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QUANTITATIVE CHROMATOGRAPHY OF HOMOLOGOUS GLUCOSE OLIGOMERS AND OTHER SACCHARIDES USING POLYACRYLAMIDE GEL

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SUMMARY

A rapid method for the separation and quantitation of mono- and oligosaccharides is described. The procedure is based on the use of Bio-Gel P-2, minus 400 mesh, in a properly designed column with water as eluent at 65°. For the colorimetric estimation of the carbohydrates the effluent is monitored by an automated analyzing system. Homologous oligosaccharides containing up to thirteen glucose units are separated within four to seven hours for analytical and preparative purposes. Separation of glucose from ribose and maltose from isomaltose has also been obtained. Quantitative calibration by peak height was performed in the range of 10 to 100 μg.

INTRODUCTION

Analytical separation of mono- and oligosaccharides has been achieved by chromatography on paper[1] and on thin layers[2]. Gas chromatography of the trimethylsilyl ether derivatives of sugars was first carried out by SWEELEY *et al.*[3]. This technique permits the separation of a wide variety of carbohydrates from mono- to tetrasaccharides.

In 1952 KHYM AND ZILL first introduced a method for the analysis of sugar mixtures utilizing an anion exchange resin column in the borate form[4]. Based on this principle and with the application of automated analyzers and elevated temperatures[5] several advanced systems have been reported for carbohydrate analysis. Resolution of multicomponent mixtures of mono- to tetrasaccharides were achieved in this way in 4 to 6 h[6–8]. Adsorption on deactivated charcoal[9] and elution with water–alcohol mixtures resulted in the separation of glucose, maltose and raffinose[10] and the fractionation of starch hydrolysis products up to polymers with 10 to 15 glucose units[11]. Partition chromatography of sugars is possible by the use of cellulose powder columns[12] as well as by either anion or cation exchange resins according to several investigators[13–15]. Fractionations of starch[16], glycogen[17], dextrans[18] and the isolation of cell wall components from yeasts[19,20] and from bacteria [21] have been achieved by means of dextran gels (Sephadex).

Polyacrylamide gel, first introduced by HJERTÉN AND MOSBACH[22], is likely to be

a useful support for the separation of carbohydrates because in contrast to dextran gels it does not split off carbohydrates and it is not attacked by bacteria. In our preliminary report we described the influence of elevated temperature on the resolution of oligosaccharides using a Bio-Gel P-2 column[23]. The present paper deals with an improved method utilizing Bio-Gel P-2, with a mesh number "minus 400" as a support. With this method we succeeded in fractionating oligomers containing two to thirteen glucose units, as well as separating a mixture of maltose, isomaltose, maltotriose and isomaltotriose. As buffer salts had no advantage, all separations were carried out with distilled water and in this way distinct saccharides were prepared with relatively high purity merely by lyophilisation.

EXPERIMENTAL

Polyacrylamide gel

Two different gels were used: Bio-Gel P-2, 200–400 mesh (control number: 3423 (9-28-65)) and Bio- Gel P-2, minus 400 mesh (control number: 3397 (8-27-65)), obtained from Calbiochem, Luzern, Switzerland. The lot-number is of importance since we found that the Bio-Gel P-2 now available, which according to the manufacturer is made by a modified process, shows poorer separation properties.

In order to obtain a more uniform particle size, the gel was passed through a 200 mesh sieve before and after hydration and fractionated by settling according to WALBORG AND LANTZ[8].

Materials

β-Amylase from barley was supplied by Serva, Heidelberg, Germany. All sugars and chemicals were analytical grade, obtained from Merck A. G., Darmstadt, Germany. Isomaltose and isomaltotriose, prepared by enzymatic hydrolysis of dextran were kindly supplied by Dipl.-Ing. D. BONSE.

Orcinol reagent was prepared by dissolving 1 g orcinol in 1000 ml cold 70% sulfuric acid in a brown glass bottle. Distilled water, degassed by boiling, was used for preparation of the columns and as eluent.

Preparation of maltodextrins

Amylomaltase (E.C. 2.4.1.3.), first described by MONOD AND TORRIANI[24], is induced in *Escherichia coli* ML 30 by maltose. The enzyme acts as a glucosyltransferase and converts maltose into α-1,4-polyglucoses (maltodextrins) and glucose[25,26].

