Journal of Chromatography, 101 (1974) 307–314

CHROM. 7834

ACCELERATED ION-EXCHANGE CHROMATOGRAPHY OF REDUCING SUGARS IN NEUTRAL BORATE BUFFERS

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(Received July 30th, 1974)

SUMMARY

A system for the ion-exchange chromatography of reducing sugars in neutral borate buffers using sulphate as the counter-ion is described. The eluted sugars were determined by a new automatic method for reducing sugars using *p*-hydroxybenzoic acid hydrazide as the colorimetric reagent. The reproducibility of the method is discussed.

INTRODUCTION

Automated methods for the estimation of reducing sugars by ion-exchange chromatography of sugar–borate complexes at neutral pH values have been described[1,2]. Although the use of neutral pH buffers, as opposed to the earlier use of alkaline eluents for chromatography of the sugar–borate complexes[3], has reduced the risk of alkaline degradation of the reducing sugars on the chromatographic column, the methods described to date still suffer a number of disadvantages. These include the use of chloride ion as the eluting counter-ion, which causes shrinkage of the resin and this obviates the application of accelerated chromatographic analysis with narrow-bore columns. The ion-exchange procedures described by Walborg and Kondo[1] and Hough *et al.*[2] require 7 h or more to resolve glucose from a simple mixture of other sugars. Another disadvantage common to most methods —apart from the colorimetric dye reagents[4,5], which are only applicable to chromatographic eluates containing ethanol— is the use of concentrated inorganic acids in the colorimetric reagents. Because of the danger from tube failure and the poor reproducibility of Acidflex tubing, the use of concentrated acids is undesirable.

We have attempted to improve the ion-exchange chromatography of sugar–borate complexes by first using sulphate as the counter-ion instead of chloride and secondly by automating the colorimetric reaction for reducing sugars described by Lever[6], who used *p*-hydroxybenzoic acid hydrazide. The choice of sulphate as the exchanging anion was made after consideration of the relative sizes of anions. The size of the sulphate ion is closer than that of the chloride ion to the size of the borate ion. Pauling[7] gave the average B → O and S → O bond lengths as 1.47 Å and 1.49 Å, respec-

tively, and the ionic radius of the chloride ion as 1.81 Å. While there are other factors which affect the swelling and contraction of the resin, this theoretical consideration is supported in practice and the pressure change during the complete cycle is never more than 40 lbs./sq.in.

EXPERIMENTAL

Materials

Sugars were obtained from BDH (Poole, Great Britain) and Sigma (St. Louis, Mo., U.S.A.). *p*-Hydroxybenzoic acid hydrazide was purchased from Fluka (Buchs, Switzerland). Anion-exchange resin (Type S) was obtained from Technicon (Tarrytown, New York, U.S.A.).

Ion-exchange chromatography

The resin was converted to the borate form by washing with 2 *M* sodium hydroxide solution, distilled water and 10% potassium tetraborate solution. A column (60 cm × 0.3 cm) widened at each end to accommodate the standard 6-mm Technicon fittings was packed in sections to give a resin bed height of 45 cm. The column was maintained at 60° by water circulating from a Haake bath. The following buffers were filtered through 0.45-μm Millipore filters before use for chromatography: (A) 0.1 *M* boric acid adjusted to pH 7.0 with 1.0 *M* sodium hydroxide solution; (B) 0.4 *M* boric acid adjusted to pH 7.0 with 50% (w/v) sodium hydroxide solution; (C) 0.4 *M* boric acid containing 0.2 *M* sodium sulphate adjusted to pH 7.0 with 50% (w/v) sodium hydroxide solution; (D) 10% (w/v) potassium tetraborate solution.

Six chambers of a Technicon Autograd were used to make the borate and sulphate gradients. The elution rate through the chromatographic column was maintained constant at approximately 30 ml/h by means of a positive displacement pump. The buffers for a 30-min regeneration (buffer D) and a 60-min equilibration (buffer A) were held in separate reservoirs which were opened and closed by electromagnetic valves which could be operated either manually, or by means of a pre-set controller*.

