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AN IMPROVED METHOD FOR THE AUTOMATED ANALYSIS OF SUGARS BY ION-EXCHANGE CHROMATOGRAPHY

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SUMMARY

A method for the separation and determination of several mono-, di- and trisaccharides is described. Step-wise elution systems with borate buffers at high temperatures have been developed for the ion-exchange chromatography of sugars, utilizing a Dowex 1 X4 resin. Total analysis time was 250, 336 and 610 min for the respective systems developed.

Analysis of the column effluent was performed with a Technicon AutoAnalyzer using the orcinol colorimetric method. It has been established that a linear relationship exists between peak area or net peak height and different varying amounts of sugars.

INTRODUCTION

At present, there are several chromatographic methods available that utilize ion-exchange resins for the quantitative analysis of sugars[1-9]. ARWIDI AND SAMUELSON[1] have been able to obtain a separation of monosaccharides by means of partition chromatography using strong anion- or cation-exchange resins. In spite of good resolution of several monosaccharides over a reasonable time-period, this chromatographic system presents considerable technical difficulties: high temperature with volatile solvents, high pressure in the column, low solubility of oligosaccharides in the water–ethanol mixture used as eluent and rapid wearing out of the manifold tubes due to the high concentration of ethanol in the eluate.

For this reason, preference has been accorded to various methods of sugar separation on the borate form of anion-exchange resins. After the pioneering work of KHYM AND ZILL[2], many authors developed chromatographic techniques with strong anion-exchange resins in the borate form for the separation and quantitative determination of a mixture of sugar–borate complexes[3-9]. The development of these chromatographic methods now permits a separation of complex mixtures of mono-, di- and trisaccharides. Most of the methods described by these authors are based on gradient elution systems.

KHYM *et al.*[3] and OHMS *et al.*[4] used a concentration gradient of borate buffers, whereas KESLER[5] and LEE *et al.*[6] described a system in which the sugars were eluted

with a pH-concentration gradient of borate buffers. SYAMANANDA *et al.*[7] carried out the chromatographic separation with a chloride-ion gradient in borate buffers at constant pH and concentration.

Separation and quantitative determination of neutral saccharides has also been achieved by step-wise elution with borate buffers, whether[8] or not[9] these contained chloride ions.

The main drawback of these chromatographic procedures was their slowness. Either the chromatography itself required a great deal of time or the column had to be regenerated after each run.

This paper deals with an improved method for automated quantitative analysis of sugars using an ion-exchange resin in the borate form. A borate buffer of fixed borate concentration or a step-wise elution system is used here for the resolution of several mono-, di- and trisaccharides.

EXPERIMENTAL

Apparatus

This work was performed with a Technicon AutoAnalyzer. The analytical train consisted of the following Technicon modules: (a) positive micropump; (b) chromatographic column; (c) proportioning pump; (d) adjustable heating bath equipped with a 1.7 mm × 12 m reaction coil; (e) colorimeter with filter at 420 mμ and 15 mm light path cuvet; (f) one-channel recorder. The chromatographic system and analytical manifold are schematically represented in Fig. 1.

The transport lines were made from glass tubes, where possible; when glass tubes could not be used, the lines consisted of acidflex transmission tubes. Chromatographic analyses were performed with 0.6 × 110 cm or 0.6 × 140 cm borosilicate

