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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ELUTION BEHAVIOUR OF OLIGOSACCHARIDES, MONOSACCHARIDES AND SUGAR DEGRADATION PRODUCTS ON SERIES-CONNECTED ION-EXCHANGE RESIN COLUMNS USING WATER AS THE MOBILE PHASE

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SUMMARY

The high-performance liquid chromatographic separation of oligosaccharides, monosaccharides, sugar degradation products and alcohols using a series-connected system of different ion-exchange columns with water as the eluent was investigated. The non-additivity of the number of theoretical plates (N), the height equivalent to a theoretical plate (H) and the resolution (R_s) on ion-exchange stationary phases is discussed.

INTRODUCTION

The rapid qualitative and quantitative analysis of oligosaccharides, monosaccharides, short-chain and heterocyclic aldehydes and also alcohols and ketones has been a central problem in food and clinical chemistry and in biotechnology. By means of liquid chromatography, mono- and oligosaccharides have been determined on amino-bonded column materials, while reversed-phase C_{18} materials have been used for the separation of aldehydes, ketones and alcohols[1–12]. More recently, ion-moderated high-performance liquid chromatography (HPLC) has been employed to separate monosaccharides, aldehydes, ketones, alcohols and oligosaccharides[13–21]. A complete single-run determination of all the classes of compounds cited has not been achieved by using these methods.

Different ion-exchange resins as stationary phases allow good separations of oligosaccharides, whereas monosaccharides and sugar degradation products are not resolved sufficiently. On the other hand, some of the stationary phases show a good ability to separate monosaccharides and further degradation products but fail with oligomeric sugars.

By choosing appropriate ion-exchange resins for combination within a single mobile phase, their separation specificities can be united to provide a system that permits the above substance groups to be separated in one analytical run. To attain this object, it seems to be possible to make a series connection of two or more columns with an initial separation step on a primary column and further separation of the

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eluted fractions in the second column using the same mobile phase[22–27]. In this arrangement the lengths of the columns and the stationary phases can be varied to achieve an acceptable elution order.

The aim of this work was the analysis of oligomeric sugars and of monosaccharides and related products in a single separation step by using a series-connected system of ion-exchange columns with water as the mobile phase.

EXPERIMENTAL

Apparatus

The liquid chromatographic equipment consisted of a pump (Beckman Model 112; Beckman, Berkeley, CA, U.S.A.), a sample injection valve (Beckman Model 210) with a 20-μl loop, a column switching valve (Whitey, OH, U.S.A.), a three-way valve (Scientific Systems, PA, U.S.A.), a Shimadzu CTO-2A column oven unit (Shimadzu, Kyoto, Japan), a refractive index detector (Altex, Berkeley, CA, U.S.A.) and an integration system [Shimadzu C/R2A (X)].

Columns

The following stationary phases and columns were employed: (I) Ca-loaded sulphonated polystyrene–divinylbenzene resin, 7.5% cross-linked, 8 μm, 300 × 7.8 mm I.D. (μSpherogel Carbohydrate N; Beckman); (II) Ca-loaded cation-exchange stationary phase, 100 × 7.8 mm I.D. (Fast Carbohydrate; Bio-Rad Labs., Richmond, CA, U.S.A.); (III) H-loaded ion-exchange stationary phase, 100 × 7.8 mm I.D. (Fast Acid, Bio-Rad Labs.); (IV) Ag-loaded cation-exchange stationary phase, 4% cross-linked, 25 μm (Aminex HPX 42A; Bio-Rad Labs.). As the pre-column ion-exclusion Micro-Guard cartridges (Bio-Rad Labs.) were used.

Samples

All reference standard solutions were prepared from analytical-reagent grade chemicals (Fluka, Buchs, Switzerland; Merck, Darmstadt, F.R.G.; Sigma, St. Louis, MO, U.S.A.; Serva, Heidelberg, F.R.G.). For injection the samples were dissolved in doubly distilled water. As a standard for oligosaccharides a starch syrup was used. The investigated compounds are listed in Table I.

TABLE I

PEAK IDENTIFICATION OF THE INVESTIGATED COMPOUNDS

Peak No.	*Compound*	*Peak No.*	*Compound*
1	D-Glucose	9	D-Fructose
2	DP 2	10	D-Arabinose
3	DP 3	11	Dihydroxyacetone
4	DP 4	12	1,6-Anhydro-β-D-glucose
5	DP 5	13	Hydroxymethylfurfural
6	DP 6	14	Furfural
7	DP 7	15	Ethanol
8	D-Xylose		

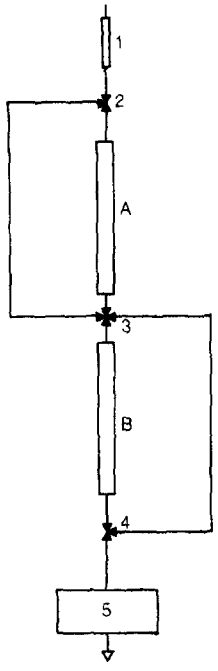


Fig. 1. Coupled system with pre-column (1) and separation columns (A) and (B); 2, 3, 4, valves; 5, refractive index detector.