Colorimetric analysis

Standard Technicon modules (Generation I), a proportioning pump, a reaction bath at 95°, a colorimeter with interference filters at 400 nm, and a single-channel recorder with a chart speed of 6 in./h, were used. The colorimetric reagent was *p*-hydroxybenzoic acid hydrazide (2% w/v) in 0.5 *M* hydrochloric acid which was made alkaline in the analytical system by mixing with 3.0 *M* sodium hydroxide. The effluent from the chromatographic column was introduced into the alkaline reagent stream via an A2 and an A1 fitting. 0.1 ml/min of the effluent were removed from the top of the A2 fitting by a tube on the proportioning pump in order to remove gas bubbles which would otherwise disturb the baseline. An additional tube on the proportioning pump was used to pump buffer A, or a standard glucose solution (25 μg/ml), in place of the chromatographic eluent. This was used to obtain a steady baseline before the start of the chromatogram and to check the sensitivity of the re-

* See Appendix.

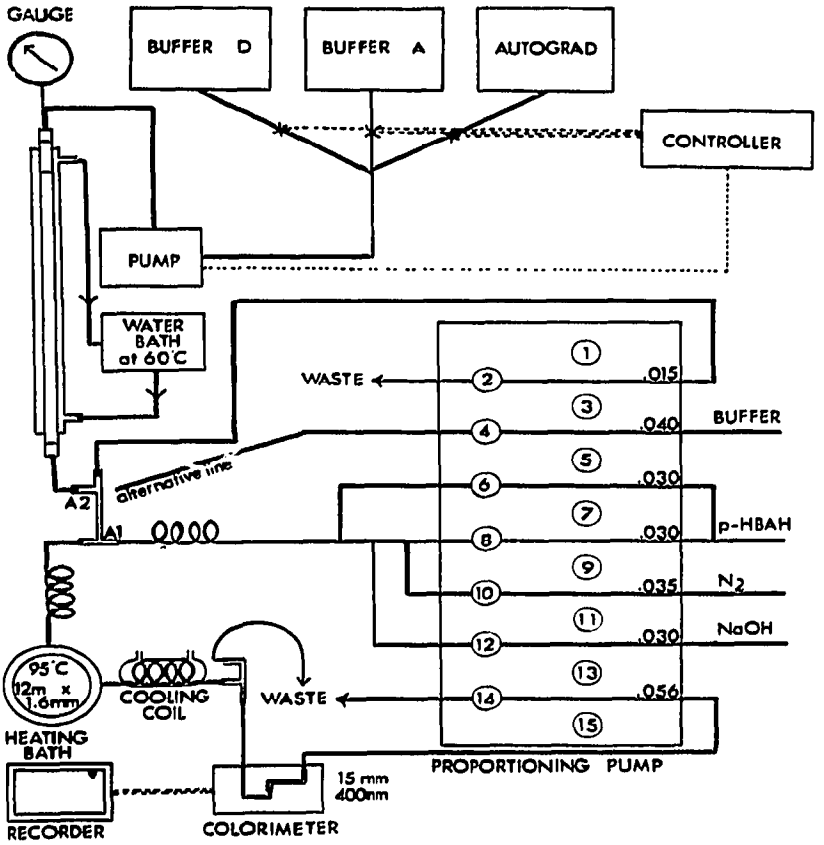

Fig. 1. Schematic diagram of the chromatographic and analytical systems. X = Solenoid valves; *p*-HBAH = *p*-hydroxybenzoic acid hydrazide. ———, Fluid lines; — — —, electrical connections.

agent. The glucose solution gave a chart reading of approximately 0.7 O.D. A diagrammatic representation of the complete chromatographic and analytical systems is shown in Fig. 1.

RESULTS AND DISCUSSION

Preliminary analyses were carried out by applying 0.2 ml of a simple sugar mixture (0.25 mg/ml of each sugar) dissolved in buffer A to the column under nitrogen pressure. By the use of various trial gradients it was possible by means of a computer program and the IBM graph plotter to calculate an initial composition for the Autograd (Table I) required to achieve a satisfactory separation of a number of sugars (Fig. 2). The sulphate counter-ion was required to produce sharp peaks of xylose and glucose, which emerged as very broad, poorly resolved peaks when borate alone was applied to the column. Investigation of the effect of temperature on the separation of the tested reducing sugars over the range 40–70° showed that 60° was the lowest temperature which produced adequate separation of arabinose from galactose. It was not possible to separate fructose from mannose and fucose, but this is unimportant

