pH, heating time, and borate ion on color development were evaluated.

Mopper and Gindler (1) developed an automated colorimetric method to detect reducing sugars in column effluents with a 2,2′-bicinchoninate reagent. Sinner and Puls (2) modified the reagent so it could be used for sugar analysis in borate buffer. During investigations on polygalacturohases, unexplained variations in the intercepts of reaction curves occurred when the Nelson (3) reducing sugar method was used. Therefore, the 2,2′-bicinchoninate reagent of Sinner and Puls (2) was modified so it could be used for manual sugar determinations. The method developed has a sensitivity comparable to the Nelson procedure for detection of monosaccharides and disaccharides.

Brown (4) and Dygert *et al.* (5) have reported reducing sugar procedures for glucose and glucose oligomers using neocuproine hydrochloride as a chelating reagent for cuprous ion. The 2,2′-bicinchoninate reagent also functions as a cuprous ion chelating agent.

MATERIALS AND METHODS

Sodium 2,2′-bicinchoninate (sodium 4,4′-dicarboxy-2,2′-biquinoline)[1] was purchased from Sigma Chemical Company. Sugars, ethylene glycol, sodium borate, and potassium phosphate salts were reagent grade.

Component Solutions and Reagent

The component solutions for the 2,2′-bicinchoninate reagent were based upon those reported by Sinner and Puls (2). However, significant modification of their solution A was required for a suitable manual procedure.

Solutions A_1 and A_2. Solution A_1[2] was

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[2] Abbreviations used: solution A_1, prepared by dissolving 160.9 g K_2HPO_4, 10.4 g KH_2PO_4, and 870 mg 2,2′-bicinchoninate in water and diluting to 1 liter, pH 8.5; solution A_2, same as A_1, but containing 333 ml/liter ethylene glycol; solution B, a solution of 25 g

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TABLE 1

COLOR FORMATION OF SINNER AND PULS' BICINCHONINATE REAGENT IN THE ABSENCE OF REDUCING SUGAR AND PRESENCE OF ETHYLENE GLYCOL

Ethylene glycol concentration[a] (% v/v in reaction mixture)	$A_{560\ nm}$
0	0.067
7.5	0.699
15	1.064
25	1.298

[a] Reaction mixture consisted of reagent (2 ml), water, and ethylene glycol in a total volume of 4 ml. The reagent containing 23 parts of a solution of 1.30 g/liter disodium 2,2°-bicinchoninate and 62.3 g/liter Na_2CO_3, pH 10.6, mixed with 1 part of solution B.

prepared by dissolving 160.9 g K_2HPO_4, 10.4 g KH_2PO_4, and 870 mg of 2,2'-bicinchoninate in water and diluting to 1 liter. The pH was 8.5. Solution A_2 was the same as A_1 except that it contained 333 ml/liter ethylene glycol.

Solution B. A solution of 25 g aspartic acid and 33.4 g Na_2CO_3 in 500 ml water was mixed with a separate solution of 6.7 g $CuSO_4 \cdot 5H_2O$ in 250 ml water. This mixture was then made to 1 liter. This is the same solution B described by Sinner and Puls. Microorganisms can grow in solution B, so it should be kept under refrigeration.

2,2'-Bicinchoninate reagent. One part of solution B was mixed with 23 parts of solution A_1 or A_2. The final reagent was allowed to stand overnight before use. The working reagents are stable for 6 weeks at room temperature.

Procedure

A 1.0-ml aqueous sample of sugar was added to 3.0 ml of the bicinchoninate reagent. The mixture was stirred vigorously on a Vortex mixer. The tube was loosely covered, heated for exactly 10 min in boiling water, and cooled in tap water. The reaction mixture was read at 560 nm with a Cary 219 spectrophotometer with water as the blank. This procedure was used with or without ethylene glycol in the reagent.

Aqueous samples of sugars were also analyzed according to the procedure of Nelson (3).

RESULTS AND DISCUSSION

The bicinchoninate reagent used by Sinner and Puls (2) gave limited color formation with glucose and galacturonic acid. The change in absorbance at 560 nm was 0.81 and 0.61 absorbance units/μmol for glucose and galacturonic acid, respectively. The curves were not linear below 30 nmol of sugar. Mopper and Gindler (1) reported that ethanol in sugar samples increased color development of their reagent. However, the low boiling point of ethanol made it unsuitable for use in a manual procedure.

Ethylene glycol has a higher boiling point than water and it was found that this alcohol also increased the color development with Skinner and Puls' bicinchoninate reagent. However, in the absence of reducing sugar, color formation increased markedly with increasing levels of ethylene glycol in the reaction mixture (Table 1). The highest level of 25% ethylene glycol in the reaction mixture, which was equivalent to 33% ethylene glycol in the reagent, was selected for use.