The application sample (gluco-oligomers) was obtained by hydrothermal treatment of poplar wood[28].

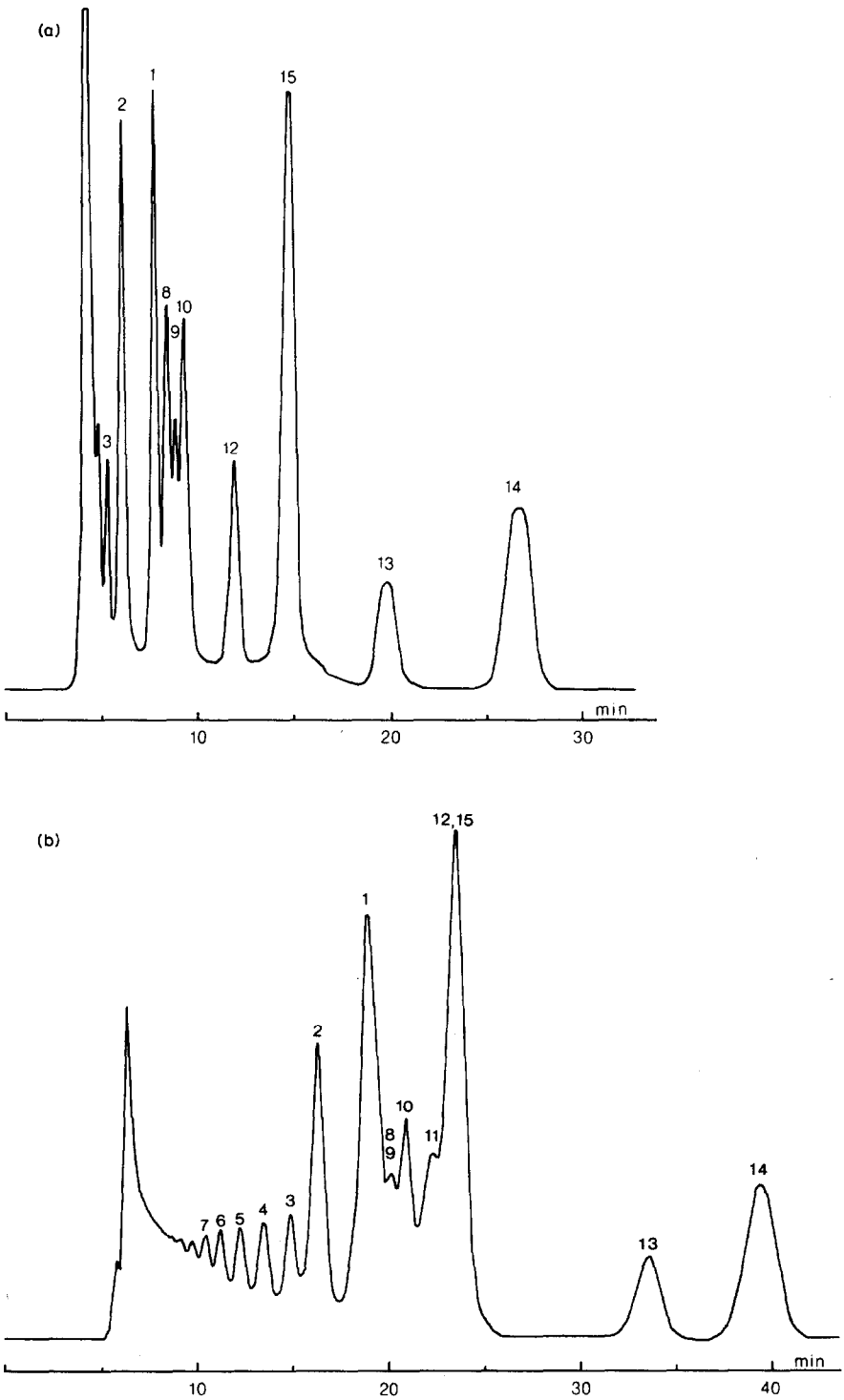
RESULTS AND DISCUSSION

The resolution per unit time decreases in order solvent programming > coupled columns > temperature programming ≈ flow programming > normal elution[24]. Solvent programming using the described stationary phases is subject to limitations because of the low sensitivity of these materials to organic solvents. Water was chosen as the mobile phase for the experiments with the coupled column systems.

As shown in Fig. 1, two columns A and B were connected in such a way as to make them available both individually and in series. The intention was that the oligomeric carbohydrates would be separated perfectly, the monosaccharides with minimum loss of resolution, and that further products of decomposition, rearrangements or fermentation would remain detectable with equally short analysis times.