TABLE I

INITIAL COMPOSITION OF THE AUTOGRAD

Chamber	*Buffer A (ml)*	*Buffer B (ml)*	*Buffer C (ml)*
1	17.5	7.5	—
2	17.5	7.5	—
3	—	25.0	—
4	—	—	25.0
5	—	—	25.0
6	—	—	25.0

to our work as fructose is not a normal constituent of glycoproteins or mucopolysaccharides. A small peak, eluted approximately 15 min after glucose, was always present on the chromatogram even when successive blank samples were applied to the column. This unidentified peak was found to be a useful marker for the chromatogram and relative elution times for the important monosaccharides calculated with respect to this peak are more consistent than the absolute elution times (Table II). The identity of this peak was not established but it appears to correspond to the replacement of borate ion by sulphate ion on the resin.

p-Hydroxybenzoic acid hydrazide is unstable in alkaline solution and insoluble in water, so it is necessary to prepare an acidic solution. This must be made alkaline, because the reaction product with reducing sugars is only formed under alkaline conditions. Although the absorption spectrum of the glucose-*p*-hydroxybenzoic acid hydrazide product in alkaline borate buffer has a maximum at 395 nm, it was found that a 400-nm interference filter was satisfactory. The effect on the colour yield of reaction time, reagent concentration, alkalinity of the reaction mixture, and the presence of air was investigated.

No increase in colour yield was observed when the heating time was doubled. Although Lever[6] reported the use of 5% w/v *p*-hydroxybenzoic acid hydrazide solution, we have found that 2.5% w/v solutions crystallise overnight and that the maxi-

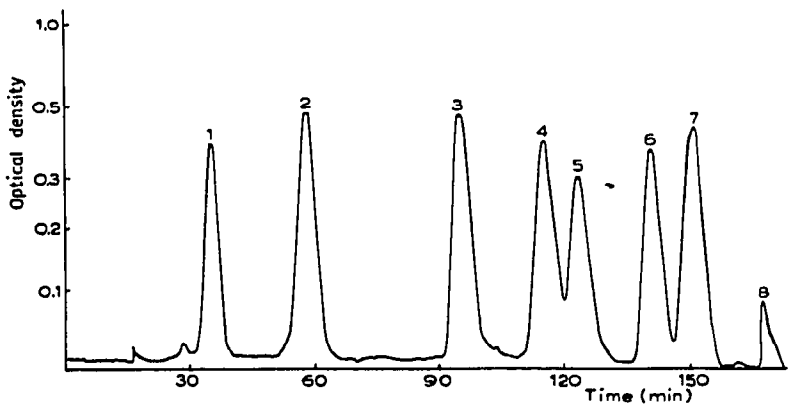

Fig. 2. Separation of sugars. (1) Maltose, (2) L-rhamnose, (3) D-mannose, (4) L-arabinose, (5) D-galactose, (6) D-xylose, (7) D-glucose, (8) unidentified. Mixed sugars 50 µg of each in 0.2 ml buffer A, were applied to the column. Flow-rate, 32 ml/min.

TABLE II

REPRODUCIBILITY OF ABSOLUTE AND RELATIVE ELUTION TIMES

Results were obtained from twelve chromatograms. Relative elution times were calculated relative to the unidentified peak. Buffer flow-rate, 32 ml/h.

Sugar	*Elution time (min)*	*Coefficient of variation of the elution time (%)*	*Relative elution time*	*Coefficient of variation of the relative elution time (%)*
Mannose	97.5	2.29	0.60	1.70
Arabinose	116.2	2.29	0.71	0.97
Galactose	123.8	2.01	0.76	1.01
Xylose	139.8	1.96	0.86	0.90
Glucose	149.8	1.30	0.92	1.24
Unidentified	163.3	1.85	1.00	—

mum usable concentration is 2% w/v. When the alkalinity of the final mixture was varied over the range 0.2 to 0.6 *M*, the colour yield of the reaction increased rapidly up to a concentration of 0.5 *M*. The use of 3 *M* sodium hydroxide produced a final alkalinity of 0.45 *M* and this was considered to be a reasonable compromise between the conflicting requirements of high colour yield and avoidance of concentrated solutions. The replacement of air by oxygen-free nitrogen for the segmentation of the reagent stream lowered the baseline by 0.1 O.D. unit. The chromatograms were quantitatively evaluated by integrating the peak areas by the usual method of multiplying the net peak height in O.D. units by the peak width at half of the net peak height. The relationships between peak area and the respective amounts of sugars shown in Fig. 3 were derived by chromatographing twelve mixtures of the sugars. These mixtures were prepared by randomly selecting weights of sugars so that duplicate analyses of each sugar at the 5-, 10-, 25-, 50-, 75- and 100-μg levels were obtained. This procedure was considered to be more realistic than the simple method of preparing different dilutions of the same mixture because sample solutions do not normally contain the same quantity of each sugar. At the highest concentrations (100 μg) a diver-

