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SEPARATION OF PERACETYLATED MONO- AND DISACCHARIDES AND QUANTITATIVE ANALYSIS OF GUARAN BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ON SILICA GEL

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SUMMARY

High-performance liquid chromatography on silica gel was successfully applied to the separation of 15 monosaccharide peracetates. The solvent system acetone–*n*-hexane gave the most efficient separations at the optimal flow-rate. Dependences of retention times or capacity factors on flow-rate or pressure were determined for all derivatives. The chromatograms obtained showed clear separations of all derivatives, including epimers and anomers. Similarly, 12 disaccharide peracetates were analysed. The best solvent system was acetone–*n*-pentane, and again the resulting chromatograms showed excellent separations. For detection both a UV photometer and a refractive index detector can be used, depending on the eluent. Acid cleavage and subsequent peracetylation of the polysaccharide guaran gave a mixture of peracetylated monosaccharides. The interpretation of the chromatogram led to qualitative results and, after careful calibration, to quantitative results for the ratio of galactose to mannose residues in guaran.

INTRODUCTION

In studies on the synthesis of natural and modified oligosaccharides, we frequently encountered the problem of the separation of various derivatized mono- and disaccharides. The efficiency of separations by thin-layer chromatography (TLC) and liquid chromatography (LC) was not satisfactory in several instances.

High-performance liquid chromatography (HPLC) has a higher sensitivity and gives more efficient separations. In carbohydrate chemistry, HPLC has so far been applied predominantly to free sugars[1–4]. There have also been some reports on the separation of benzoylated and *p*-nitrobenzoylated sugars[5–8], with UV detection. However, the detection of peracetylated carbohydrates, which frequently occur in synthetic work, and which contain only a weak chromophore, is still a problem. In earlier studies, we demonstrated[9] that some peracetylated monosaccharides could be sepa-

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rated by high-performance liquid adsorption chromatography, with UV detection at 223 nm. In this paper, we describe the optimization of this type of adsorption chromatography when applied to monosaccharide acetates and its extension to acetylated disaccharide derivatives. In addition, we show that this method is particularly suitable for the qualitative and quantitative analysis of natural polysaccharides after hydrolytic cleavage and subsequent peracetylation of the monosaccharide mixture.

EXPERIMENTAL

Apparatus and materials

An S 100 liquid chromatograph (Siemens, Karlsruhe, G.F.R.) equipped with an MK 00 pumping system (Orlita, Giessen, G.F.R.) (maximal pressure 300 bar) was employed. For sample injection, a pneumatic micro syringe (10 μl) was used. Detection was effected with a Zeiss PLC 2 DLC UV photometer (Zeiss, Oberkochen, G.F.R.) at 220 nm and an SR 210 refractive index detector (Siemens).

The columns were made of V-4-A steel, 25 cm long and of I.D. 3 mm. The packing material was silica gel Si 60, LiChrosorb, 5 μm (Merck, Darmstadt, G.F.R.). The columns were filled according to a modified balanced slurry procedure[10], using dioxane as the slurry medium. The packing was effected by use of an S 15 pumping system (Orlita) (maximal pressure 1000 bar). During the filling process, the pump lift was continuously increased from 70% to 100% of the pump capacity. The average filling time was *ca.* 20 min.

Elution was performed with acetone–*n*-pentane and acetone–*n*-hexane mixtures of various compositions. All solvents were purified before use by careful distillation via a 50-cm Vigreux column. The separations were carried out at room temperature.

Sample preparation

Monosaccharide derivatives. 1,2,3,4-Tetra-O-acetyl-β-D-ribo- (5), -α/β-D-arabino- (6/3) and -α-L-rhamnopyranose (1), and also 1,2,3,4,6-penta-O-acetyl-α-D-gluco- (8), -β-D-manno- (15) and -β-D-allopyranose (12) were obtained from the free monosaccharides by acetylation with acetic anhydride in dry pyridine[11]. 1,2,3,4,6-Penta-O-acetyl-β-D-gluco- (10) and -β-galactopyranose (11), and also 1,2,3,4-tetra-O-acetyl-α-D-lyxopyranose (2), were prepared from the free monosaccharides by employing sodium acetate and acetic anhydride[12]. 1,2,3,4,6-Penta-O-acetyl-α-D-mannopyranose (9) was prepared by acetolysis with acetic acid and sulphuric acid from mannan[13]. 1,2,3,4,6-Penta-O-acetyl-α-D-galactopyranose (7) and 1,2,3,4-tetra-O-acetyl-α-D-xylopyranose (4) were obtained by anomerization of the corresponding β-anomers with acetic anhydride and zinc chloride[12]. 1,2,3,4,6-Penta-O-acetyl-α-D-ido- (13) and -α-D-talopyranose (14) were synthesized by acetoxonium rearrangements from 2,3,4,6-tetra-O-acetyl-β-D-gluco- and -β-D-galactopyranosyl chloride, respectively[14].