Series connection of a Ca-loaded ion-exchange resin (8 μm) (I) with an Ag-loaded ion-exchange resin (25 μm) (IV)

In Fig. 2a and b, the chromatographic characteristics of the reference compounds (Table I), under the same conditions, are compared for both of the columns alone, with an optimum temperature in the range 85–95°C. On the Ca-loaded 8-μm resin the resolution (R_s) is good for the monosaccharides glucose, xylose and arabi-


(a)
1
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(b)
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min
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40

Fig. 2.

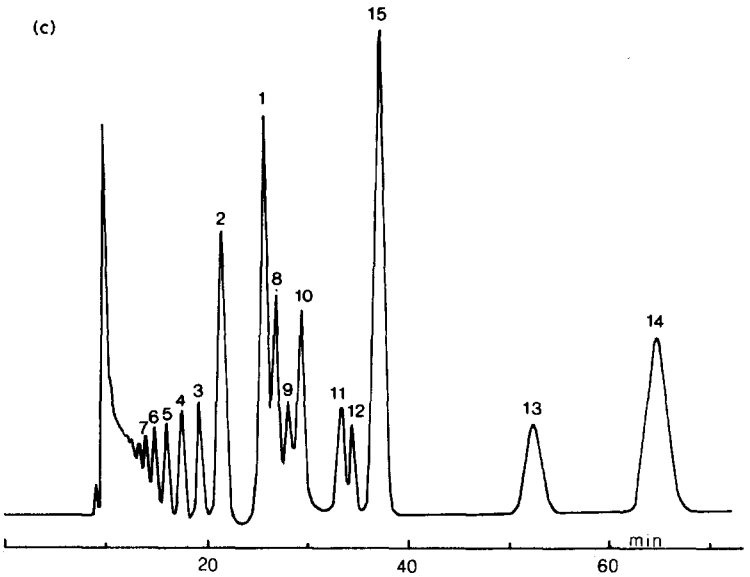

Fig. 2. Optimized separations of a standard mixture. For peak identification, see Table I. Chromatographic conditions: column temperature, 85°C; mobile phase, water; flow-rate, 0.6 ml/min; refractive index detection. Column: (a) I; (b) IV; (c) coupled I + IV.

nose, but poor for the pair fructose and arabinose. The 25-μm resin provides a good separation within the oligomeric part, up to a degree of polymerization (DP) of 9, but it is poor for glucose, xylose, fructose, arabinose, dihydroxyacetone and ethanol.

Fig. 2c shows the chromatogram of the coupled columns I + IV at 85°C and a flow-rate of 0.6 ml/min. On account of the very similar elutions for the monosaccharides, the R_s value was determined for the pairs glucose–xylose, glucose–fructose and xylose–fructose for both the single columns and the coupled system (Table II). A comparison of the calculated R_s values for these pairs of monosaccharides shows an insufficient resolution of the monosaccharides using the Ag-loaded ion-exchange resin as a single column; with the coupled system the separation of monosaccharides is good enough in all instances, but xylose–fructose can be separated more completely with the coupled system than with the separate columns alone.

TABLE II

RESOLUTION (R_s) OF MONOSACCHARIDES ON ION-EXCHANGE STATIONARY PHASES I AND IV

Chromatographic conditions as in Fig. 2.

Monosaccharide pair	*Column I*	*Column IV*	*Coupled columns I + IV*
Glucose–xylose	1.3	0.4	0.9
Glucose–fructose	2.2	1.1	1.9
Xylose–fructose	0.9	0.6	1.0

TABLE III

NUMBER OF THEORETICAL PLATES (N) AND HEIGHT EQUIVALENT TO A THEORETICAL PLATE (H) OF MONOSACCHARIDES ON A Ca-LOADED ION-EXCHANGE STATIONARY PHASE (I), AN Ag-LOADED ION-EXCHANGE STATIONARY PHASE (IV) AND ON THE COUPLED COLUMNS

Monosaccharide	*Column I*		*Column IV*		*Coupled columns I + IV*		
	N	H (mm)	N	H (mm)	N_c	H (mm)	N_c*
Glucose	3943	0.08	4515	0.07	7498	0.08	7463
Xylose	4157	0.07	4412	0.07	7417	0.08	7478
Fructose	3937	0.08	4315	0.07	7334	0.08	7322

* N_c calculated using eqn. 2.

Table III compares the calculated number of theoretical plates (N) and the height equivalent to a theoretical plate (H) of the columns alone and coupled. For the calculation of the number of theoretical plates in a coupled column system (N_c) the following expression is applicable for the connection of column A (N_A) and column B (N_B):

$$N_c \leqslant (N_A + N_B) \tag{1}$$

For the different combined stationary phases the numbers of theoretical plates (N_c) are not additive, but are related to the retention volumes (V_A, V_B) and the plate numbers (N) for the ion-exchange stationary phases used[27]:

$$N_c = \frac{(V_A + V_B)^2}{\frac{V_A^2}{N_A} + \frac{V_B^2}{N_B}} \tag{2}$$

In Table III the calculated N_c values are compared with the measured values; the agreement is good.