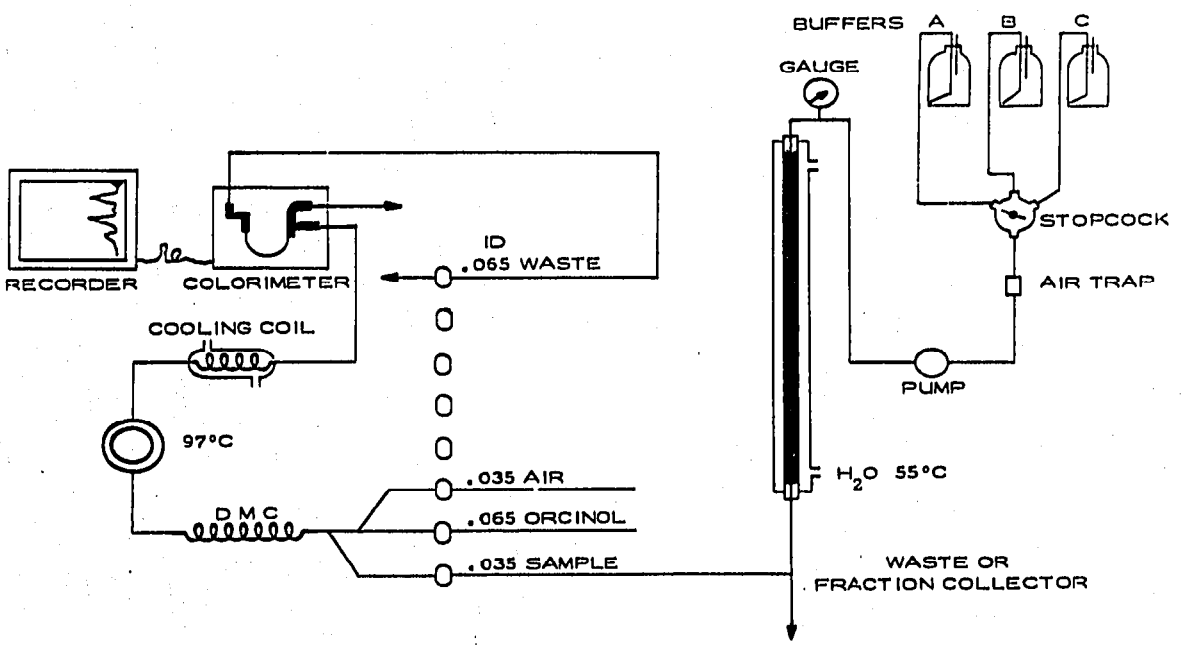

Fig. 1. Diagram of the chromatographic and analytical systems.

glass columns. The columns were glass jacketed; the water circulating within the jacket at 55° came from a superthermostat, Bühler type A5.

Resins

The resin used for the chromatographic analysis described in this paper was Dowex 1 X4 (200–400 mesh: Dow Chemical Co.). Other resins such as AG 1 X8 and AG 1 X4 (minus 400 mesh: Bio-Rad Laboratories); Dowex 1 X2 and Dowex 2 X8 (200–400 mesh: Dow Chemical Co.) and De-Acidite FF (3–5 cross-linked, minus 200 mesh: Permutit Co. Ltd.) were also tested.

Chemicals and reagents

Sugars were supplied by Nutritional Biochemicals Corp.; all were of 96% purity minimum. The other chemical products used were purchased from B. D. H. Laboratories. The colour reagent for the autoanalyzer consisted of 1 g of orcinol per litre of sulphuric acid 70% (v/v); this solution was prefiltered in a G4 glass filter and stored in a brown glass bottle.

Buffers

Buffer A: 0.11 *M* potassium tetraborate–0.17 *M* boric acid, pH 8.80; buffer B: 0.0255 *M* potassium tetraborate–0.125 *M* boric acid, pH is adjusted to 8.40 with 1 *M* KOH; buffer C: 0.053 *M* potassium tetraborate–0.088 *M* boric acid, pH 8.80.

Preparation of ion-exchange resins and columns

A large batch of resin was fractionated by repeated settling in aqueous ethanol (50%) to obtain a narrower distribution of uniform beads; the finest particles were isolated and used for chromatography. The purified resin (about 200 ml of settled beads) was treated on a büchner funnel with 2 *N* NaOH until no further chloride ions were detectable in the filtrate (with silver nitrate–nitric acid); after washing with deionized water to remove excess NaOH, the resin was converted to the borate form by washing with 0.5 *M* boric acid until the pH reached 4.2–4.3 and it then was equilibrated with a suitable buffer (A or C).

The resin, de-aerated under vacuum at 55°, was poured into the column and packed in 3–5 sections. During the packing of the columns, buffer A or C was pumped through the resin at a flow rate of 45 ml/h. The final height of the packed resin bed was 100–102 cm for the shorter column and 130–132 cm for the longer one. Final equilibration of the column was carried out with 200 ml of the selected buffer (A or C).

