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THE FULLY AUTOMATIC ION-EXCHANGE AND GEL-PERMEATION CHROMATOGRAPHY OF NEUTRAL MONOSACCHARIDES AND OLIGOSACCHARIDES WITH A JEOLCO JLC-6AH ANALYSER

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ABSTRACT

Experiences with a Jeolco JLC-6AH carbohydrate analyser used to provide a fully automated analysis service for neutral mono- and oligo-saccharides have been summarised Modifications have been made to the equipment to extend its application, and a method for routine separations of oligosaccharides (d p 2–15) has been established The degree of separation achieved is far superior to that which may be obtained by gel-filtration techniques under gravity- or peristaltic-pump-aided flow The versatility and adaptability of equipment for use with assays other than the standard orcinol–sulphuric acid reaction shows that it is not too inconvenient to interchange between various assays and separations

INTRODUCTION

Analytical separations of monosaccharides and oligosaccharides have been achieved by a number of methods including paper chromatography, t l c , charcoal columns, ion-exchange resin columns, and g l c on a variety of liquid phases on solid supports The most versatile methods have proved to be ion-exchange chromatography and g l c their various forms are reviewed annually[1] For the analysis of solutions of monosaccharides and smaller oligosaccharides, liquid phase ion-exchange chromatography is more convenient, as derivatisation or removal of water is not necessary Moreover, good separations may be achieved conveniently by using the borate complexes of the carbohydrates[2] Accordingly, for the handling of large numbers of samples automatically, such a system was adopted for carbohydrate analysis at the inception of the University of Birmingham Macromolecular Analysis Service We now describe our results with, and adaptations of, the Jeolco JLC-6AH carbohydrate analyser

EXPERIMENTAL AND RESULTS

Equipment. — A Jeolco JLC-6AH standard amino acid analyser (Jeol, Japan Electron Optics Laboratory Co. Ltd., Tokyo) was converted for use as an ion-exchange chromatographic analyser for carbohydrates by installation of (*a*) a piston pump suitable for conc. sulphuric acid, (*b*) absorbance filters for 510 and 425 nm, and (*c*) a second column to duplicate the action of the first. A schematic diagram of the analyser is shown in Fig. 1. The series of borate buffers (A) are selected by two eight-way valves (B_1 and B_2) and are pumped by two double-action, high-pressure

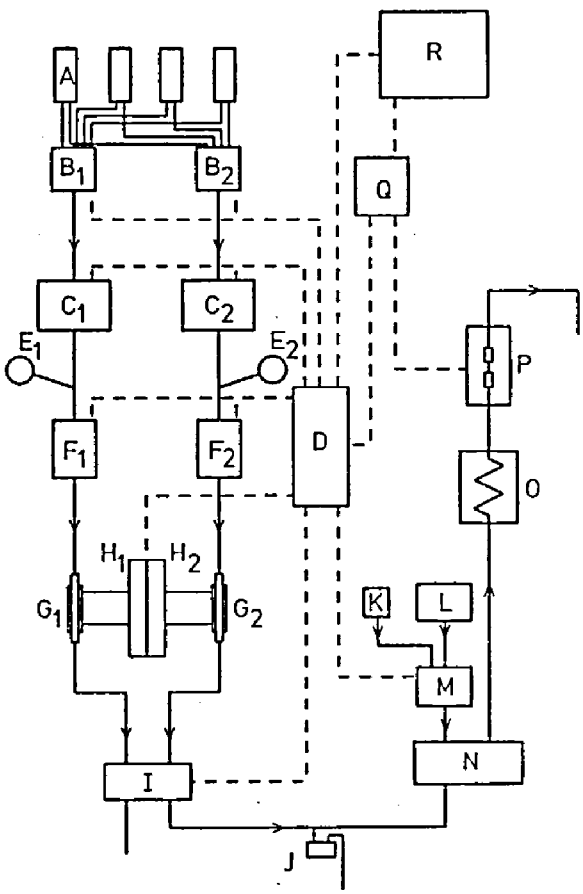

Fig. 1. Diagram of the modified automatic carbohydrate analyser.

pumps (C_1 and C_2). Buffer and sample selection and flow switching are performed by a tape programmer (D) and the output from the pumps passes the pressure gauges E_1 and E_2. The samples, previously injected into the storage loops F_1–F_2, are pumped on to columns G_1 or G_2 maintained at 55° by independent heating circulating pumps H_1 and H_2. The eluant from these columns passes to a two-way selector (I), which enables either of the two columns to be connected to the detection system.