In Fig. 3, the dependence of retention time (t_R) on the use of single or coupled columns is demonstrated for the investigated substances; the resolution is good over the entire range for the coupled systems. For the strongly retained aldehydes, hydroxymethylfurfural and furfural, to be eluted more rapidly, the flow-rate was increased from 0.6 to 1.2 ml/min 35 min after the start. By this means, their retention times were reduced by about 7 min.

To reduce the analysis time further, the temperature was raised to 95°C and the pressure to flow-rate ratio of the eluent was optimally controlled so as to achieve flow-rates of up to 1.2 ml/min. Fig. 4 shows the chromatograms of the reference compounds obtained with columns I, IV and I + IV under the conditions described. Compared with the experiment at 85°C and a flow-rate of 0.6 ml/min the analysis time was more than halved (30 min).

In order to examine the sensitivity of the refractive index detector towards the coupled system, glucose and dihydroxyacetone were measured at various concentra-

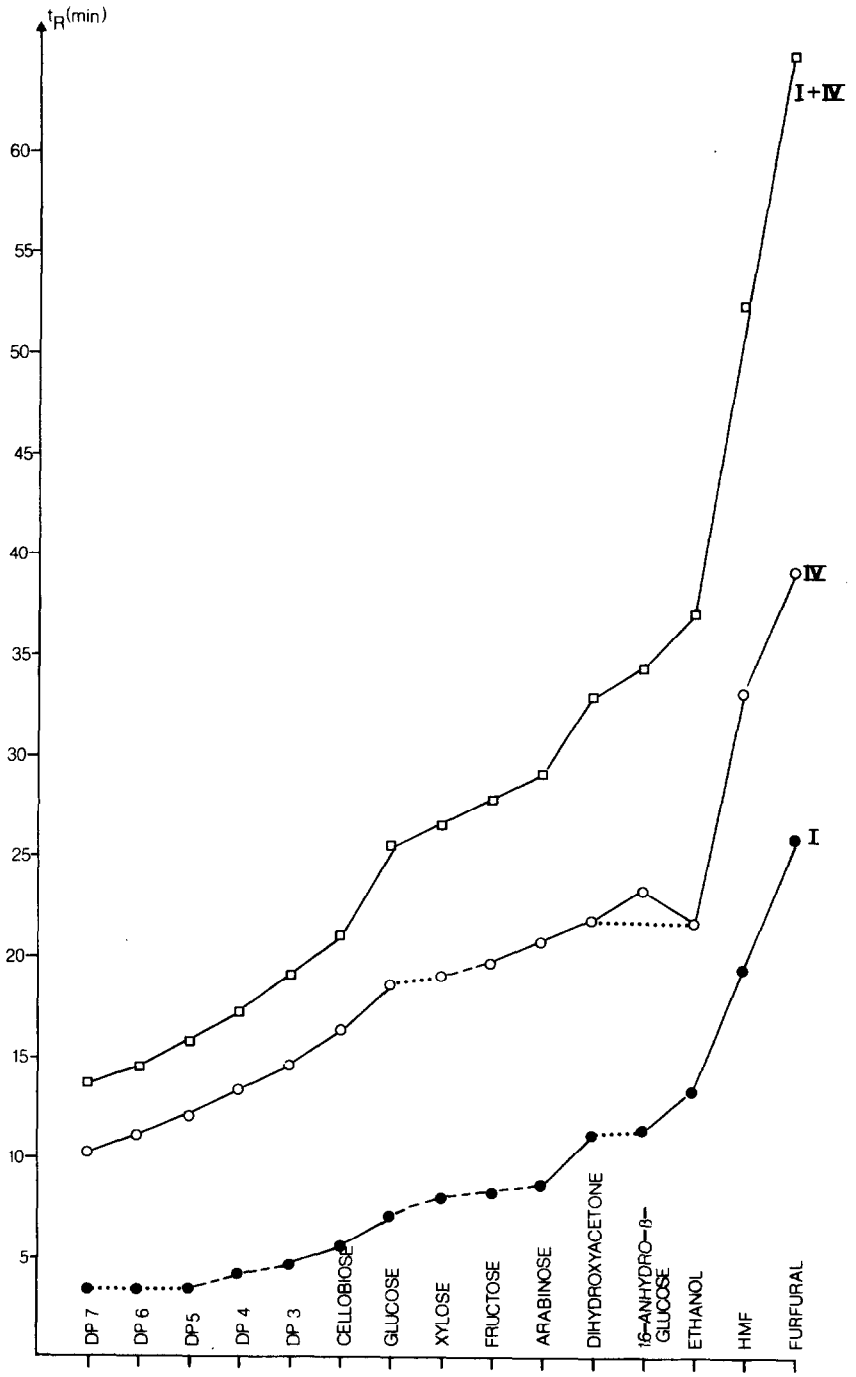

Fig. 3. Dependence of retention time, t_R (min), on the use of single and coupled columns (I and IV) for the investigated substances. ———, Good resolution; – – –, minimum loss of resolution; ·····, no resolution. Chromatographic conditions as in Fig. 2.