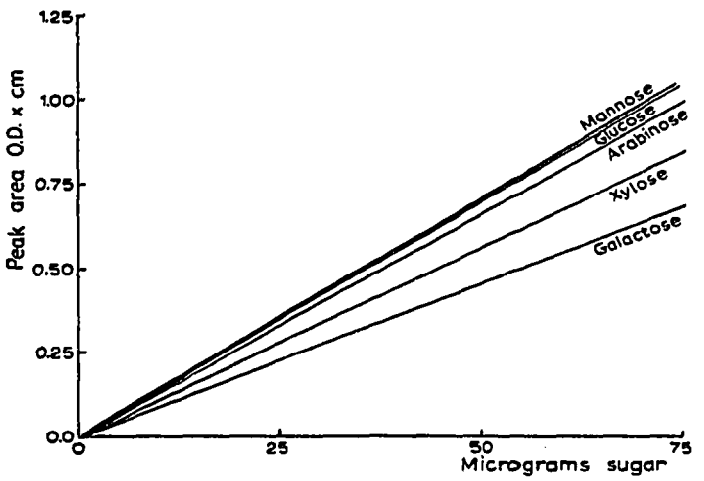

Fig. 3. Relationship of peak area to sugar concentration.

TABLE III

CONFIDENCE INTERVALS FOR PREDICTED QUANTITIES OF SUGARS

The predicted quantities were calculated from the correlations shown in Fig. 3.

Sugar	*Peak area (O.D. × cm)*	*Predicted quantity (μg)*	*Confidence interval at the 5% level of probability*
Mannose	0.15	10.8	9.5–12.0
	0.55	39.4	38.4–40.3
	1.00	71.5	69.8–73.2
Arabinose	0.15	12.4	11.1–13.7
	0.50	38.7	37.7–39.7
	0.95	72.4	70.7–74.2
Galactose	0.10	12.1	8.8–15.3
	0.35	39.1	36.5–41.6
	0.65	71.5	67.3–75.8
Xylose	0.10	9.6	8.2–11.0
	0.45	40.6	39.5–41.6
	0.80	71.5	69.7–73.3
Glucose	0.15	11.2	9.9–12.4
	0.55	39.7	38.7–40.7
	1.00	71.8	70.0–73.5

gence from Beer's Law was apparent and these results have been excluded. Table III gives the calculated confidence intervals at the 5% level of probability for various concentrations of sugar. The larger variation shown by galactose is thought to be due to the proximity of the galactose peak to the arabinose peak. Because of the difference in colour yield for these sugars (Fig. 2) galactose does not interfere with the measurement of the arabinose peak. It is recommended that a standard graph should be used only to obtain approximate results. Because of variations in reagent sensitivity and pump tubes accurate determinations of sugars can only be obtained by comparison with recent determinations of known similar mixtures.

As there was very little shrinkage of the resin when sulphate was used, it has been possible by use of the controller to carry out an analysis and automatic regeneration with a narrow-bore column in 4 h 30 min. This affords a considerable improvement over other methods[1,2], which require at least 7 h for analysis alone, and at this rate it is possible with the present equipment to carry out two analyses per day. If automatic sample addition and buffer-gradient production were added to the system, similar to methods used for amino acid analysis[8], it should be possible to analyse up to thirty-five samples per week. Lever[6] reported fluorescence spectra for the glucose-*p*-hydroxybenzoic acid hydrazide product and it is possible that the sensitivity of the method could be increased by the use of fluorometric analysis.

Maltose was well resolved from the monosaccharides (Fig. 3) and it is likely that the system could be used for the separation of oligosaccharides. Other experiments have shown that uronic acids were eluted at approximately the same position as the unidentified peak at the end of the chromatogram. It seems to us that it may be possible, by starting a pH gradient after the elution of glucose, to resolve and estimate uronic acids, which would be a useful extension of the procedures described in this communication.