E. coli ML 30 was grown aerobically in a maltose salt medium, washed with ice-cold water and freeze-dried. 0.6 g freeze-dried cells were suspended in 20 ml 0.02 *M* phosphate buffer, pH 6.8 and 1.4 g maltose were added. After incubation at 28° for 3 h excess trichloroacetic acid was added, the solution was centrifuged (30,000 × *g*, 20 min) and the supernatant was freeze-dried.

Preparation of the columns

Two types of water-jacketed columns were used: a 2.5 × 100 cm column for preparative work was obtained from Pharmacia, Sweden. For analytical work a Duran-glass tube, 1.5 × 127 cm, threaded at both ends, was used. Screw caps with hose couplings were attached to both ends, the lower cap containing a nylon sieve as support

for the gel. To avoid wall effects the columns were coated by rinsing a solution of 1% dichlorodimethylsilane in benzene[27].

The column was charged in the following way: It was filled with water and air bubbles were removed carefully. A glass tube was attached to the top of the column by means of a screw joint. Sufficient gel suspension, previously degassed in a vacuum flask was poured into the lengthened column and the gel was allowed to settle overnight. Tight and close packings were obtained by pumping water at 30° through the column at a rate of 28 ml/h, till settling of the gel was complete. An exactly fitting, punched plastic cylinder was then inserted on the surface of the gel in order to minimize the dead volume and the upper screw cap was threaded on the top of the column. The use of this column adapter (Fig. 1) together with a Swagelok Union Tee (1/8 in.) permitted the injection of the samples, by means of a 100 μl Hamilton syringe through the rubber septum immediately into the eluent stream. For analytical purposes the best results were obtained when not more than 10 tot 20 μl were injected.

All separations were carried out at a column temperature of 65° and a pressure of 6 kp/cm^2.

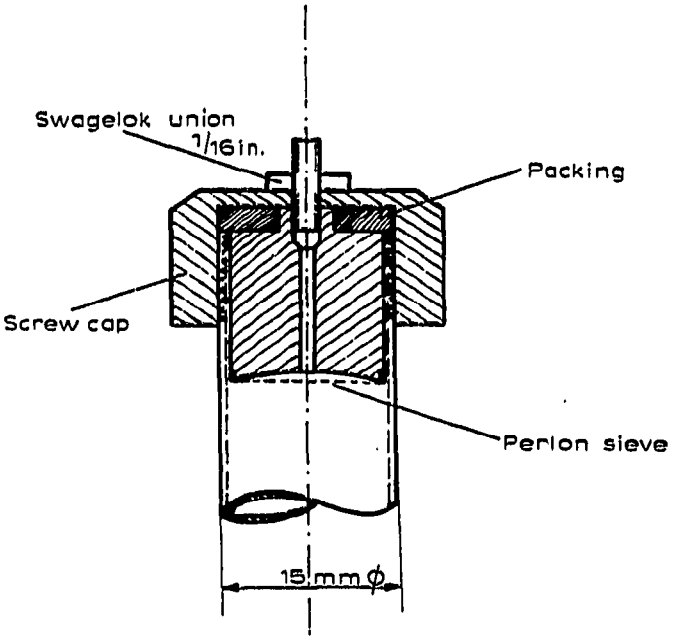

Fig. 1. Design of the upper end of the analytical column with punched plastic cylinder and screw cap.

Analytical procedure

Fig. 2 shows the flow scheme for the separation and analyzing system which was essentially an Auto-Analyzer using orcin–H_2SO_4 for the detection of the carbohydrates[6]. A Milton-Roy Minipump was used to pump water through the column at a fixed rate of 28 ml/h. 18 ml/h of this effluent was fed to the detection system. For colour development a double glass coil (24 meter) in a 95° heating bath was used. The absorbance pattern at 420 nm was followed by means of a Zeiss colorimeter (Elko III) with a 10 mm flow cell and a logarithmic converter (Zeiss) and was recorded by a Moseley/Hewlett-Packard strip-chart recorder 7101 B. Reaction coils, glass-fittings and acidflex tubings were obtained from Technicon.