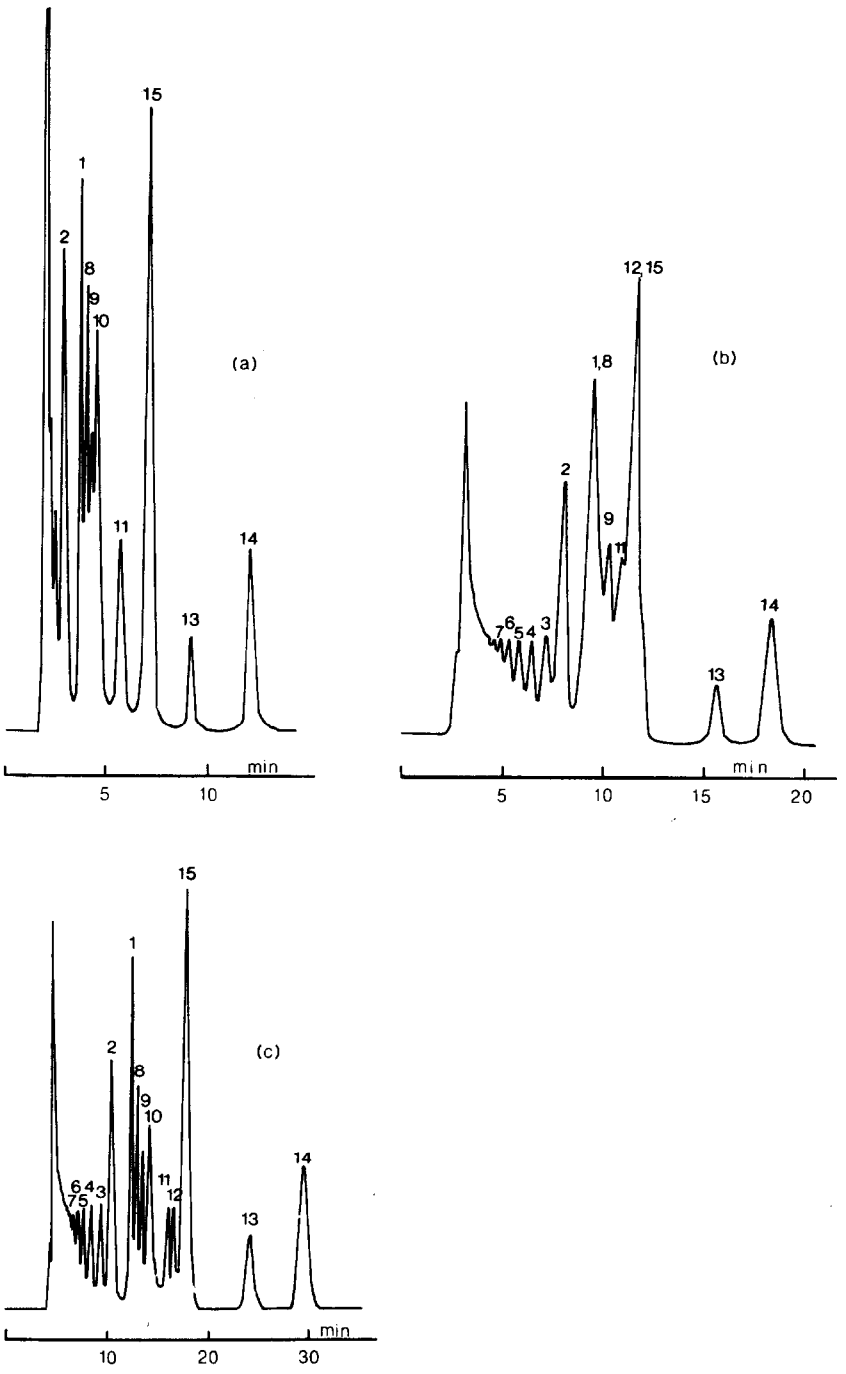

Fig. 4. Optimized separations of a standard mixture. For peak identification, see Table I. Chromatographic conditions: column temperature, 95°C; mobile phase, water; flow-rate, 1.2 ml/min; refractive index detection. Column: (a) I; (b) IV; (c) coupled I + IV.