Disaccharide derivatives. By reaction with acetic anhydride in anhydrous pyridine[11], the following peracetylated disaccharide derivatives were obtained from the free disaccharides: 1,3,4,6-tetra-O-acetyl-2-O-(2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl)-β-D-fructofuranoside (16), 2,3,4,6-tetra-O-acetyl-1-O-(2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl)-α-D-glucopyranoside (25), 2,3,4,6-tetra-O-acetyl-1-O-(2,3,4,-6-tetra-O-acetyl-β-D-glucopyranosyl)-β-D-glucopyranoside (27), 1,2,3,6-tetra-O-ace-

tyl-4-O-(2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl)-β-D-glucopyranose (20), 1,2,3,6-tetra-O-acetyl-4-O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-α-D-glucopyranose (26) and 1,2,3,6-tetra-O-acetyl-4-O-(2,3,4,6-tetra-O-acetyl-β-D-mannopyranosyl)-α/β-D-mannopyranose (17/19).

Reaction of the free sugars with sodium acetate and acetic anhydride[12] gave the following acetylated derivatives: 1,2,3,6-tetra-O-acetyl-4-O-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl)-β-D-glucopyranose (23) and 1,2,3,4-tetra-O-acetyl-6-O-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl)-β-D-glucopyranose (24).

Acetolysis[13] of the corresponding glucans gave 1,2,4,6-tetra-O-acetyl-3-O-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl)-α-D-glucopyranose (18), 1,2,4,6-tetra-O-acetyl-3-O-(2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl)-α-D-glucopyranose (21) and 1,2,3,6-tetra-O-acetyl-4-O-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl)-α-D-glucopyranose (22).

Cleavage of guaran

Guaran[15] (500 mg) was dissolved in 1 *N* hydrochloric acid (30 ml) and heated at 80° for 24 h. The reaction mixture was neutralized with sodium hydrogen carbonate and evaporated to dryness. The resulting mixture of mannose, galactose and salts was taken up in anhydrous pyridine (10 ml) and acetylated with acetic anhydride (5 ml). The general work-up gave a mixture of two anomeric pairs of pentaacetates 7, 9, 11 and 15.

RESULTS AND DISCUSSION

Monosaccharide derivatives

The solvent mixture diethyl ether–*n*-pentane gave a partial separation of some monosaccharide derivatives in TLC and was tried first in the HPLC separation[9]. However, extensive studies with various solvent systems demonstrated that solvent mixtures such as acetone–*n*-hexane and acetone–*n*-pentane were the most suitable for the HPLC separation of this class of compounds, even though there was virtually no separation in corresponding TLC tests. In Fig. 1 the height equivalent to a theoretical plate (HETP) is plotted as a function of solvent composition and flow-rate. The test compound for these measurements was 1,2,3,4,6-penta-O-acetyl-β-D-mannopyranose (15). The resulting graphs show a typical course[16]: with increasing flow-rate the HETP decreases, reaching a minimum in all graphs at a flow-rate of *ca.* 0.5 ml/min. With further increase in flow-rate, a simultaneous and nearly linear increase in HETP is observed. It is clear from Fig. 1 that application of the solvent mixtures acetone–*n*-hexane (1:9 and 1:10) can be expected to give the most efficient separation. An increase in the amount of *n*-hexane (decrease in polarity) results in considerably longer retention times. In addition, it causes poorer separations because of the lower solubility of peracetylated monosaccharides in these eluents.

In Table I the retention times (t_R) and the resulting capacity factors (k') are listed for the eluent system acetone–*n*-hexane (1:10) as a function of pressure (p) or flow-rate (v).

