A Colorimetric Estimation of Sugars Using Benzidine

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Many colorimetric methods using organic reagents are available for the estimation of sugars, a number of which have been utilized in conjunction with chromatography. For example, a method using aniline phthalate has been described (Blass, Macheboeuf & Núñez, 1950; Bartlett, Hough & Jones, 1951). This method, however, consists of several stages, which is a disadvantage. Methods using concentrated mineral acids are to be avoided if possible, in order to simplify practical procedure, and for this reason the method described by Dubois, Gilles, Hamilton, Rebers & Smith (1951), using a phenol-sulphuric acid reagent, was considered unsuitable for routine analysis.

In connexion with work on gums, it was considered necessary to have available a simple colorimetric estimation applicable to all the monosaccharide constituents of complex polysaccharides and their methylated derivatives.

Fig. 1. Absorption spectra for: A, reaction of 100 $\mu\text{g.}$ D-galactose with the benzidine reagent; B, the blank determination. Curves for aldopentoses, methyl aldopentoses, hexuronic acids, and methylated sugars were similar to A.

Benzidine is used as a chromatographic spray reagent for reducing sugars (Horrocks & Manning, 1949) and for the estimation of pentoses by an indirect method involving furfural (McCance, 1926). An attempt was therefore made to estimate sugars directly using a solution of benzidine in glacial acetic acid. Preliminary experiments showed that aldopentoses, methyl aldopentoses, aldohexoses, hexuronic acids, methylated sugars, and oligosaccharides all gave an orange-yellow coloration when heated with the reagent, and that the colour in each case had a similar maximum absorption band (Fig. 1). Further investigation showed that the relationship between absorption and concentration was linear in all cases studied.

It was observed that the benzidine reagent in glacial acetic acid gradually became pale yellow when exposed to air and light. However, the addition of a small amount of stannous chloride prevented this discoloration, and also increased the sensitivity, enabling the determination of much lower concentrations of sugar. Stannous chloride is also used in this laboratory to stabilize the *p*-anisidine hydrochloride spray reagent (Hough, Jones & Wadman, 1950).

The composition of polysaccharides was determined using the benzidine reagent, after hydrolysis and separation of the monosaccharide constituents on paper chromatograms.

EXPERIMENTAL

Reagent. This consists of a 0.2% (w/v) solution of benzidine (A.R.) in glacial acetic acid (A.R.) containing 0.1% (w/v) SnCl_2 .

Owing to the slight instability of the reagent, the determination of sugars is not based on a standard curve, but estimations of standard sugar solutions are carried out together with the unknown. The solution of benzidine in glacial acetic acid should be freshly prepared. The addition of stannous chloride produces a cloudy precipitate which is filtered off. The clear, colourless solution is then ready for use.

Method

The estimations are carried out in test tubes (7 $\times$ 1 in.) provided with small glass spheres to prevent the entry or loss of water. Sugar solution to be determined (1 ml.), containing not more than 600 $\mu\text{g.}$ of sugar, is added from a pipette into a tube, followed by the benzidine reagent (5 ml.) from a burette. After a thorough mixing, the tubes are heated in a vigorously boiling water bath for a period depending on the class of the sugar (Table 1 and Fig. 2). At

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least two standards of suitable concentration are included and also a blank determination, i.e. 1 ml. of water and 5 ml. of benzidine reagent.

After being heated, the tubes are cooled in running water for not less than 3 min. The colour is then measured with a Hilger 'Spekker' photoelectric absorptiometer using a violet filter (Ilford no. 601, wavelength 425 m μ .) and a 0.5 cm. glass cell. The concentration of the unknown can then be calculated from the standards by simple proportion, or, better still, if three or more standards are employed, by plotting absorption against concentration and reading the unknown from the resulting graph. The relation between concentration and absorption was found to be linear over the range 100–600 μ g./ml. for the sugars listed in Table 1.

Concentrations as low as 20 μ g./ml. can also be determined satisfactorily, provided that standards are included within the range 20–100 μ g./ml., and the calculation is based on these standards. The reason for this is that below a concen-

tration of 100 μ g./ml. the slope of the concentration/absorption graph changes slightly (Fig. 3). The method is not suitable for concentrations of less than 100 μ g./ml. of methylated sugars and disaccharides or less than 50 μ g./ml. of hexuronic acids. The determination may be carried out with errors of less than $\pm 3\%$.

The absorption spectra of the coloured compounds produced by interaction of the benzidine reagent with sugars were plotted at intervals of 5 m μ . using a Unicam S.P. 500 spectrophotometer, and a 1 cm. glass cell. The maximum absorption with all the sugars tested was in a wavelength band ranging from approximately 340 to 420 m μ . (Fig. 1). Small peaks were evident within the wavelength band, with a small variation depending on the nature of the sugar. The main absorption plateau, however, was of a similar nature in every case. The use of the 'Spekker' with a violet filter to measure the colour produced, instead of a spectrophotometer employing a diffraction grating for the selection of a definite wavelength, was found to be quite satisfactory. The former instrument was easier to operate, especially when dealing with many determinations, and the violet filter allowed absorption to be measured over a wavelength band where the absorption maximum of the blank determination was at a minimum.