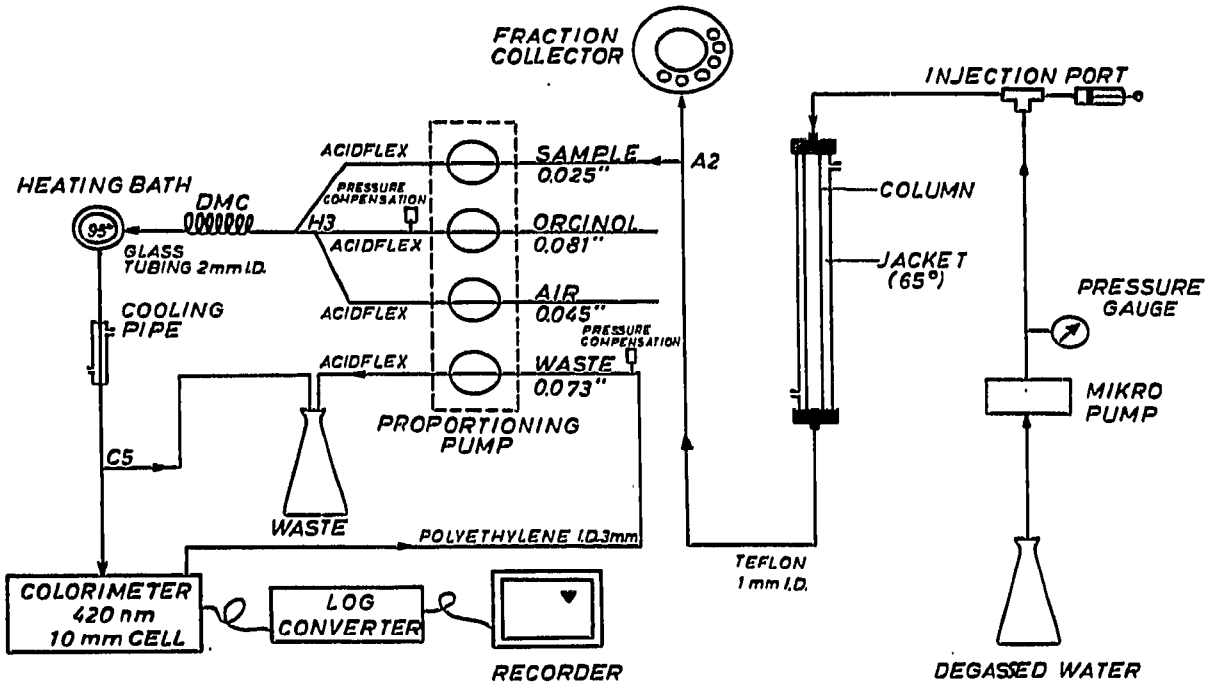

Fig. 2. Flow scheme for the separation and analyzing system.

Fractionation of oligosaccharides

For preparative separation of oligosaccharides 1 g of the freeze-dried product obtained from the action of *E. coli* cells on maltose was dissolved in 3 ml water and applied to a column (Bio Gel P-2, 200–400 mesh, 2.5 × 100 cm) through the injection equipment. Because of the high sugar concentration the sensitivity of the orcinol reaction had to be decreased by reducing the reaction temperature to 50° and by using a photometer cell with 2 mm path length. 60 ml/h of water were pumped through the column and 42 ml/h of effluent were collected in five fractions which were freeze dried and stored below 0°. A sample of each fraction was rechromatographed.

RESULTS AND DISCUSSION

Bio-Gel P-2 exhibited excellent properties for the separation of glucose, maltose and higher oligosaccharides up to eleven glucose units — Glc_1 to Glc_{11} — (Fig. 3). The sugars were eluted from the gel bed in a sequence of decreasing molecular weight and as expected the concentration of higher homologs decreased exponentially.

Attempts were made to resolve higher glucose homologs on a Bio-Gel P-2, minus 400 mesh column. Equal amounts of the five freeze-dried fractions from the 200 to 400 mesh column (Fig. 3) were dissolved in water and the mixture injected into a column minus 400 mesh (1.5 × 127 cm). With this gel oligomers up to a chain length of 13 glucose units were well resolved (Fig. 4). Products with a still higher molecular weight were excluded from the gel pores and appear within a single peak after passing the outer volume (V_o) through the column. This fraction stains blue on addition of iodine.