As shown in Fig. 2, which gives plots of capacity factors (k') and pressures (p) for all individual peracetylated monosaccharides, the k' values remain almost constant, as expected, and only below a flow-rate of 0.5 ml/min does a deviation

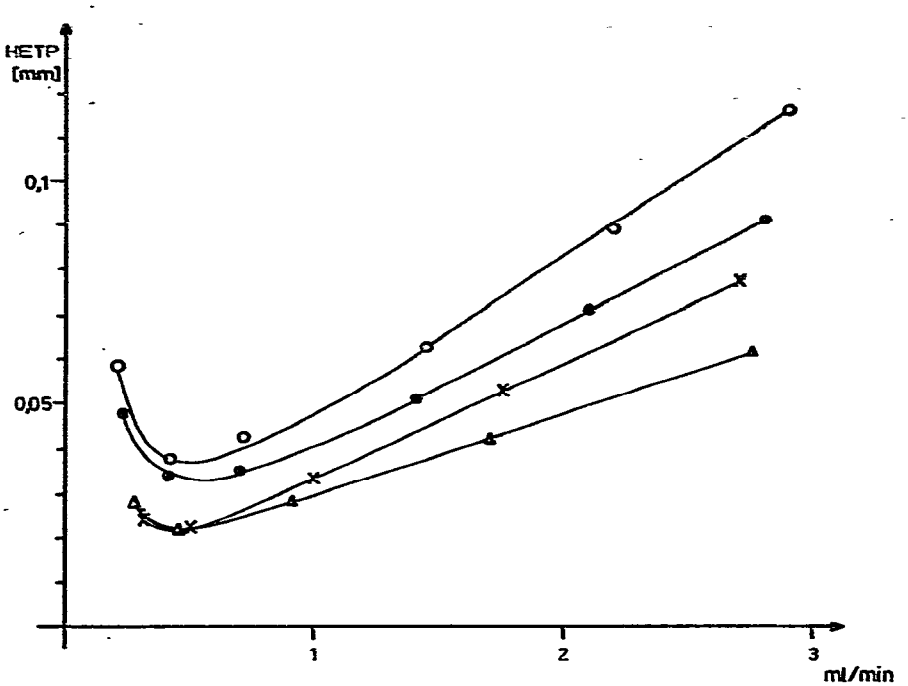

Fig. 1. HETP as a function of flow-rate. Test compound, 15. Eluent, acetone–*n*-hexane: ○, 1:7; ●, 1:8; ×, 1:9; △, 1:10.

TABLE I

CAPACITY FACTORS (k') AND RETENTION TIMES (t_R) FOR PERACETYLATED MONOSACCHARIDES 1–15 AS A FUNCTION OF PRESSURE (p, bar) AND FLOW-RATE (v, ml/min)

Eluent: acetone–*n*-hexane (1:10). N = number of plates; HETP = height equivalent to a theoretical plate.

Compound	$p = 200$, $v = 2.75$		$p = 150$, $v = 1.7$		$p = 100$, $v = 0.91$		$p = 50$, $v = 0.46$		$p = 30$, $v = 0.28$	
	t_R (min)	k'	t_R (min)	k'	t_R (min)	k'	t_R (min)	k'	t_R (min)	k'
1	5.41	5.6	8.82	5.68	15.5	5.74	31.0	5.97	53.0	6.46
2	6.2	6.56	10.14	6.68	17.9	6.78	35.7	7.02	61.1	7.61
3	6.76	7.24	11.0	7.33	19.4	7.43	38.9	7.74	66.2	8.32
4	7.22	7.8	11.8	7.94	20.75	8.02	41.5	8.33	71.0	9.0
5	8.9	9.85	14.64	10.11	25.5	10.13	51.2	10.51	88.0	11.39
6	9.65	10.77	15.84	11.0	26.0	11.17	55.6	11.49	96.1	12.54
7	10.89	12.29	17.82	12.5	31.5	12.7	62.5	13.04	108.1	14.23
8	12.45	14.18	20.42	14.47	36.1	14.7	71.8	15.13	125.0	16.61
9	13.42	15.37	22.04	15.7	38.95	15.93	77.3	16.37	135.0	18.01
10	14.5	16.68	23.7	16.95	42.0	17.26	83.2	17.7	146.1	19.58
11	14.84	17.1	24.5	17.56	43.35	17.85	85.9	18.3	150.3	20.17
12	15.9	18.39	26.14	18.8	46.1	19.04	91.8	19.63	161.0	21.68
13	17.67	20.55	28.36	20.48	50.0	20.74	99.6	21.38	174.2	23.54
14	17.67	20.55	28.96	20.94	51.35	21.33	102.0	21.92	178.2	24.1
15	19.59	22.89	32.2	23.39	57.0	23.78	113.5	24.51	199.1	27.04
HETP (mm)	0.062		0.042		0.028		0.022		0.028	
N	4050		5980		8900		11420		8780	