ACKNOWLEDGEMENTS

The authors are grateful to Mr. R. Stansfield for computing and statistical assistance and Mr. E. H. A. Prescott for the construction of the electro-mechanical controller.

APPENDIX

Automatic Controller for the Carbohydrate Analyser, by E. H. A. Prescott, R. Couchman and A. M. C. Davies (A.R.C. Food Research Institute, Colney Lane, Norwich NR4 7UA, Great Britain)

In order to obtain automatic regeneration and shut-down of the analyser described in the main paper, a controller was constructed. The basic circuit is shown diagrammatically in Fig. A1.

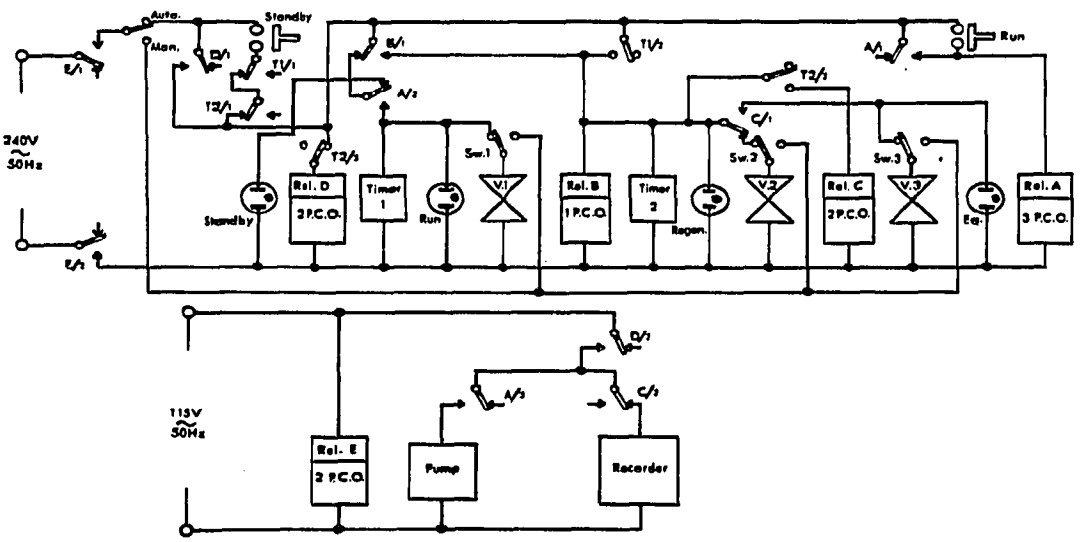

Fig. A1. Circuit diagram of the controller.

The controller was based on the use of two cam timers operating micro-switches. The parts operated by the controller were linked to the micro-switches via relays. The 240-V power supply to the controller was switched on by relay E when the power supply to the colorimeter on the analyser was operational. The solenoid valves V1–V3, which controlled the buffer flow, could be operated manually by means of the "mode" switch and switches SW1–SW3. The cam timers were used to select the times of the run and regeneration steps by means of the setting of the scaled drum on the timer, which is graduated into sixty divisions. The time of equilibration was determined by the position of the cam operating contact T2/3.

The controller was energised by re-setting both timers to zero and depressing the "stand-by" push-button. After the timers had been set to the required settings, the

TABLE AI

OPERATIONAL SEQUENCE OF THE CONTROLLER

Time (h)	*Manual operation*	*Timer 1 setting divisions (1 division = 12 min)*	*Timer 2 setting divisions (1 division = 6 min)*	*Operation*
0	Set timers to zero.	0	0	
0	Set 'Mode' to 'Auto'.	0	0	
0	Press 'Stand-by'.	0	0	Recorder powered on.
0	Set timers.	25	35	Set for 3-h run with a 30-min regeneration.
0	Press 'Run'.	25	35	Relay A energised. V1 opened, buffer from Autograd to pump. Pump started. Timer 1 started.
3		40	35	Contact T1/2 closed. Relay B energised. Timer 1 off. V1 closed, V2 opened, 10% borate to pump. Timer 2 started.
3½		40	40	Contact T2/2 closed. Relay C energised. V2 closed, V3 opened, 0.1 *M* borate to pump. Recorder off.
4½		40	50	Contact T2/3 closed. All relays A to D de-energised. V3 closed. Timer 2 off. End of sequence.

run was started by pressing the "run" button. The complete sequence of manual and automatic operations is shown in Table AI.

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