Chromatographic conditions

Sugar mixtures of known quantities were dissolved in 0.05 *M* potassium tetraborate solution and adjusted to pH 7.2–7.3 with boric acid; appropriate volumes of this solution (0.05 – 1.5 ml) were used for chromatography.

In this work, three chromatographic systems were used.

System 1: One-buffer system. The column was equilibrated with buffer A; after absorption of the sample under nitrogen pressure and washing with buffer A, elution was started with the same buffer at 60 ml/h. We were able to obtain a good separation of two disaccharides and seven monosaccharides in 250 min.

System 2: Two-buffer step-wise elution system. The sample was poured on the

TABLE I

ELUTION TIMES OF SUGARS (min)

Sugar	*System 1*	*System 2*	*System 3*
Sucrose	55	63	96
Trehalose	—	—	107
Melezitose	—	77	125
Raffinose	—	95	149
Maltose	74	118	206
Rhamnose	—	—	220
Lactose	—	130	240
Stachiose	—	—	270
Ribose	87	155	291
Mannose	115	190	338
Fructose	136	211	380
Arabinose	151	222	391
Galactose	169	241	412
Xylose	191	267	438
Glucose	230	306	484
Gentiobiose	—	—	505
Melibiose	—	—	585

column equilibrated with buffer A; elution was started with buffer B at 45 ml/h. After 90 min, elution was continued with buffer A at 60 ml/h. These operating conditions allowed a good chromatographic resolution of twelve sugars in 336 min. Regeneration of the column was not required.

System 3: Three-buffer step-wise elution system. In this case, chromatographic conditions were as follows: buffer C was used for equilibration of the column (the complete regeneration of the resin after each run required about 180 min); elution was started with buffer B at 60 ml/h; the buffer was changed after 225 min to buffer C, increasing the flow rate to 75 ml/h, and after 260 min to buffer A with the same flow rate. This separation was carried out with the longer column.

All separations were carried out at a column temperature of 55°.

RESULTS

The elution time of the sugars investigated in this paper was established by adding two components at a time and at different concentrations, to a synthetic mixture of sucrose, mannose and glucose, the elution time of which had previously been determined. The elution times of the seventeen sugars, examined in the three systems, are given in Table I. The values are given as the number of minutes required to elute the peak of each sugar on the chromatogram.

The resolution possibilities that can be obtained with chromatographic system 1 are shown by the chromatogram in Fig. 2. A typical chromatogram of a mixture of two trisaccharides, three disaccharides and seven monosaccharides obtained under the elution conditions of system 2 is shown in Fig. 3. In addition to these, it has been established that, under the same experimental conditions, trehalose presents an elution volume similar to sucrose; stachiose is eluted with ribose, and rhamnose is only partially separated from lactose. The chromatographic behaviour of seventeen sugars can be seen in Fig. 4. The separation of the complex mixture components was possible by using the chromatographic conditions of system 3.