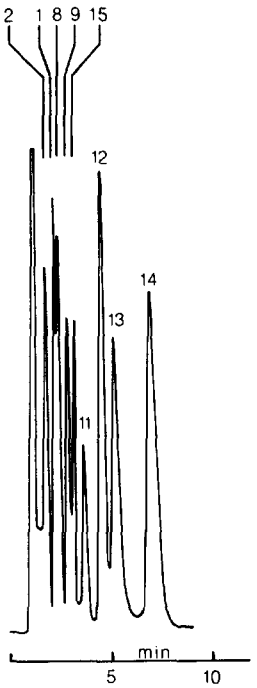

Fig. 5. Optimized separation of a standard mixture. For peak identification, see Table I. Chromatographic conditions: column, II; column temperature, 95°C; mobile phase, water; flow-rate, 1.2 ml/min; refractive index detection.

tions without a substantial loss of sensitivity. A column temperature of 95°C was not found to favour chemical reactions of the compounds under examination.

Using the columns in inverse sequence, *i.e.* IV + I instead of I + IV, a markedly poorer elution behaviour was observed.

Series connection of a Ca-loaded ion-exchange resin (II) with an Ag-loaded ion-exchange resin (25 μm) (IV)

In order to shorten the times required for the separation of monosaccharides,

TABLE IV

RESOLUTION (R_s) OF MONOSACCHARIDES ON ION-EXCHANGE STATIONARY PHASES II AND IV

Chromatographic conditions as in Fig. 6.

Monosaccharide pair	*Column II*	*Column IV*	*Coupled columns II + IV*
Glucose–xylose	0.5	0.4	0.7
Glucose–fructose	1.8	0.8	1.6
Xylose–fructose	1.3	0.4	1.0

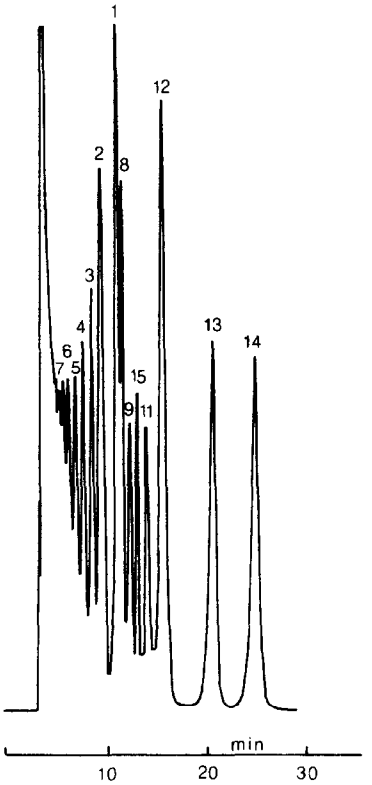

Fig. 6. Optimized separation of a standard mixture. For peak identification, see Table I. Chromatographic conditions: coupled columns II + IV; column temperature, 95°C; mobile phase, water; flow-rate, 1.2 ml/min; refractive index detection.

aldehydes, ketones and alcohols, a short Ca-loaded column (100 × 7.8 mm I.D.) was investigated and optimized. The oligomeric part cannot be detached from the monosaccharides on this stationary phase (Fig. 5). However, cellobiose, glucose, xylose, fructose, dihydroxyacetone, 1,6-anhydro-β-D-glucose and heterocyclic aldehydes such as hydroxymethylfurfural and furfural are separated within 7 min on account of the high flow-rate and the column temperature of 95°C. Calculated R_s values for glucose–xylose, glucose–fructose and xylose–fructose are given in Table IV.

In the coupled system II + IV (Fig. 6), oligosaccharides of DP up to 7 are well resolved, as are the monosaccharides in the presence of dihydroxyacetone, 1,6-anhydro-β-D-glucose, hydroxymethylfurfural and furfural (Table IV). Ethanol can also be determined in the same analytical run. In Fig. 7, retention times (t_R, min) of the various substances on columns II, IV and II + IV are depicted. In Table V the theoretical plate numbers as calculated for the single columns and the heights equivalent to a theoretical plate for the single columns and the coupled system are shown. The non-additivity of the number of theoretical plates (N_c) in coupled column chromatography was calculated using eqn. 2 and corresponded well with the experimental results.