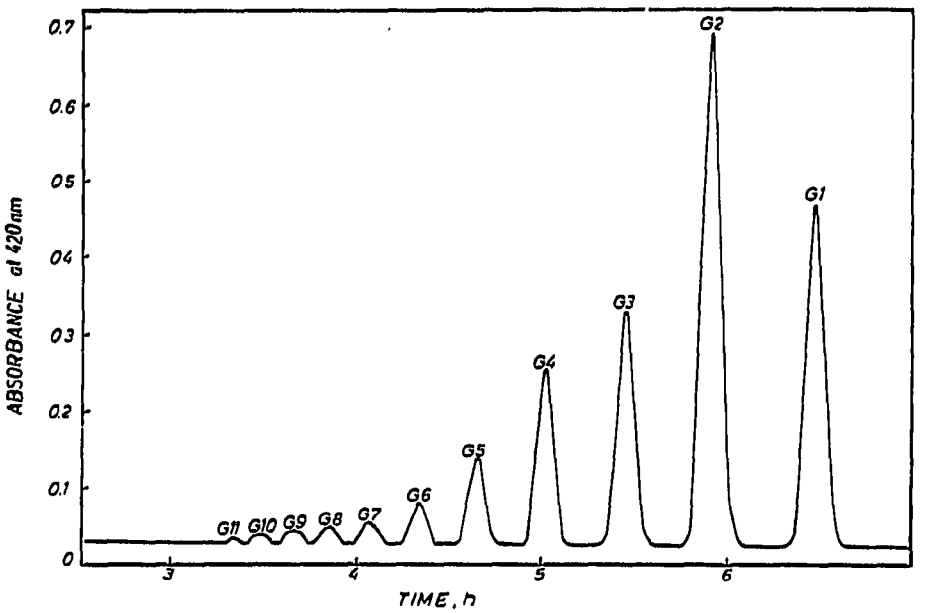

Fig. 3. Separation of glucose and oligosaccharides synthesized by the action of *E.coli* ML 30 on maltose. Bio-Gel P-2, minus 400 mesh, 65°; column 1.5 × 127 cm; eluent, water; flow rate, 28 ml/h. Peaks G1–G11 = glucose to maltoundecaose.

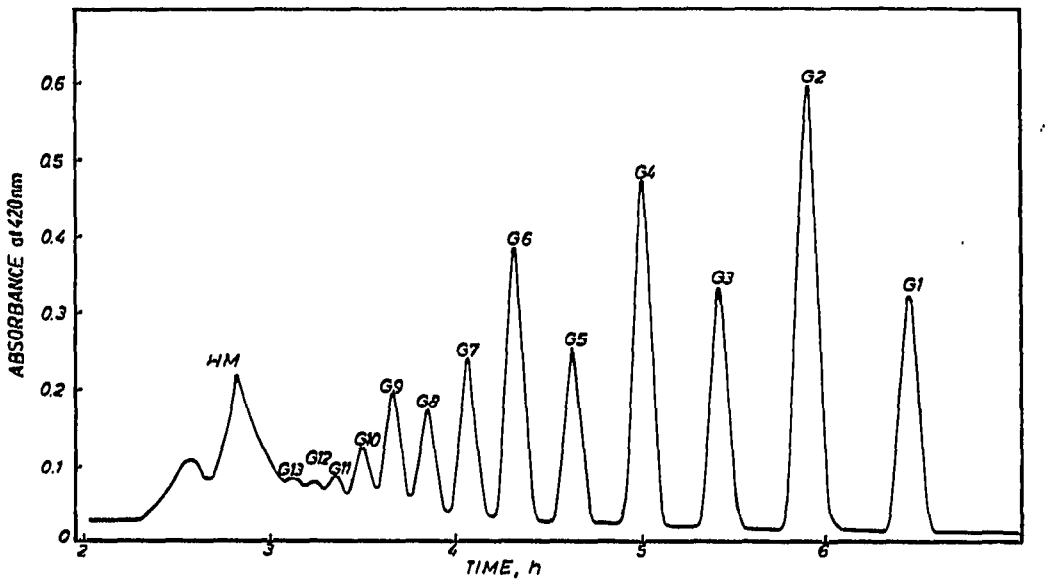

Fig. 4. Rechromatography of oligosaccharides from Fig. 3 (see text) under same conditions as mentioned there. HM = high molecular weight fraction. Outer volume, V_o = 2.58 h × 28 ml/h = 72 ml.