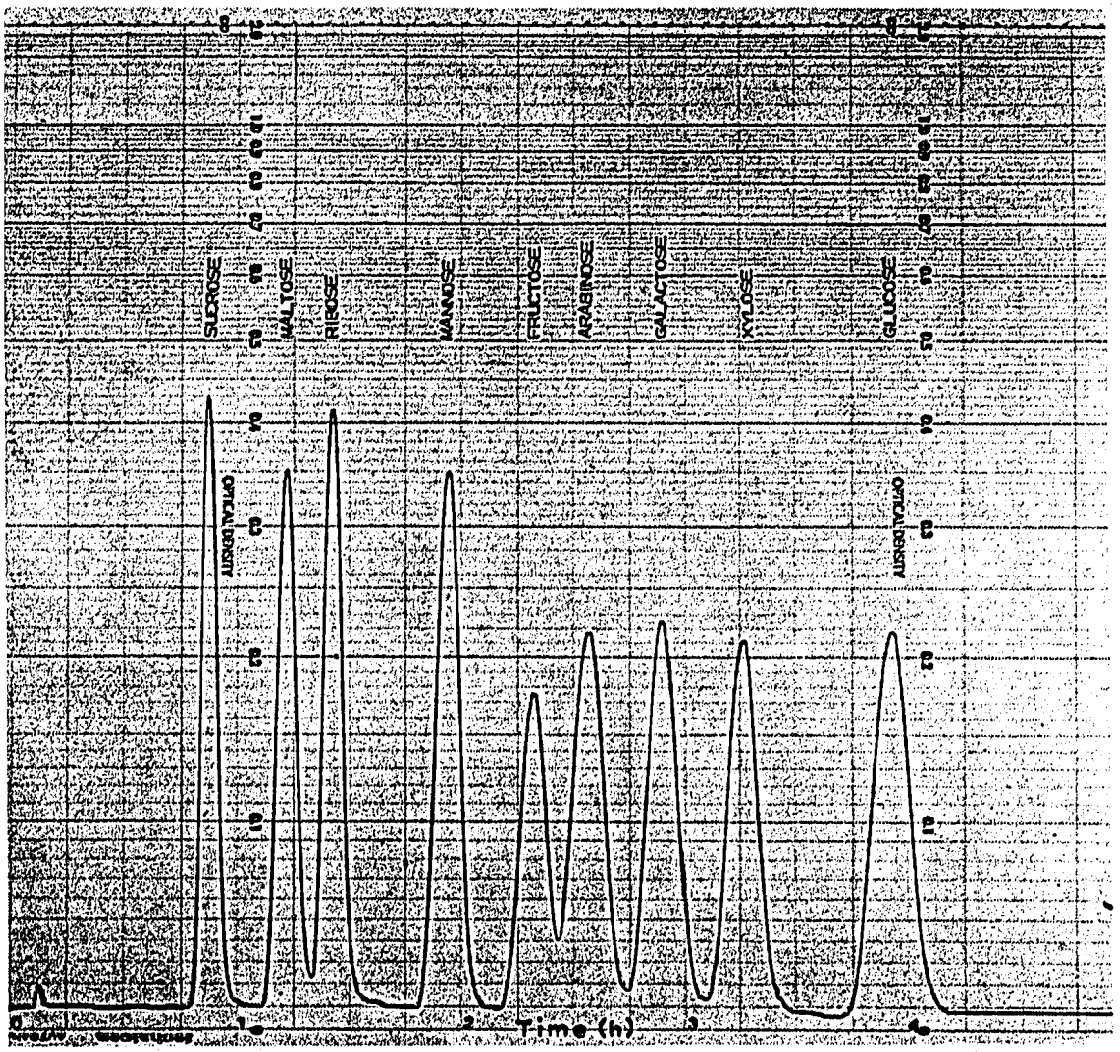

Fig. 2. Chromatogram of a nine-component synthetic mixture (system 1). Pentoses: 20 μg; other sugars: 50 μg.

Quantitative estimation

In the quantitative evaluation of the chromatograms, the areas of the chromatographic peaks (optical density *vs.* time) were usually integrated by multiplying the height (net optical density) of the peak by the width (mm) at half of the nett height[10].

The ratio of the integrated values relative to different quantities of sugars was determined by a series of five chromatograms of a nine-component standard solution performed under the conditions of system 1. Quantities of each sugar added to the column ranged from 5 to 50 μg.

The relationship between peak area and the respective amounts of sugars is shown in Fig. 5. It must be emphasized that since each peak width at half height is constant and independent of the amount of sugar, the net peak height is proportional to the concentration: thus a quantitative calibration of the chromatograms can also be obtained from the nett absorbance measured from base line to peak-tip (Fig. 6).