An all-polytetrafluoroethylene (PTFE) pressure pump (N) draws the sample stream from I (with excess running to waste J) and mixes it with the orcinol–sulphuric acid reagent which is drawn from storage K *via* a valve M which can also admit water L. The reagent–sample mixture is then pumped through a reaction coil (O) at 95°. The resulting colour is measured by two flow-cells (P) at 425 and 510 nm. The data are then printed out onto a point–plot chart recorder (Q). The data may be calculated and quantitated by means of a Nova 1220 computer (R) which is on-line to the analyser.

Ion-exchange chromatography. — The short-type jacketed columns (17 × 0.8 cm i.d.) were packed with LC-R-3 quaternary ammonium ion-exchange resin (Jeol) (bed height, 12 cm), and regenerated and equilibriated at 30 ml/h (see elution programme, Table I). Solutions of samples (1–1000 μg of each component) were centrifuged to remove any insoluble material, loaded into the storage loops (capacity, 800 μl) for automatic loading in turn on to one of the ion-exchange columns, and eluted in descending fashion at 55° at the 30 ml/h flow-rate using a modified buffer programme (Table I) which gave improved separations (Fig. 2). The elution programme was made repetitive by using an appropriately punched tape in the elution programmer. Recrystallised orcinol dissolved in conc. sulphuric acid (1.5 g/l) was used as assay reagent; when stored in the equipment at 4–6°, the reagent is stable for

TABLE I

RECOMMENDED ELUTION PROGRAMME AND ELUTION POSITIONS FOR ION-EXCHANGE CHROMATOGRAPHY OF CARBOHYDRATES ON A JEOLCO JLC-6AH ANALYSER

Molarity and pH of potassium tetraborate buffer[a]	*Duration of pumping (min)*	*Function of eluant*	*Elution time*[b]	
0.13M (pH 7.50)	110	Separation of di- and tri-saccharides and some deoxymonosaccharides	2-Deoxyribose	0.47
			Sucrose	0.62
			Cellobiose	0.80
			Maltose	1.13
			Lactose	1.38
			Rhamnose	1.58
0.25M (pH 9.08)	90	Separation of monosaccharides	Ribose	2.75
			Mannose	3.22
0.35M (pH 6.0)	190	Separation of monosaccharides	Fucose	4.03
			Arabinose	4.12
			Fructose	4.33
			Galactose	4.46
			Xylose	4.75
			Glucose	5.38
0.50M (pH 9.60)	90	Regeneration of column	—	—
0.13M (pH 7.50)	120	Equilibration of column	—	—

[a]All eluant solutions are filtered through a Whatman cylindrical filter after preparation. [b]These times may vary over a period according to differences in the length, state, and age of the resin bed, but without alteration of the elution order.