Degradation with β-amylase

In order to prove the exclusive existence of α-1,4-glucosidic linkages, the oligosaccharides synthesized by the action of *E. coli* cells on maltose were incubated with purified β-amylase from barley in phosphate buffer (0.02 *M*, pH 6,5, 40°) before application to the column. Fig. 5 shows that the higher homologs were completely degraded within 2 h, maltose, maltotriose and glucose being the only degradation products.

Whenever other glucosidic linkages were formed by the cells in appreciable amounts, for instance 1,6 linkages, the degradation products (isomaltose and isolmaltotriose) would have been detected in the effluent as shown below. These findings agree with the results published by BARKER AND BOURNE[25] who also established that only α-1,4-linkages are formed by the action of amylomaltase.

Relation of elution volume to molecular weight

Separations of high molecular weight substances by gel chromatography are considered to be based on the exclusion principle, small molecules being able to diffuse

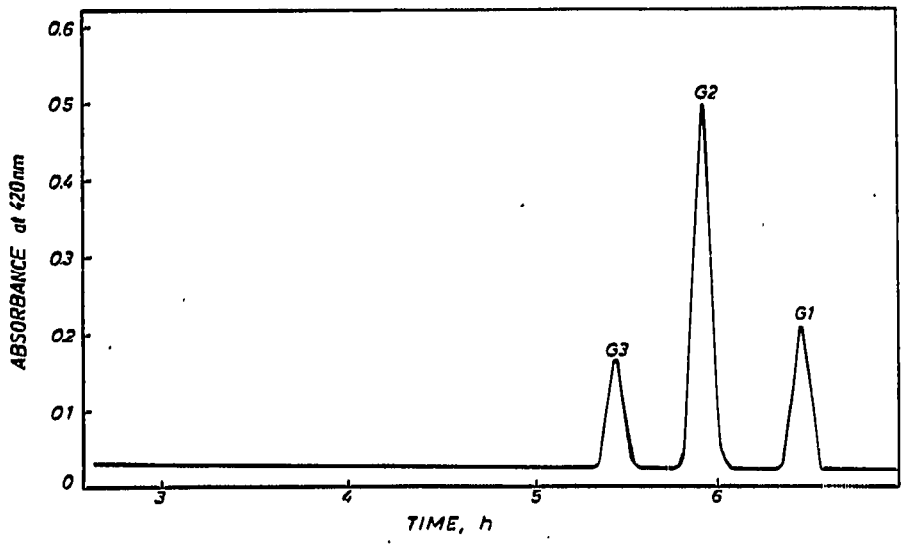

Fig. 5. Oligosaccharides synthesized by the action of *E.coli* ML 30 on maltose were degraded by β-amylase and chromatographed as in Fig. 3. G1, G2, G3 represent glucose, maltose and maltotriose respectively.

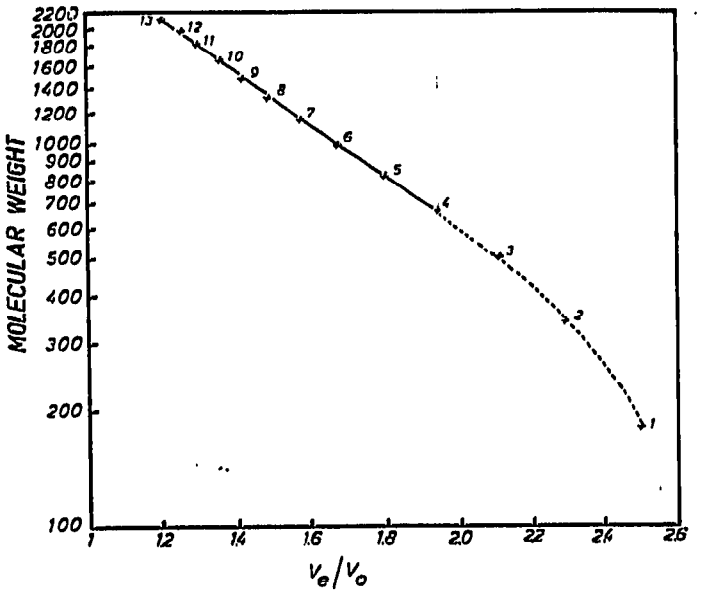

Fig. 6. Semi-logarithmic plot of molecular weight of oligosaccharides *versus* relative elution volume (V_e/V_o).