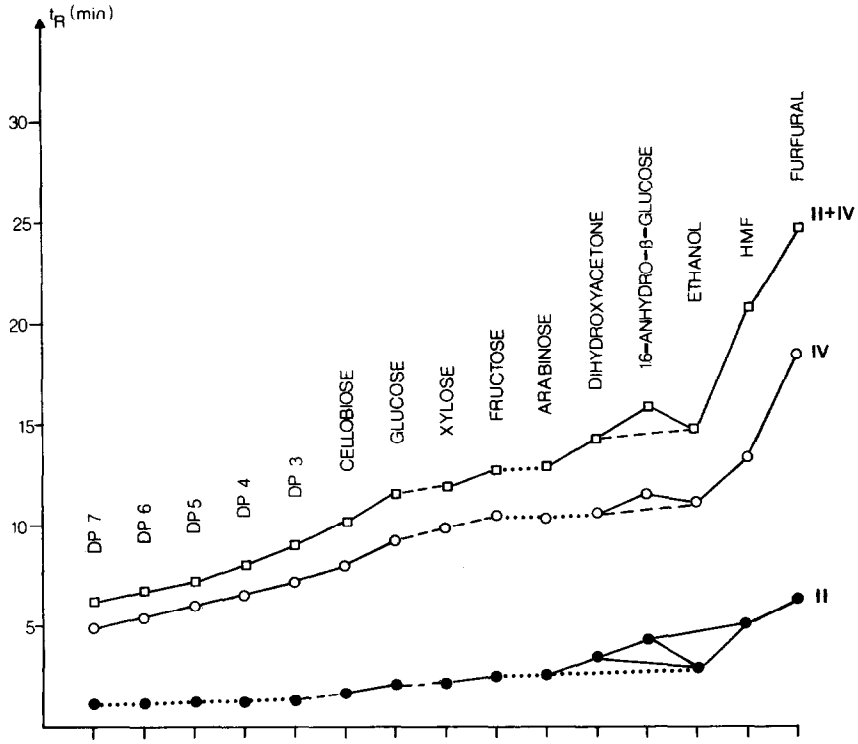

Fig. 7. Dependence of retention time, t_R (min), on the use of single and coupled columns (II and IV) for the investigated substances. ———, Good resolution; – – –, minimum loss of resolution; ·····, no resolution. Chromatographic conditions as in Fig. 6.

Series connection of an H-loaded ion-exchange resin (III) with an Ag-loaded ion-exchange resin (IV)

Fig. 8 shows the chromatogram obtained on the coupled system III + IV. This arrangement, at 95°C and with a flow-rate of 1.2 ml/min, gives the shortest retention times for the reference substances at the cost of the resolution of the monosaccharides (Fig. 9).

TABLE V

NUMBER OF THEORETICAL PLATES (N) AND HEIGHT EQUIVALENT TO A THEORETICAL PLATE (H) OF MONOSACCHARIDES ON A Ca-LOADED ION-EXCHANGE STATIONARY PHASE (II), AN Ag-LOADED ION-EXCHANGE STATIONARY PHASE (IV) AND ON THE COUPLED COLUMNS

Monosaccharide	*Column II*		*Column IV*		*Coupled columns II + IV*		
	N	*H (mm)*	*N*	*H (mm)*	N_c	*H (mm)*	N_c*
Glucose	703	0.14	3412	0.08	3995	0.1	4110
Xylose	806	0.12	3038	0.10	3870	0.1	3841
Fructose	926	0.11	2766	0.10	3613	0.1	3675

* N_c calculated using eqn. 2.

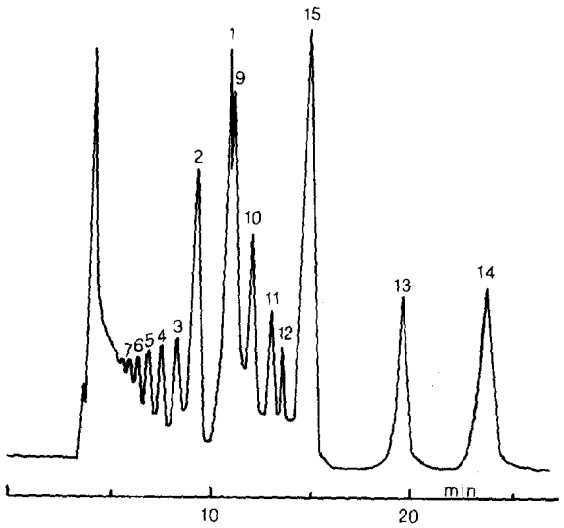

Fig. 8. Optimized separation of a standard mixture. For peak identification, see Table I. Chromatographic conditions: coupled columns III + IV; column temperature, 95°C; mobile phase, water; flow-rate, 1.2 ml/min; refractive index detection.