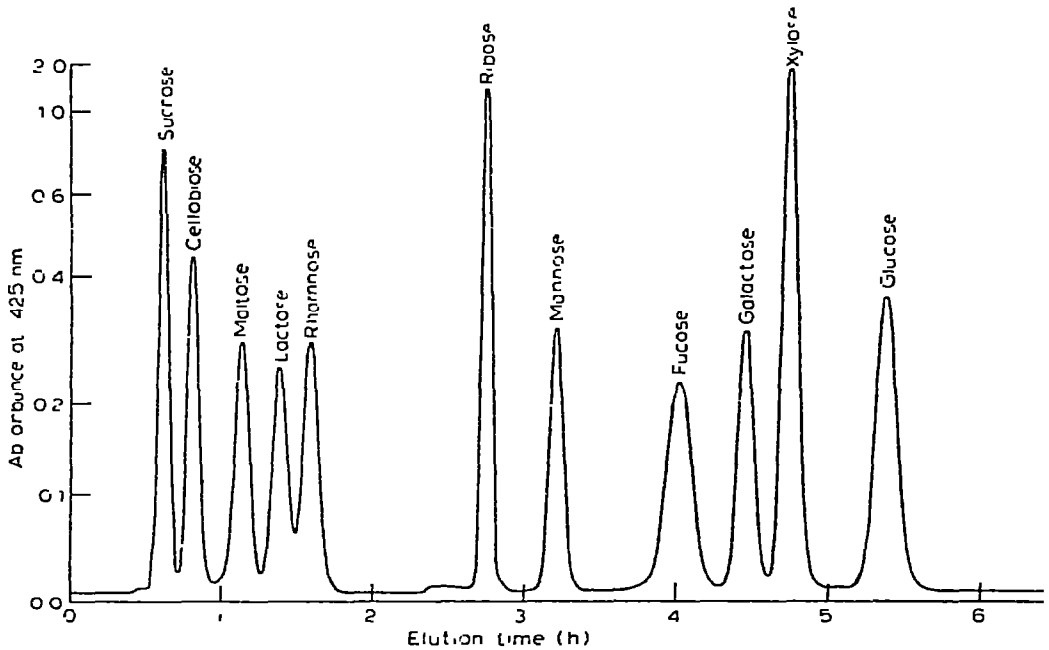

Fig 2 Chromatography of neutral carbohydrates on Jeol LC-R-3 ion-exchange resin (borate form)

at least 2–3 weeks This reagent is mixed (24 ml/h) with the sample taken from the column eluate (sampling rate, 12 ml/h) in the assay manifold The mixed reagent and sample was passed through the heating coil maintained at 95° (residence time, 15 min)

The ion-exchange columns were used as a pair running out of phase, so that analysis on one column was being carried out whilst the second column was being regenerated This gave a turn-round time of 6 5 h per analysis

Components were identified by their elution positions compared with those for standards run under the same conditions, and were quantitated from peak areas

Gel-permeation chromatography — The ion-exchange resin columns described above were replaced by a column (> 1 5 m × 2 cm i d) which was jacketed The column was packed at 20° with an aqueous slurry of Bio-Gel P2 (400 mesh) (Bio-Rad Laboratories), pre-swollen in water and vacuum-deaerated under water at 20°, to give a bed height of 1 5 m, and then fitted at the top with a standard Jeol adapter The column was slowly heated *via* the jacket to 65°, whilst being eluted at 16 ml/h in ascending fashion with deaerated water which had been impregnated with toluene as a bacteriostat (see Fig 3) This was achieved by using one side of a spare pump identical to that integral with the analyser and used for pumping the ion-exchange columns (see Discussion) Samples of carbohydrate (1–1000 μg of each component) were centrifuged, transferred to the storage loops, and automat-

ically loaded on to the column as before The column was eluted in ascending fashion at 65 with deaerated water at 16 ml/h to give separations on a basis of molecular size A typical elution profile for a starch hydrolysate is shown in Fig 4 The orcinol reagent, reagent and column sampling rates, heating of the assay mixture, and identification and quantitation, were as before The turn-round time was 20–22 h per analysis