into the gel matrix, while big molecules are not able to do so, thus the small ones become retarded in the column. A consequence of this theory is the fact that the elution volume of a substance is inversely proportional to the logarithm of its molecular weight[28]. As shown in Fig. 6 the logarithm of molecular weight is a linear function of elution volume. This, however, holds true only for oligosaccharides with more than four glucose units. Mono-, di- and trisaccharides do not fit into this function and one might assume that a mechanism other than gel exclusion is the basis for the separation of sugars with mol. wt. less than 600. Experiments on this point are in progress and will be published later.

Resolution of other carbohydrates

Based on these considerations we tested the chromatographic separation of isomeric di- and trisaccharides. A solution of glucose, maltose and maltotriose, together with a mixture containing mainly isomaltose and isomaltotriose (α-1,6-glucosidic) from a degradation of dextran were injected into a "minus 400"-column. Fig. 7 shows the resolution of the 5 sugars, though there is no difference in molecular weight of the two di- and the two trisaccharides. The incomplete separation of maltose from isomaltose may probably be improved by increasing the column length. Another example is shown in Fig. 8 where ribose, glucose, sucrose and raffinose are separated. The pentose is almost completely separated from the hexose although their molecular weights differ only by 30 units.

Quantitative estimations

Provided that the liquid flow through the monitoring system is kept constant by the use of precision pumps the method is suitable for the quantitative estimation of carbohydrates. As each peak width at half height is constant and independent from the amount of substance, the peak height, measured from the baseline should be proportional to the concentration. In Fig. 9 the peak heights are plotted against the

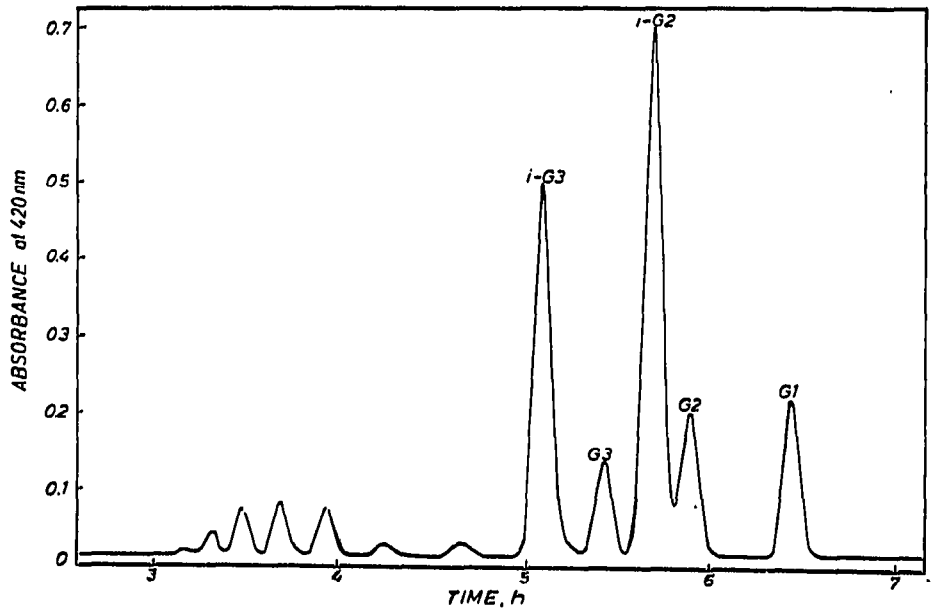

Fig. 7. Resolution of glucose (G1), maltose (G2), isomaltose (i-G2), maltotriose (G3) and isomaltotriose (i-G3) on Bio-Gel (conditions as in Fig. 3).