Table 1. *Times of heating sugars with the benzidine reagent*

Sugar	Heating time necessary for maximum colour production (min.)
Arabinose	15
Ribose	
Lyxose	
Xylose	
Glucose	30
Galactose	
Mannose	
Rhamnose	
Fucose	
Glucuronic acid	
2:3:4-Trimethylxylose	60
2:4:6-Trimethylglucose	
2:3:6-Trimethylglucose	
2:3:4:6-Tetramethylglucose	
Maltose	
Sucrose

Fig. 2. Variation in optical density (400 m μ .) with time of heating for: A, D-glucurone (300 μ g.); B, D-ribose (250 μ g.); C, 2:4:6-trimethyl-D-glucose (160 μ g.) with benzidine reagent. Methyl aldopentoses, aldohexoses, and galacturonic acid gave curves similar to A. All aldopentoses resembled curve B and all methylated sugars curve C. Measurements were made with a Unicam S.P. 350 spectrophotometer using a 1 cm. glass cell.

Specificity of coloration

The benzidine reagent was observed to give colours with hexose diphosphate, 2-deoxy-D-galactose, dihydroxyacetone, and with ketonic compounds with free or easily accessible carbonyl groups, e.g. acetone, formaldehyde, α -oxoglutaric acid, etc. D-Fructose, however, only gave a colour when in high concentration.

No colour was observed with glycine (in concentration less than 1.0 mg./ml.), tryptophan, tyrosine, asparagine, cystine, hydroxyproline, urea, hexitols, or calcium gluconate. D-Glucosamine hydrochloride gave a faint colour in a concentration of 500 μ g./ml.

Fig. 3. Concentration/absorption graphs showing change of slope below a concentration of 100 μ g./ml. for: A, D-mannose; B, D-xylose; C, D-glucurone. Other sugars show a similar change. The filter used (Ilford 601) transmits light in the range 385–470 m μ ., max. transmission 425 m μ .

Table 2. *Analysis of polysaccharides*

Polysaccharide	Ratio of D-glucose or D-galactose to D-mannose		Recovery based on ribose recovery (%)
	Determined by benzidine method	Results of previous workers	
Lucerne seed galactomannan	1:1.35	1:1.25*	101.8
<i>Lilium umbellatum</i> glucomannan	1:1.88	1:1.83†	94.4

* Andrews, Hough & Jones (1952).

† Andrews, Hough & Jones (unpublished results).

Table 3. *Recoveries of sugars after partition on paper*

Sugar	Amount added to paper (μ g.)	Recovery (μ g.)	Recovery (%)
L-Arabinose	309	309	100
	233	235	101
	155	165	106
D-Xylose	998	930	93
	750	750	100
	499	457	92
	250	229	92
D-Galactose	99	99	100
	398	365	92

Analysis of polysaccharides

Two polysaccharides have been hydrolysed, their constituent sugars separated on paper chromatograms (Flood, Hirst & Jones, 1948) and analysed using the benzidine reagent. The results obtained (Table 2) were comparable with those of other workers.

The sugars were eluted from the chromatograms by shaking the paper strips with suitable volumes of distilled water in tubes fitted with ground-glass stoppers. The sugar solutions were then poured off, centrifuged to remove small particles of cellulose, and samples taken for analysis (cf. Roudier, 1951).

The recovery from the paper using the above technique was examined by applying standard sugar solutions to chromatograms with an 'Agl'a' micrometer syringe (Burroughs Wellcome and Co., London), developing with ethyl acetate-acetic acid-water solvent (9:2:2, v/v) and eluting the sugar as described. The error was found to be within $\pm 9\%$ (Table 3).

Lucerne seed galactomannan. The polysaccharide (11.38 mg.) was hydrolysed with $N-H_2SO_4$ in a sealed tube overnight at 100° . D-Ribose (2.925 mg.) was added to the hydrolysate and then the acid neutralized with barium carbonate. After filtration, the solution was evaporated under reduced pressure to a small volume. The three component sugars were then separated chromatographically using *n*-butanol-ethanol-water solvent (40:11:19, v/v) (see Table 2).

Lilium umbellatum glucomannan. The glucomannan (20.27 mg.) was hydrolysed in a sealed tube with 98% formic acid at 100° overnight. The acid was removed by evaporation under reduced pressure, and the residue taken up in $N-H_2SO_4$. D-Ribose (6.318 mg.) was then added and the solution heated at 100° for 30 min. to hydrolyse formyl

esters. The acid was then neutralized as before with barium carbonate, and the remainder of the procedure repeated as described for lucerne seed galactomannan (see Table 2).

SUMMARY

1. A new method of determining sugars colorimetrically is described, the reagent consisting of a solution of benzidine in glacial acetic acid. The method is suitable for the determination of aldopentoses, methyl aldopentoses, aldohexoses, hexuronic acids, methylated sugars, and disaccharides. Ketoses and amino sugars cannot be estimated with this reagent.

2. From 20 to 600 μ g./ml. of aldopentose, methyl aldopentose, and aldohexose may be determined by the method. Hexuronic acids can be estimated in concentrations ranging from 50 to 600 μ g./ml. Methylated sugars and disaccharides cannot be estimated satisfactorily below a concentration of 100 μ g./ml. The sugars may be determined with errors less than $\pm 3\%$.

3. The analysis of a glucomannan and galactomannan has been carried out using this procedure.

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