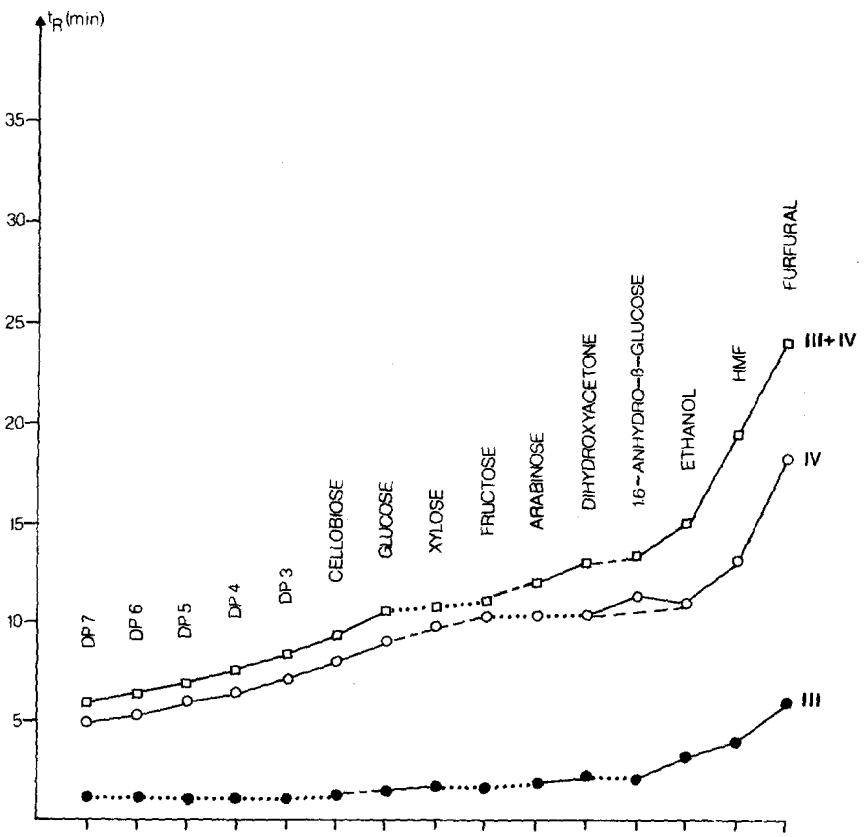

Fig. 9. Dependence of retention time, t_R (min), on the use of single and coupled columns (III and IV) for the investigated substances. ———, Good resolution; – – –, minimum loss of resolution; ·····, no resolution. Chromatographic conditions as in Fig. 8.

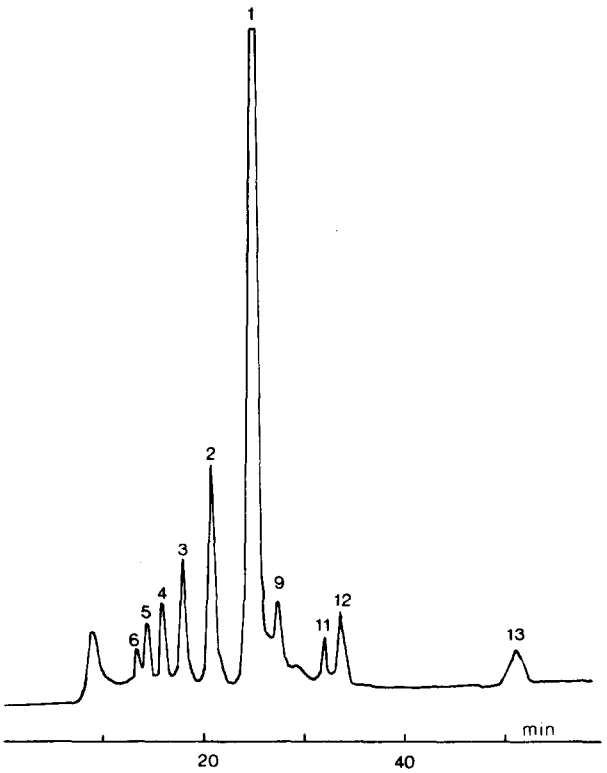

Fig. 10. HPLC trace of a hydrothermally degraded poplar wood sample. Conditions as in Fig. 2. For peak identification, see Table I.

Several other arrangements of coupled columns were tested, including heavy metal-loaded ion-exchange resins (Pb counter ion), but failed to produce major improvements for the classes of substances concerned.

Application

The described systems of series-connected ion-exchange columns are especially suitable for the analysis of biomass degradation solutions[29–33]. On the one hand the extent of degradation, which corresponds to the concentration of the oligosaccharidic part, can be determined, and on the other the monosaccharides and conversion and degradation products and also, in a fermentation process, alcoholic compounds can be separated in one analytical run. Fig. 10 shows the chromatogram of a hydrothermally degraded poplar wood sample[28,34]. The separation was obtained under the described chromatographic conditions by using the coupled column system I + IV.

CONCLUSION

Series connection of various ion-exchange stationary phases with different counter ions and with water as the eluent provides the attractive possibility of recording the entire range of substances in a carbohydrate process in one analytical

run, *e.g.*, starting from yet undegraded oligosaccharides, comprising monosaccharides and anhydro-sugars, and extending to products of subsequent conversions. The refractive index detection sensitivity apparently was not seriously impaired by the use of coupled columns. Another advantage of these systems over the use of solvent or temperature gradients is that the columns need not be regenerated after analysis. This is desirable in routine operations whenever a complete range of carbohydrate chemistry has to be elucidated in a short time.

Depending on the specific separation problem, the most suitable column-coupling combination can be chosen from Figs. 3, 7 and 9. Together with the short analysis times, the reduction in technical expenditure compared with the use of single columns should be emphasized.

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