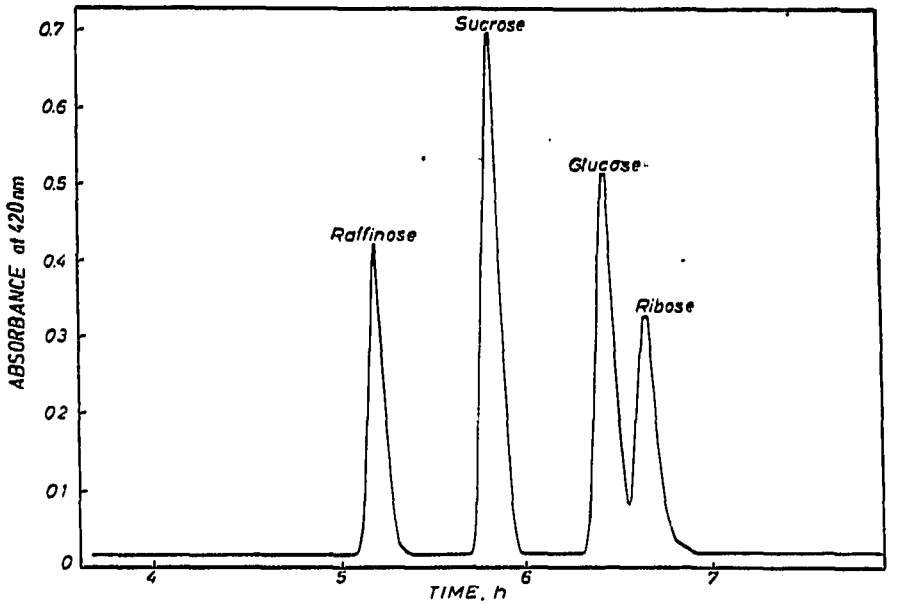

Fig. 8. Chromatography of a mixture of 38 μg ribose, 110 μg glucose, 125 μg sucrose and 85 μg raffinose on Bio-Gel (conditions as in Fig. 3).

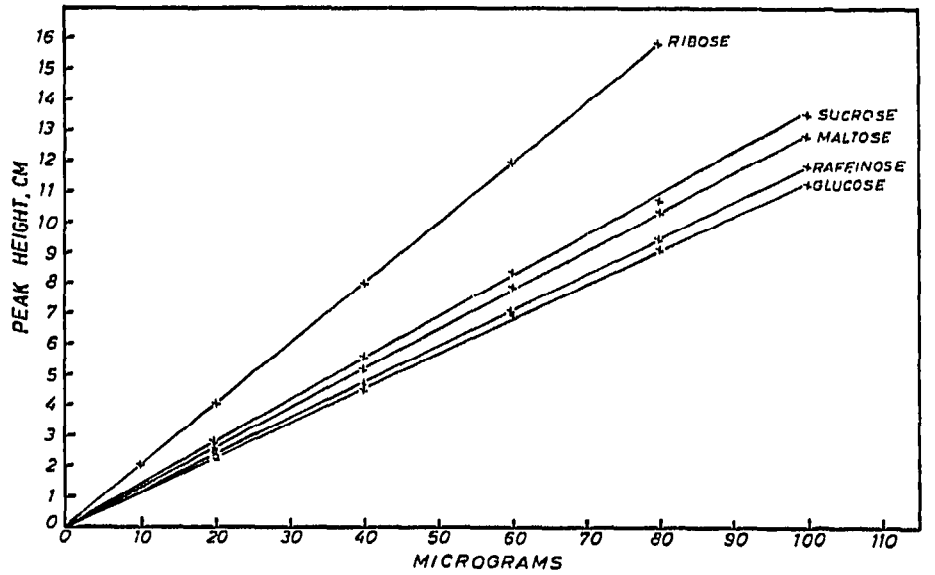

Fig. 9. Calibration curves for different sugars from their peak heights on Bio-Gel P-2, minus 400 mesh.

amounts of sugars, injected into an analytical column (1.5 × 127 cm, minus 400 mesh). For five different carbohydrates linear calibration curves were obtained in the range of 10 to 100 μg.

CONCLUSIONS

The results indicate that separations and quantitative determinations of carbohydrates can be obtained using polyacrylamide gel chromatography. This technique permits direct analysis of sugars in biological materials without extensive preliminaries. Moreover, continuous practice is possible, because the procedure is not based on gradient elution and the column must not be regenerated. The sample is injected directly

into the stream of eluent without interrupting the process. In this way the analysis of a mixture of three sugar components can be achieved by applying a sample every 90 min to the analytical column, though the entire time for passing glucose through the length of the column amounts to about 400 min. We currently use this method to study the consumption of sugars by yeasts in common fermentation broths.