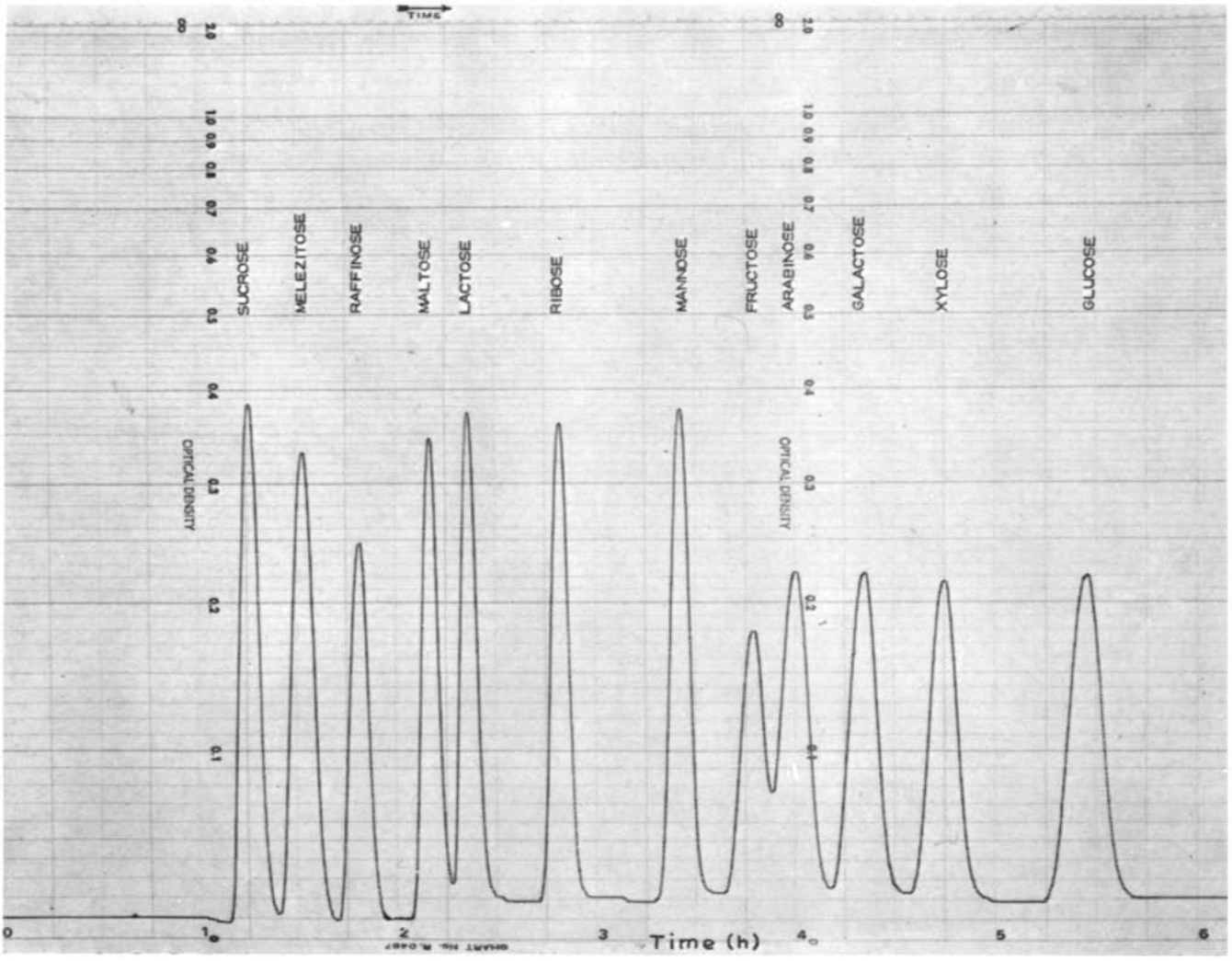

Fig. 3. Chromatography of a mixture of twelve sugars (system 2). Concentrations as in Fig. 2.

A linear relationship between sugar amount and optical density exists in the range investigated of 5–50 μg.

Reproducibility of the results

Reproducibility of the results with known mixtures of sugars was checked from chromatograms similar to that reported in Fig. 2. The reproducibility test was carried out by six runs made with the same amount of each sugar; the results are summarized in Table II. The values refer to the calculation of the mean areas and peak heights corresponding to 20 μg of pentoses and 50 μg of all the other sugars present in the mixture.

The pH change of the eluate during a chromatographic analysis performed with systems 2 and 3 is recorded in Fig. 7.