TABLE 2

EFFECT OF pH OF REAGENT WITH 33% ETHYLENE GLYCOL ON THE SLOPE AND INTERCEPT OF A GALACTURONIC ACID STANDARD CURVE

Reagent pH	Slope ($\Delta A_{560\ nm}$/μmol galacturonic acid)	Intercept ($A_{560\ nm}$)
7.5	1.30	0.235
8.0	2.00	0.221
8.5	2.21	0.183
9.0	2.70	0.288
9.5	5.50	1.439

aspartic acid and 33.4 g Na_2CO_3 in 500 ml H_2O mixed with a separate solution of 6.7 g $CuSO_4 \cdot 5H_2O$ in 250 ml H_2O, then made to 1 liter.

TABLE 3

SENSITIVITY OF THE 2,2'-BICINCHONINATE REAGENT CONTAINING 33% ETHYLENE GLYCOL TO VARIOUS REDUCING SUGARS

Sugar	$A_{560\ nm}/\mu mol$	95% confidence interval	Correlation coefficient	Maximum sugar assayed (nmol)
Galacturonic acid	2.21	0.08	0.9975	360
Glucuronic acid	5.85	0.38	0.9956	120
Glucose	3.60	0.26	0.9939	240
Galactose	2.47	0.08	0.9983	360
Fructose	9.85	0.48	0.9983	60
Mannose	4.82	0.18	0.9986	120
Xylose	4.62	0.08	0.9997	120
Rhamnose	1.58	0.05	0.9987	360
Fucose	1.32	0.03	0.9991	360
Arabinose	3.00	0.11	0.9984	240
Melibiose	4.32	0.22	0.9965	240
Maltose	3.09	0.15	0.9971	240
Cellobiose	3.85	0.16	0.9977	240

The color level in the absence of sugar could be reduced by lowering the pH and changing to a phosphate buffer. Table 2 shows the effect of reagent pH on the slope and intercept of a galacturonic acid standard curve. The reagent with ethylene glycol for pH 7.5 to 9.0 was the same as that described under Materials and Methods except that the ratio of K_2HPO_4 and KH_2PO_4 was varied. For pH 9.5, 1.0 M carbonate buffer was substituted for phosphate buffer. The sensitivity of the reagent increased with increasing pH. The large increase in both slope and intercept at pH 9.5 indicated that a specific buffer effect as well as a pH effect occurred when carbonate was used.

A 1.0 M phosphate buffer at pH 8.5 was selected for the reagent to give a sensitivity similar to that of the Nelson (3) reagent. The high phosphate concentration was used to provide reasonable buffering capacity at a pH well above the pK of phosphate. An attempt was made to replace phosphate buffer with 0.1 M Tris or aspartic acid in order to use a buffer with a pK nearer to the desired pH for the reaction. However, neither buffer resulted in a useful level of color development.

Since color development varies with pH, care must be taken to be sure that the pH of sugar samples is the same as that for the standard curve. This was not a problem with unbuffered samples of sugars or sugar acids. However, the addition of 1.0 ml of pH 4.5, 0.1 M acetate buffer to 3.0 ml of reagent dropped the pH of the reaction mixture 0.15 units.

Color development increased with duration of heating in a boiling water bath up to 20 min. However, a 10-min heating time gave 75% as much color as 20 min, color development was reproducible, and the sensitivity was similar to that of the Nelson reagent. Therefore, 10 min was selected as a convenient heating time. The color was quite stable. The slope of galacturonic acid standard curves differed by less than 1% when measurements were made immediately after cooling compared to when the samples were held for 22 h at room temperature prior to taking the readings.

For the reagent containing ethylene glycol, Table 3 shows the absorbance change per micromole of sugar, the 95% confidence interval, and the correlation coefficient for the linear regression line through the standard curve. The maximum quantity of each sugar which could be

TABLE 4

SENSITIVITIES OF THE 2,2'-BICINCHONINATE REAGENT CONTAINING NO ETHYLENE GLYCOL TO VARIOUS REDUCING SUGARS

Sugar	$\Delta A_{560\ nm}/\mu mol$	95% confidence interval	Correlation coefficient	Maximum sugar assayed (nmol)
Galacturonic acid	2.19	0.07	0.9988	360
Glucuronic acid	4.56	0.08	0.9997	240
Glucose	1.53	0.08	0.9965	360
Galactose	1.24	0.04	0.9987	360
Fructose	10.76	0.52	0.9983	120
Mannose	2.04	0.06	0.9989	360
Xylose	2.90	0.07	0.9994	360
Rhamnose	1.41	0.04	0.9991	360
Fucose	0.54	0.03	0.9952	360
Arabinose	2.01	0.05	0.9993	360
Melibiose	2.23	0.08	0.9985	360
Maltose	1.46	0.05	0.9983	360
Cellobiose	1.21	0.03	0.9995	360

measured is also given. With freshly prepared reagent, a blank without added sugar generally had an absorbance between 0.2 and 0.25.