In contrast to several other methods based on adsorption or ion-exchange the widths of peaks in gel chromatography are constant and independent of concentration and therefore quantitative estimations are possible on the base of peak height without integration. The use of distilled water as an eluent is another advantage because separated sugars can be obtained in pure form by evaporation or lyophilisation.

Polyacrylamide gels are not attacked by the usual microorganisms and little care need be taken to provide for sterile conditions. A column, once packed in the manner described and handled with care gives absolutely reproducible results for a period of several months.

Thus polyacrylamide gel chromatography of carbohydrates, being rapid and economical, offers a useful procedure equivalent or even superior to other usual methods.

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REFERENCES

1 K. MACEK, in I. M. HAIS AND K. MACEK, *Handbuch der Papierchromatographie*, Vol. 1, VEB Fischer Verlag, Jena, 1963, p. 332.
2 C. E. WEILL AND P. HANKE, *Anal. Chem.*, 34 (1962) 1736.
3 C. C. SWEELEY, R. BENTLEY, M. MAKITA AND W. W. WELLS, *J. Am. Chem. Soc.*, 85 (1963) 2497.
4 J. X. KHYM AND L. P. ZILL, *J. Am. Chem. Soc.*, 74 (1952) 2090.
5 J. G. GREEN, *Natl. Cancer Inst. Monograph.* No. 21 (1966) 447.
6 R. B. KESLER, *Anal. Chem.*, 39 (1967) 1416.
7 J. I. OHMS, J. ZEC, J. V. BENSON AND J. A. PATTERSON, *Anal. Biochem.*, 20 (1967) 51.
8 E. F. WALBORG AND R. S. LANTZ, *Anal. Biochem.*, 22 (1968) 123.
9 R. L. WHISTLER AND D. F. DURSO, *J. Am. Chem. Soc.*, 72 (1950) 677.
10 R. L. WHISTLER AND J. N. BEMILLER, in R. L. WHISTLER AND M. L. WOLFROM, (Editors), *Methods in Carbohydrate Chemistry*, Vol. 1, Academic Press, New York and London, 1962, p. 42.
11 D. FRENCH, J. F. ROBYT, M. WEINTRAUB AND P. KNOCK, *J. Chromatog.*, 24 (1966) 68.
12 J. A. THOMA, H. B. WRIGHT AND D. FRENCH, *Arch. Biochem. Biophys.*, 85 (1959) 452.
13 J. K. N. JONES, R. A. WALL AND A. O. PITTET, *Can. J. Chem.*, 38 (1960) 2285.
14 P. JONSSON AND O. SAMUELSON, *Anal. Chem.*, 39 (1967) 1156.
15 R. M. SAUNDERS, *155th Am. Chem. Soc. Meeting*, 1968.
16 P. NORDIN, *Arch. Biochem. Biophys.*, 99 (1962) 101.
17 J. HELLER AND M. SCHRAMM, *Biochim. Biophys. Acta*, 81 (1964) 96.
18 T. C. LAURENT AND K. A. GRANATH, *Biochim. Biophys. Acta*, 136 (1967) 191.
19 Y. C. LEE AND C. E. BALLOU, *Biochemistry*, 4 (1965) 257.
20 T. S. STEWART, P. B. MENDERSHAUSEN AND C. E. BALLOU, *Biochemistry*, 7 (1968) 1843.
21 J. C. SPECK AND D. J. RYNBRANDT, *Anal. Biochem.*, 19 (1967) 426.
22 S. HJERTÉN AND R. MOSBACH, *Anal. Biochem.*, 3 (1962) 109.
23 G. TRÉNEL, M. JOHN AND H. DELLWEG, *FEBS-Letters*, 2 (1968) 74.
24 J. MONOD AND A. M. TORRIANI, *Compt. Rend.*, 227 (1948) 240.
25 S. A. BARKER AND E. J. BOURNE, *J. Chem. Soc.*, (1952) 209.
26 H. WIESMEYER AND M. COHN, *Biochim. Biophys. Acta*, 39 (1960) 427.
27 A. SCHWARTZ AND B. A. ZABIN, *Anal. Biochem.*, 14 (1966) 321.
28 H. DETERMANN AND W. MICHEL, *J. Chromatog.*, 25 (1966) 303.