DISCUSSION

The ion-exchange chromatographic systems reported in this paper can be

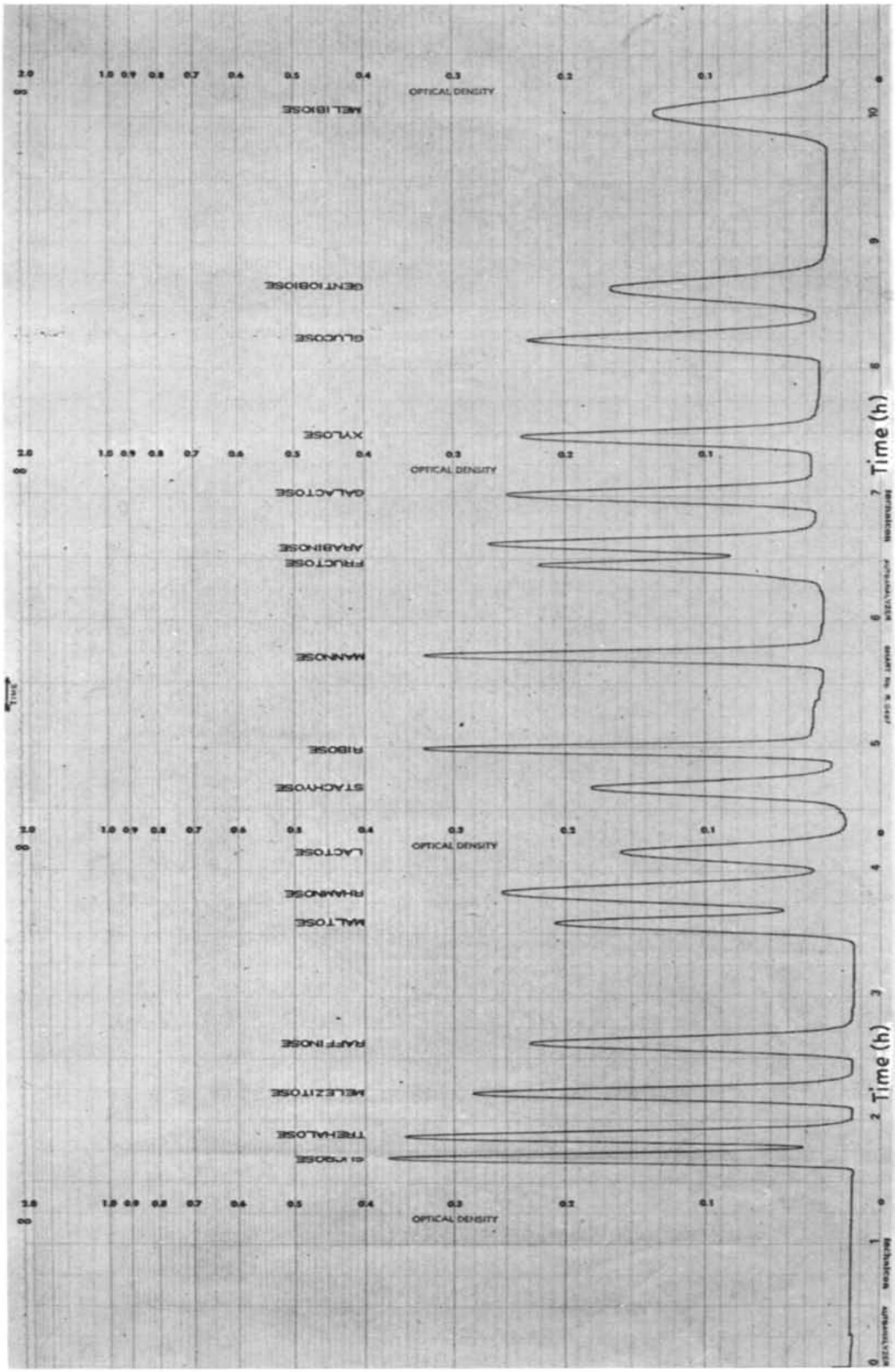

Fig. 4. Chromatographic separation (system 3) of a seventeen-component mixture. Concentration of each sugar as in Fig. 2.