It was subsequently found that color development also occurred when ethylene glycol was not added to the phosphate-buffered reagent at pH 8.5. In this case, the reagent was prepared by mixing solution A_1 with solution B. The data for the absorbance change per micromole, 95% confidence interval, correlation coefficient, and maximum level of sugar which was measured are given in Table 4. The absorbance for blank solutions with this reagent was about 0.05 as compared to water.

For the purpose of comparison, standard curves were done for a few sugars using the Nelson (3) reagent. These data are shown in Table 5.

The use of a bicinchoninate reagent with or without ethylene glycol resulted in standard curves with high correlation coefficients. A minimum of 12 nmol of sugar was determined with both reagents. For sugars where the absorbance change is large enough, it may be possible to measure somewhat lower levels. The reagent with ethylene glycol was more sensitive by a factor of 1.5 to 3-fold than when ethylene glycol was excluded. Galacturonic acid did not change. Fructose and rhamnose showed only small changes when ethylene glycol

TABLE 5

SENSITIVITY OF THE NELSON REAGENT TO VARIOUS REDUCING SUGARS

Sugar	$\Delta A_{600\ nm}/\mu mol$	95% confidence interval	Correlation coefficient	Maximum sugar assayed (nmol)
Galacturonic acid	2.37	0.05	0.9993	360
Glucose	3.16	0.07	0.9993	360
Galactose	2.40	0.13	0.9957	360
Fructose	3.03	0.08	0.9990	360
Xylose	2.56	0.06	0.9992	360
Rhamnose	0.98	0.11	0.9833	360

was not used. The fact that the color yield with fructose was considerably higher than that with the other sugars suggested that bicinchoninate was particularly sensitive with keto sugars. This limits the amount of fructose which can be measured.

Since borate ion is commonly used in the chromatography of sugars, the effect of borate on the development of color was investigated. Sodium borate solution adjusted to pH 8.5 was added to samples to give a borate concentration in the sample of 0.16 and 0.32 M. Table 6 shows the change in absorbance per micromole of glucose and galacturonic acid with and without ethylene glycol in the bicinchoninate reagent. In all cases, the correlation coefficients for the standard curves were 0.99 or higher. The results showed that these sugars can be measured in the presence of borate. In reagent with ethylene glycol borate decreases the sensitivity over 50%. Otherwise, there was only a slight decrease in color development due to borate.

In summary, a procedure has been developed for the manual assay of reducing sugars using 2,2′-bicinchoninate as the chelating agent for cuprous ion. With 33% ethylene glycol in the reagent, the sensitivity was comparable to that found with the Nelson reagent (3). Galacturonic acid, fructose, and rhamnose could be assayed in bicinchoninate reagent without ethylene glycol, without loss in sensitivity, and with a lower blank absorbance. The procedure can be used with samples which contain borate ion.

The bicinchoninate assay method is more convenient to use than the Nelson reagent because the working reagent is stable for 6 weeks and because only one reagent needs to be added to a sample. The Nelson method requires the addition of one solution prior to the heating step and a second solution after cooling. When the bicinchoninate method was used to measure the reducing sugar released by polygalacturonases, variations in the intercepts of the reaction curves were not observed. Therefore, this method may be a useful alternative for reducing sugar assays.

TABLE 6

EFFECT OF BORATE ION CONCENTRATION ON COLOR DEVELOPMENT OF 2,2′-BICINCHONINATE REAGENT WITH AND WITHOUT ETHYLENE GLYCOL

Sugar	Borate concentration (M)	2,2′-Bicinchoninate reagent ($\Delta A_{560\ nm}/\mu mol$)	
		+Ethylene glycol	−Ethylene glycol
Glucose	0	3.60	1.53
	0.16	1.64	1.46
	0.32	1.55	1.35
Galacturonic acid	0	2.21	2.19
	0.16	2.20	1.83
	0.32	2.01	1.74

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REFERENCES

1. Mopper, K., and Gindler, E. M. (1973) *Anal. Biochem.* **56**, 440–442.
2. Sinner, M., and Puls, J. (1978) *J. Chromatogr.* **156**, 197–204.
3. Nelson, N. (1944) *J. Biol. Chem.* **153**, 375–380.
4. Brown, M. E. (1961) *Diabetes* **10**, 60.
5. Dygert, S., Li, L. H., Florida, D., and Thoma, J. A. (1965) *Anal. Biochem.* **13**, 367–374.