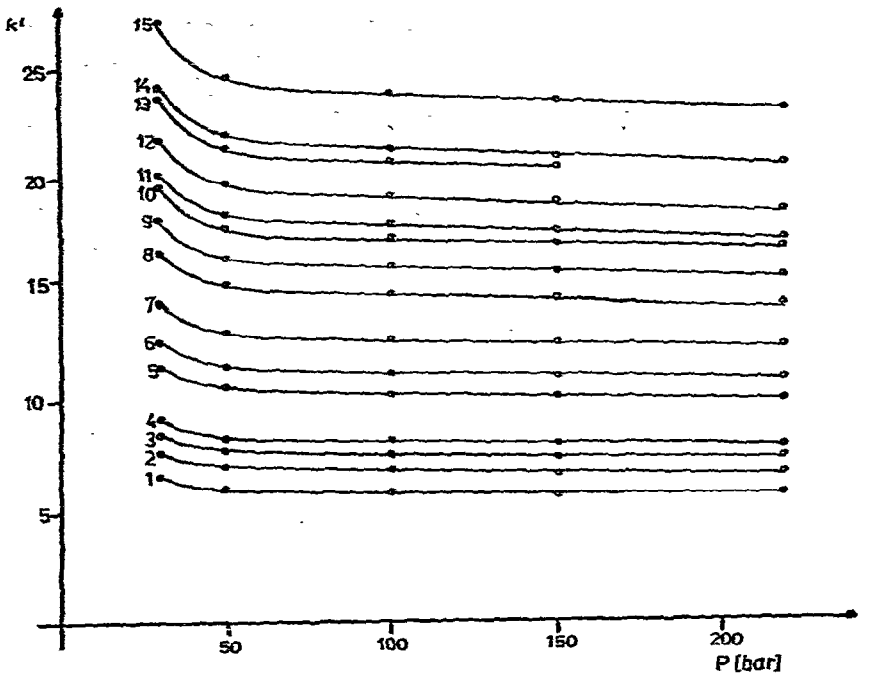

Fig. 2. Capacity factors (k') of peracetylated monosaccharides 1–15 as a function of pressure (p). Eluent: acetone–*n*-hexane (1:10).