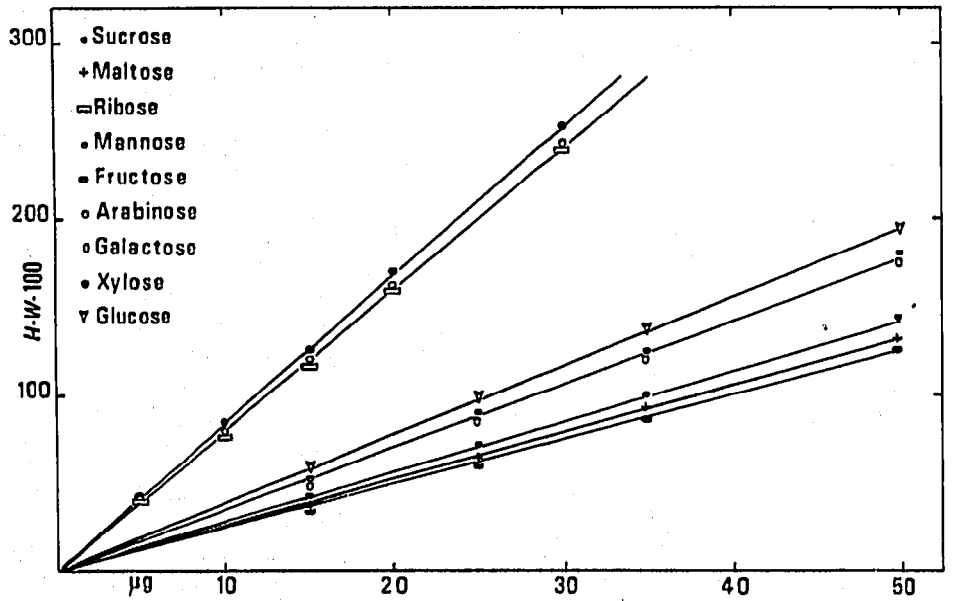

Fig. 6. Relationship between amount of sugar and peak height.

employed successfully for the complete separation of complex mixtures of sugars.

Elution system 1 does not provide a good resolution of di- and trisaccharides, but it can be used for the quantitative analysis of natural monosaccharides or monosaccharides released by chemical or enzymatic hydrolysis of polysaccharides or glycoproteins. System 2 is a versatile tool for the separation of multicomponent sugar mixtures in less than 6 h. Resolution, particularly for di- and trisaccharides, is very high. Moreover, under these conditions, the column need not be regenerated and a series of analyses can be carried out continuously.

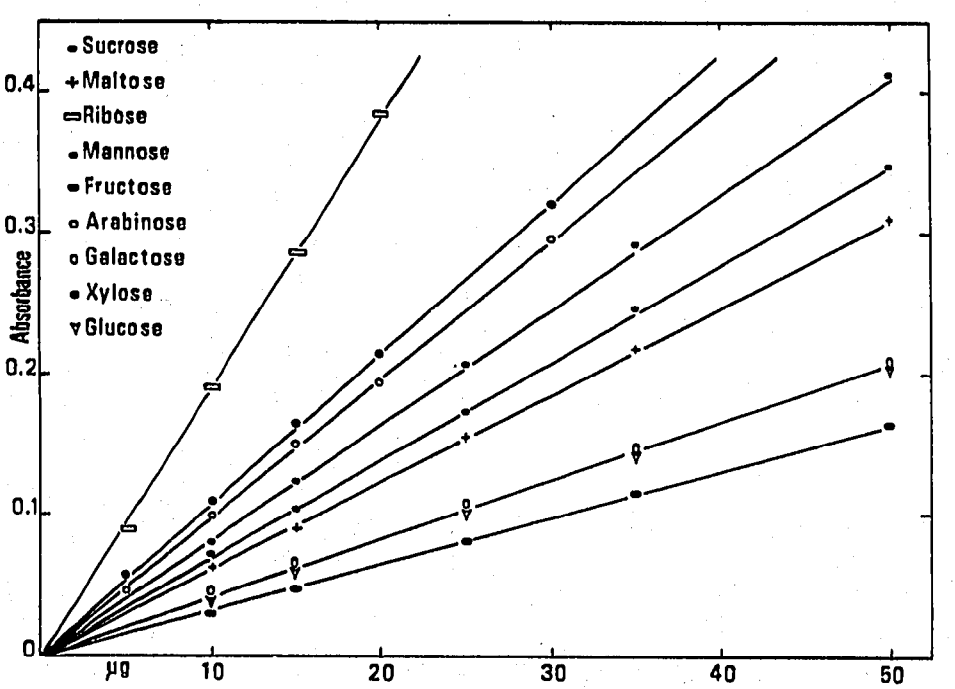

Fig. 6. Relationship between amount of sugar and peak height.