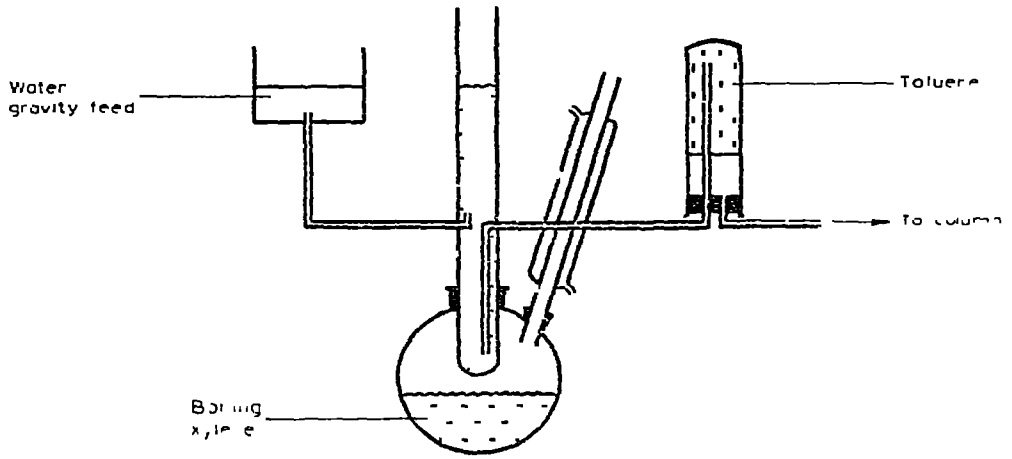

Fig 3 Deaeration and toluene-impregnation system for water input to column

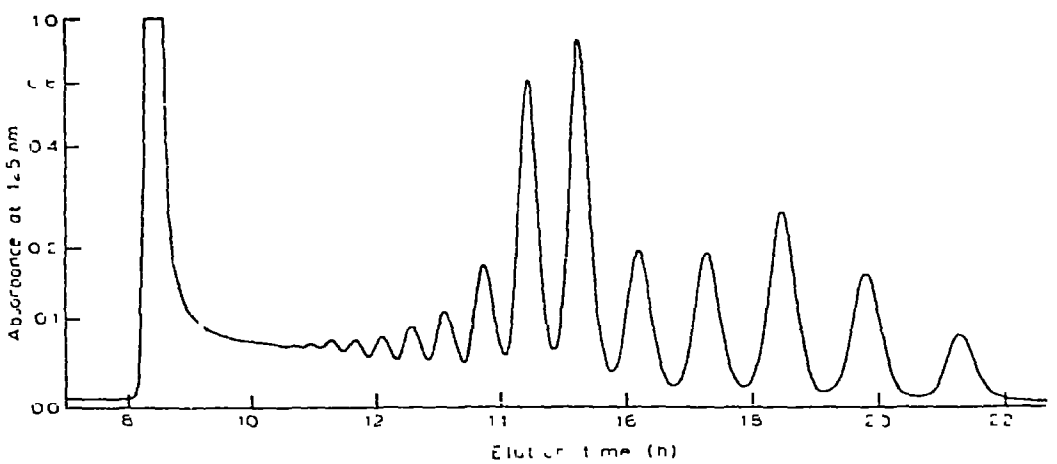

Fig 4 Chromatography of starch and oligosaccharides therefrom on Bio-Gel P2

DISCUSSION

In the first year of use of the Jeolco JLC-6AH carbohydrate analyser, repeated failure of the O-rings of the sulphuric acid pump occurred However, a modification has now been introduced by Jeol, in which the O-ring is dispensed with and the cylinder is packed with PTFE chevrons which can be tightened to give a good fit on the piston and cylinder wall As wear progresses, the chevrons are tightened further to maintain the fit Ultimately, the chevrons are replaced (life-time, at least one year) Nevertheless, the use of conc sulphuric acid remains a hazard, in view of the extensive damage which can arise from a small leak A matrix programmer is now available as a replacement for the punched-tape programming unit, and is more convenient to operate

A further problem has been the deposition of purple material in the heating coil which carries the mixed reagent and column eluate This colour continued to develop, even after extensive purification of the orcinol and use of high quality conc sulphuric acid, and ultimately blocked the coil and made replacement necessary

Flushing of the sample storage loops with 0 02% sodium azide solution before filling is essential to avoid microbial degradation of samples awaiting analysis Azide at this concentration has no effect on the orcinol–sulphuric acid assay Gel-filtration columns, which do not have an alkali wash, should also be flushed from time to time with azide

Although usually used with the standard 2-mm flow-cells, the sensitivity of response of the analyser may be increased by using 10-mm cells

The absorption maximum (λ_{max}) for the orcinol chromophore arising from most neutral sugars occurs at 420 nm, and accordingly the filter to measure absorbance at 425 nm is acceptable The trace from the 510-nm filter is rarely used, it being intended for low sensitivity–high proportion in sample However, it is useful for confirmation of peak identification for fucose or rhamnose or carbohydrates containing these structures, as the relative absorbances of the chromophore at 425 and 510 nm are different from those of the other monosaccharides The ratios of A_{425} A_{475} A_{510} are glucose 1 00 0 45 0 46, fructose, 1 00 0 48 0 45, fucose, 1 00 0 71 0 34, rhamnose, 1 00 0 49 0 27)