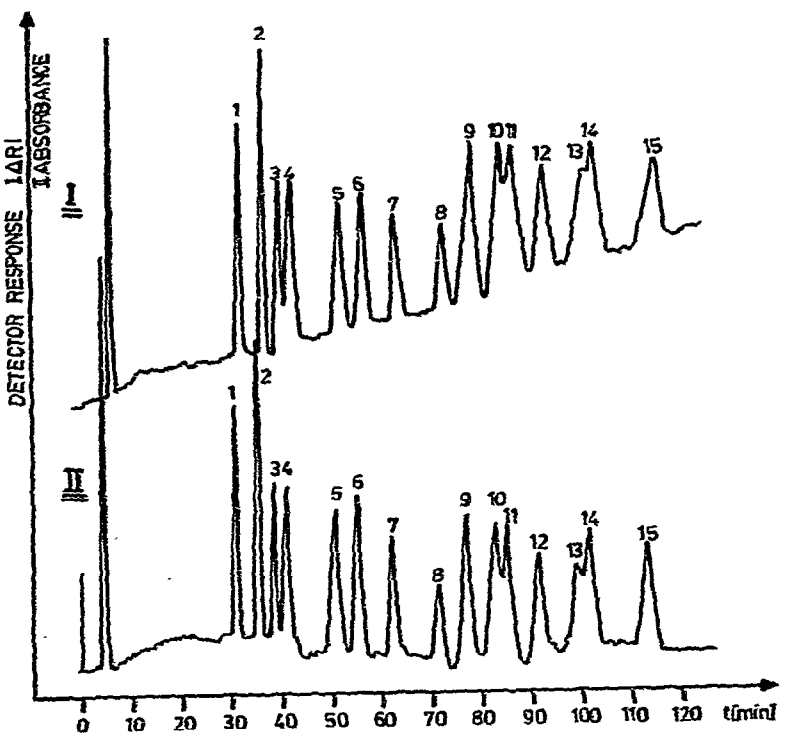

Fig. 3. Chromatogram of peracetylated monosaccharides 1–15. Eluent: acetone–*n*-hexane (1:10). Flow-rate: 0.46 ml/min. Pressure: 50 bar. Detection: (I) refractive index (ΔRI) and (II) UV

from this ideal pattern occur. This deviation seemed to be of minor importance in relation to our work and was not considered further, but it cannot be explained at present. The difference in the k' values proved to be large enough, and we succeeded in obtaining satisfactory separations of all 15 peracetylated monosaccharides, with a sufficient number of plates.

The influences of flow-rate and eluent composition on the efficiency of the separation discussed above can be used to deduce optimal conditions for the separation of all 15 peracetylated monosaccharides. Fig. 3 shows a chromatogram that was obtained in the separation of 15 peracetylated pento- and hexopyranoses at 50 bar (0.46 ml/min) using acetone–*n*-hexane (1:10) as the eluent. It clearly shows a baseline separation for almost all of the monosaccharide acetates. Only in the pairs 3/4, 10/11 and 13/14 are slight overlaps of the peaks observed. It seems to be more important that all of the anomeric pairs (3/6, 7/11, 8/10 and 9/15) are completely separated. In addition, it is noteworthy that even with the epimeric pairs 8/9, 10/15 and 7/14 a clear-cut baseline separation proved to be feasible. Whereas the total time required for the chromatogram shown in Fig. 3 was almost 2 h, the time required for the separation of only a few monosaccharide peracetates which exhibit different capacity factors can be reduced considerably. For instance, by applying a pressure of 150 bar and a flow-rate of 1.7 ml/min, the total time of analysis is reduced to 30 min. Even under these conditions we observed an almost complete baseline separation, with the exception of partial overlapping in different pairs such as 3/4, 10/11 and 13/14.

Disaccharide derivatives

Following the experiments in the monosaccharide series, we started with TLC tests in order to find the optimal eluent composition for the separation of disaccharide derivatives. Again, it soon became obvious that TLC results cannot be transferred easily to HPLC. Whereas in TLC separations diethyl ether is a suitable eluent, it causes considerable tailing effects in HPLC, which prevents the efficient separation of disaccharide derivatives which show similar R_F values. For HPLC, the most suitable eluent was acetone–*n*-pentane.

For testing the efficiency of the separation, we used the same column as in the monosaccharide series. The compound applied was 1,2,3,4-tetra-O-acetyl-6-O-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl)-β-D-glucopyranose (24), and Fig. 4 shows a plot of HETP against flow-rate for three different eluent compositions. With all three solvent systems the optimal separation would be obtained at a flow-rate of 0.3–0.4 ml/min, and obviously the best results would be obtained with acetone–*n*-pentane (2:7), which exhibits the lowest HETP.

Table II lists the retention times and the resulting capacity factors for 12 disaccharide derivatives and the eluent acetone–*n*-pentane as a function of pressure and flow-rate. By comparison with the values in Table I it can be seen that the capacity factors for similarly linked disaccharide derivatives are smaller than for isomeric monosaccharide derivatives. In particular, the pairs 17/23 and 18/27 exhibit rather small differences in the k' values, which results in incomplete separations.

A further improvement in the separation could not be achieved, because an additional decrease in the polarity of the eluent mixture leads to a poorer solubility of the peracetylated disaccharides.