TABLE II

REPRODUCIBILITY OF THE AREAS AND HEIGHTS OF THE ELUTION PEAKS

Sugar	*Mean areas* $H \cdot W \cdot 100$	*S.D.%*	*Mean peak heights absorbance*	*S.D.%*
Sucrose	136	0.7	0.405	2.4
Maltose	128	3.1	0.319	1.8
Ribose	150	2.0	0.390	2.0
Mannose	172	1.1	0.351	3.8
Fructose	97	3.8	0.161	3.2
Arabinose	131	3.0	0.190	2.7
Galactose	143	3.3	0.204	2.8
Xylose	145	1.3	0.208	2.9
Glucose	168	0.5	0.209	2.9

Analysis of more complex mixtures of sugars requires the three-buffer step-wise system. We were able to obtain the complete separation of seventeen sugars, in about 600 min with a 0.6 × 140 cm column. After each run the column had to be re-equilibrated with buffer C. It must be emphasized that the elution volumes of trehalose, rhamnose and stachiose were greatly dependent on the pH changes occurring in the column whenever the ionic strength of the buffer changed (Fig. 7).

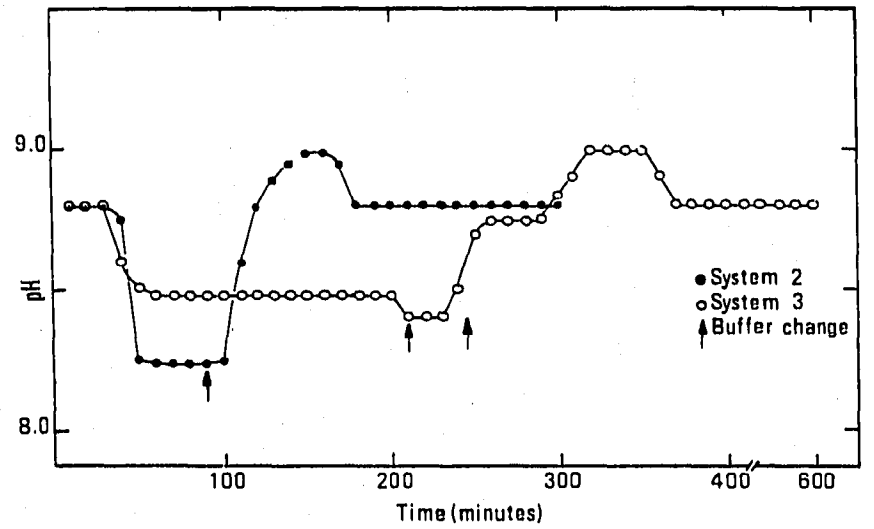

Fig. 7. pH changes of column effluent in step-wise chromatographic systems.

It was observed that, if the pH value is lower than 8.4, trehalose overlaps sucrose, and rhamnose overlaps lactose. Similarly stachiose was eluted with ribose at pH values higher than 8.8. In the step-wise elution systems, a slight shift of the chromatogram baseline was constantly observed.

The resolution power of the resin remained constant even after several months; nevertheless the column had to be repacked after 70–80 analyses, since after a while lactose undergoes transformation to a new compound which was eluted between

lactose and ribose. This drawback is not remedied by periodic replacement of the top centimetre of the blackened resin bed.

The other resins investigated presented low or very low separation power. Under the chromatographic conditions described here, AG 1 X8, Dowex 2 X8 and Amberlite CG 400 did not demonstrate any valid chromatographic resolution whatsoever. Moderate resolution was shown in a progressive order by Permutit, AG 1 X4 and Dowex 1 X2.

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