The ion-exchange chromatographic system described herein is excellent for identification and quantitation of the common mono- and di-saccharides (Fig 3), including the components of the carbohydrate moieties of glycoproteins The elution pattern is not directly discernible from the structures of the saccharides, since it arises from different preferred forms of each reducing unit and from the number of borate ions complexed per molecule Trisaccharides and higher oligosaccharides are not readily separable since they are almost unabsorbed by the column The overlap of the elution positions of arabinose, fucose, and fructose is not a serious disadvantage, since it is uncommon for these sugars to occur together in a sample Complete separation could be achieved by alteration of the eluant programme with concomitant lengthening of the turn-round time Extension of the technique to

neutral sugars other than those in Fig 2 may require modification of the buffer programme The turn-round time cannot be reduced for a full analysis, but shorter runs for the separation of selected carbohydrates may be conducted with modification of the buffer programme For example, mixtures of D-fructose and D-glucose may be analysed using a buffer programme in which only the pH 9 08 and pH 9 60 buffers (Table I) are used for column elution (regeneration and equilibration steps unchanged), this gives a turn-round time of less than 4 h

With the standard 2-mm flow-cells, as little as 0 1 μg of carbohydrate can be detected It is recommended that one in twelve runs is a standard, *i e* one standard run every cycle of the samples The resin packings were stable for at least 1 year, but columns gradually accumulate a brown coloration/deposit at the top When this is very noticeable, the separations are impaired by peak broadening Replacement is the only satisfactory remedy, but only the affected part of the bed need be changed

The gel-permeation chromatography mode of operation of the analyser provides an excellent separation of oligosaccharides and monosaccharide in the range d p 1–15 (Fig 4) Such separations in our experience, cannot be achieved with the same column by gravity-flow or peristaltic-pump elutions Deaerated input water and ascending elution are necessary to avoid " cracking" of the gel bed Normally, the flow in the column is maintained even when the column is not in use Variation of the column operating-temperature showed that 65° is optimal, insulation of the long column being necessary

No regeneration or equilibration steps are necessary in the running of the gel-permeation column However, to achieve separation of the higher oligosaccharides, 20 h are required when the analyser is operated in the mode described above Each of the two double-action high-pressure pumps (C1 and C2) consists of two piston (*e g* $C1_a$ and $C2_a$) pumps working 180° out of phase to even out the pulse effect, for normal operation, the outputs from $C1_a$ and $C1_b$ are joined to give the final output from C1 Since $C1_a$ and $C1_b$ cannot be decoupled to leave one stationary, and since the minimum flow rate of C1 is 32 ml/h, one side of a spare pump was used to achieve the slow flow-rate It was not necessary to introduce any additional damping device to compensate for the fact that only one side of the pump was being employed

In order to maximise time-saving, the equipment was modified to allow the use of one column in which the minimum residence time of 8 h is used as a means of overlapping runs Thus, the second sample is introduced to the column not more than 8 h before the completion of the first run, *i e* , the runs are 240°, rather than 360°, out of phase The relevant modification to the equipment requires breaking the pipe connection between F_1 and G_1 and linking it into the pipe between F_2 and G_2 (G_2 is the column location being used) In this mode, C_1 and C_2 stop and start together (thereby avoiding spurs on the chart) and act as non-return valves when not actually operating The sample loader automatically becomes more versatile since samples can be loaded at will by selecting F_1 or F_2, whichever is not being pumped through to the column (samples cannot normally be introduced when column elution is in

progress) By sacrificing marginally on the separations achieved, the convenient flow-rate of 32 ml/h is retained This gives a final turn-round time of 13 h per run, with overlapping for the last 5 h of each run