Fig. 5 shows three chromatograms for 12 disaccharide peracetates obtained

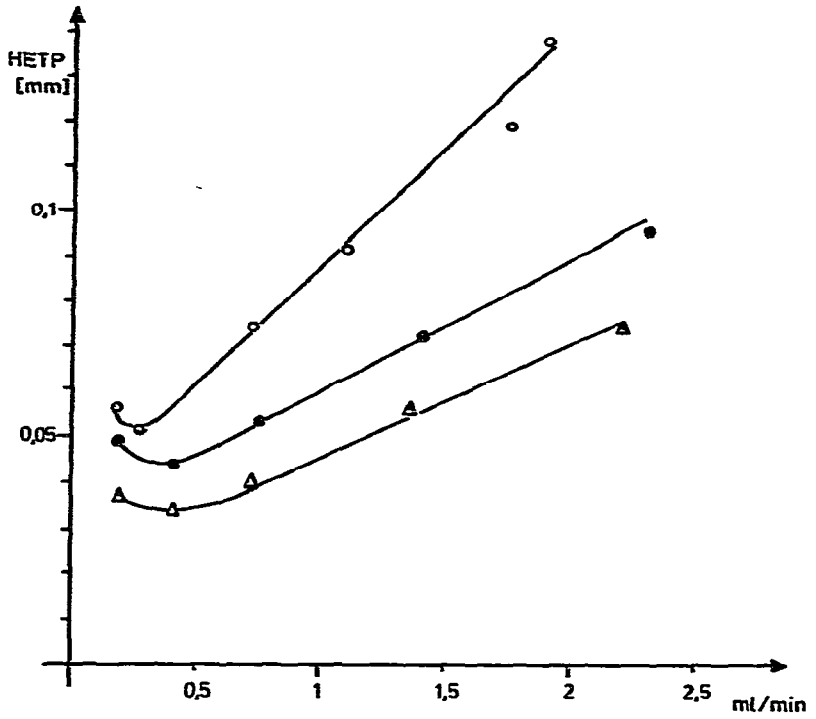

Fig. 4. HETP as a function of flow-rate. Test compound 24. Eluent, acetone–*n*-pentane: ○, 2:5; ●, 2:6; △, 2:7.

TABLE II

CAPACITY FACTORS (k') AND RETENTION TIMES (t_R) FOR PERACETYLATED DISACCHARIDES 16–27 AS A FUNCTION OF PRESSURE (p, bar) OR FLOW-RATE (v, ml/min) Eluent: acetone–*n*-pentane (2:7). N = number of plates; HETP = height equivalent to a theoretical plate.

Compound	$p = 215$, $v = 2.2$		$p = 150$, $v = 1.35$		$p = 100$, $v = 0.75$		$p = 50$, $v = 0.39$		$p = 22$, $v = 0.19$	
	t_R (min)	k'	t_R (min)	k'	t_R (min)	k'	t_R (min)	k'	t_R (min)	k'
16	6.12	5.65	10.32	5.79	15.3	5.91	36.4	6.14	73.4	6.06
20	6.62	6.2	11.18	6.36	18.6	6.4	39.4	6.73	79.6	6.65
25	6.93	6.53	11.8	6.76	20.9	6.89	41.5	7.14	83.5	7.03
21	7.13	6.75	12.06	6.93	21.2	7.0	42.3	7.29	85.3	7.2
22	8.49	8.23	14.38	8.46	25.2	8.51	50.8	8.96	101.2	8.73
23	8.89	8.86	15.06	8.91	26.35	8.94	53.4	9.47	105.9	9.18
17	8.92	8.7	15.2	9.0	26.85	9.13	53.7	9.53	108.7	9.45
18	9.7	9.54	16.2	9.66	28.9	9.91	57.8	10.33	117.2	10.27
26	9.89	9.75	16.82	10.07	29.8	10.26	59.3	10.63	120.5	10.59
27	9.9	9.76	16.9	10.12	29.9	10.28	59.8	10.73	121.4	10.67
24	10.03	9.9	17.0	10.18	30.0	10.32	60.3	10.82	121.6	10.69
19	10.5	10.41	18.0	10.84	31.6	10.92	63.9	11.53	128.1	11.32
HETP (mm)	0.093		0.068		0.039		0.035		0.062	
N	2690		3680		6410		7140		4030	

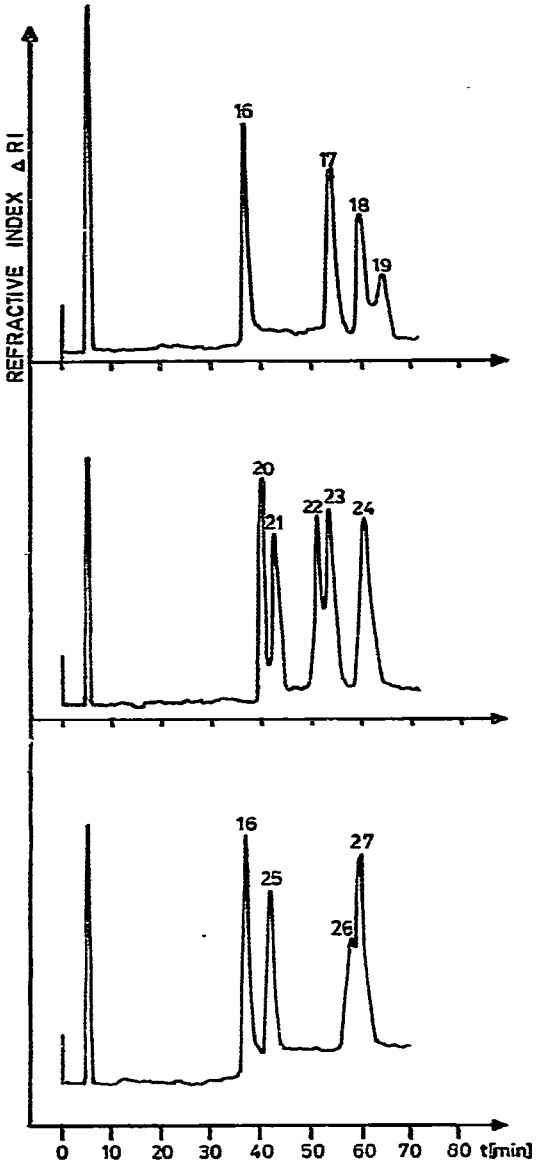

Fig. 5. Chromatograms of peracetylated disaccharides 16–27. Eluent: acetone–*n*-pentane (2:7). Flow-rate: 0.39 ml/min. Pressure: 50 bar. Detection: refractive index (ΔRI).

at a pressure of 50 bar with the solvent system acetone–*n*-pentane (2:7). The baseline separation of the anomeric pair 17/19 and the evident partial separation of the peaks of 22/23 is of interest.

The slightly inferior separations of the disaccharide derivatives in comparison with the monosaccharide peracetates have been related *inter alia* to the molecular size[17]. It should be pointed out that this effect supports our approach of making

use of the peracetylated derivatives rather than the perbenzoylated or per-*p*-nitrobenzoylated saccharides[5–8].

Detection

Both a UV photometer and a refractive index detector were used. In the separation of the monosaccharide derivatives both detector systems could be applied simultaneously, as shown in Fig. 3. For the peracetylated pentoses and hexoses, the absorption maximum at 220 nm could be used. UV detection can be applied successfully only if the eluent composition does not exhibit considerable absorption in this region. This applies to the eluent diethyl ether, as shown previously[9]. However, the solvent systems used here with better results permit UV detection only when there is a very small proportion of acetone present, as it absorbs considerably in this region. Consequently, in the separation of the pentose and hexose peracetates, UV detection was feasible, whereas in the separation of disaccharide peracetates only the refractive index detector could be used.

Guaran

The above results encouraged us to make use of the advantages of HPLC, which are its high separation capacity and sensitivity, rapidity and simplicity, in the qualitative and quantitative analysis of polysaccharides. In order to study the efficiency of this method, we chose the polysaccharide guaran from guar seeds for preliminary experiments, because its structure and composition are well known (Fig. 6)[15]. The main chain of guaran is known to be built up of D-mannopyranose, β,1→4 linked, and to roughly every second mannose residue a D-galactopyranose is attached by an α,1→6 interglycosidic linkage[18]. The ratio of D-mannose to D-galactose has been determined to be approximately 2:1 (ref. 15).