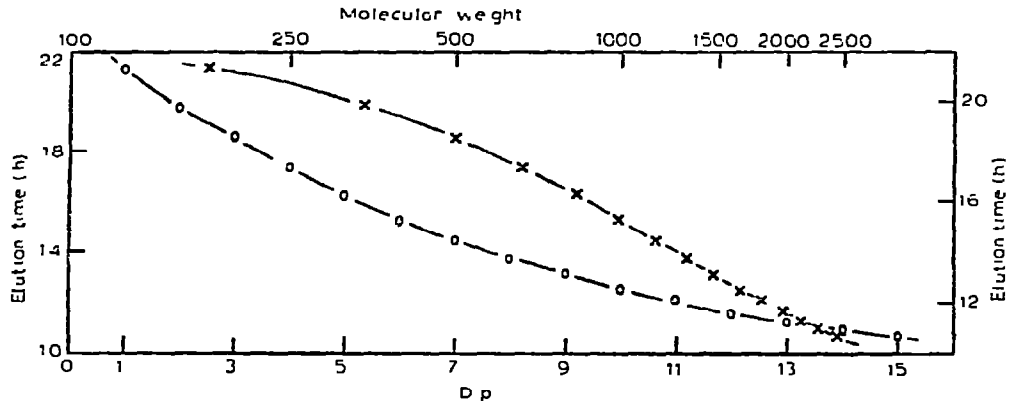

Fig 5 Relationships between the molecular weight and elution position for a series of maltosaccharides fractionated on Bio Gel P2 —×—, log scale, —○—, linear scale

In the maltosaccharide series, plots of molecular weight against elution position gave curves (Fig 5) for which the shapes are commensurate with the accepted theories of gel-filtration techniques As reported[3] elsewhere, isomaltose is eluted from Bio-Gel P2 in a slightly different position to maltose For the analysis of hydrolysates of amylose, amylopectin, and starch, the presence of one or two (1→6)-linkages in the otherwise maltosaccharides does not affect the elution position of the oligosaccharides This is advantageous for quantitation of starch oligosaccharides, but disadvantageous for identification purposes

For most series of oligosaccharides pure standards are not readily available for the higher members, but for many applications to starch hydrolysates it is sufficient to express the amount of each component as a percentage of the total carbohydrate present For D-glucose, maltosaccharides, and starch, the colour yield for each D-glucose residue is, within experimental limits, identical Using the standard 2-mm flow-cells, as little as 0 1 μg of carbohydrate can be detected The stability of the P2 gel has proved to be excellent Undoubtedly, the regular washing with azide aids in this respect, but columns maintained and used at 65° for a year or more have not shown any decrease in performance level

The successful adaptation of the Jeolco JLC-6AH analyser to automated gel-permeation indicated that the machine could also be used for the separation of orcinol-sulphuric acid positive carbohydrates by the ion-exclusion phenomena using ion-exchange resin as the column packing[4] We have found that a satisfactory performance in this mode can be achieved Furthermore, the sensitivity of the orcinol

reagent to hexuronic acids (λ_{max} 518 nm, but also measurable at 425 and 440 nm) allows their fractionation and quantitation

In practice, samples for carbohydrate analysis usually involve mixtures of free saccharides from industrial processes such as sucrose D-glucose and D-fructose production, and starch hydrolysis, from clinical specimens and physiological fluids, from macromolecules such as glycoproteins and polysaccharides and from synthetic work For ion-exchange chromatography, samples should normally be dissolved in 0 13M boric acid for column loading, this is recommended to ensure that the sample is not loaded at neutral or higher pH which gives rise to peak broadening Where hydrolysis is necessary to release the carbohydrate, the optimum conditions for a glycoprotein are 2 0M trifluoroacetic acid in a sealed tube at 100° for 6 h, after which the acid is removed by rotary evaporation *in vacuo* However, the most accurate results can only be obtained *via* a range of hydrolysis conditions to take account of the different stabilities of monosaccharides and their glycosyl bonds For gel-permeation chromatography, it is desirable to adjust the pH of sample solutions to 5–8 before loading

Although the Jeolco JLC-6AH carbohydrate analyser, when modified and used as described above, can provide an acceptable analytical service, instruments designed to utilise the reactions of carbohydrates with 4-anisyltetrazolium chloride (Tetrazolium Blue)[5], or with periodic acid followed by determination of the liberated formaldehyde/acetaldehyde[6, 7], would avoid the use of conc sulphuric acid Fluorimetric assays give greater sensitivity Although fluorimetric assays of carbohydrates have received little attention, the chromophore of the periodate–formaldehyde assay is fluorescent, and an automated version has been developed[6, 7]

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