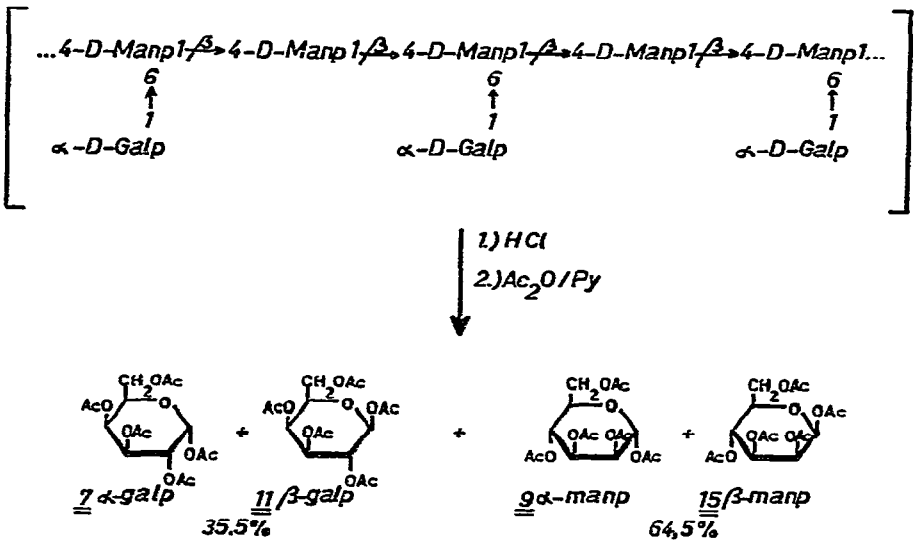

Fig. 6. Structure[15,18] and degradation of guaran.

We first tried to obtain peracetylated monosaccharides directly from guaran by acetolysis[13]. However, this resulted in the formation of various decomposition products which accompanied the mannose and galactose peracetates. Hence, this method can be applied in qualitative but not quantitative studies. Therefore, we turned to hydrolytic cleavage of the material, the completion of which could be observed easily by TLC. After work-up, the resulting mixture was peracetylated in the usual manner and subjected to the HPLC separation.

Fig. 7 shows the chromatogram of the peracetylated reaction mixture. Four peaks can be observed, the assignment of which could be based on the capacity factors and comparison chromatograms. By this means, α- and β-galactopyranose pentaacetate (7 and 11) and α- and β-mannopyranose pentaacetate (9 and 15), uncontaminated with by-products, were identified.

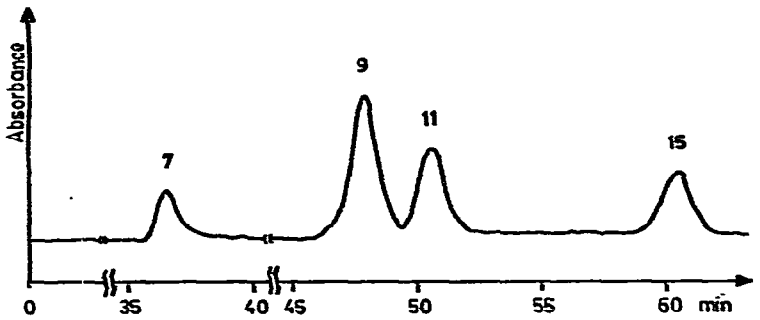

Fig. 7. Chromatogram of the peracetylated hydrolysis mixture of guaran. Eluent: acetone–*n*-hexane (1:8). Flow-rate: 0.28 ml/min. Pressure: 30 bar. Detection: UV absorption at 220 nm.

Quantitative information can be obtained only if the dependence of UV absorption on the amount of material applied is known. In other preliminary studies we noticed that the UV absorptions of various anomeric pairs were identical. Subsequently, we studied the dependence of UV absorption on the amount of material applied for three hexopyranose pentaacetates (10, 11 and 15). This was performed by application of 5, 10, 15...50 μg of each of 10, 11 and 15 to HPLC, elution with acetone–*n*-hexane (1:8) and UV detection. The calibration graph is given in Fig. 8, which shows the dependence of the integrated peak area (determined by cutting out the peak and weighing) on the amount of material applied. The results demonstrate clearly that in the chosen range of concentrations there is a linear dependence between UV absorption and concentration. Further, all three hexopyranose pentaacetates obviously exhibit the same UV absorption at the same concentration.

Having obtained this information, the relative ratio of mannose to galactose in guaran could be determined directly from the chromatogram (Fig. 7). By the same procedure as described above, we found a ratio of galactose to mannose of 35.5:64.5, which is in agreement with the result determined previously.

CONCLUSIONS

The results demonstrate that HPLC on silica gel is a versatile, fast and ac-

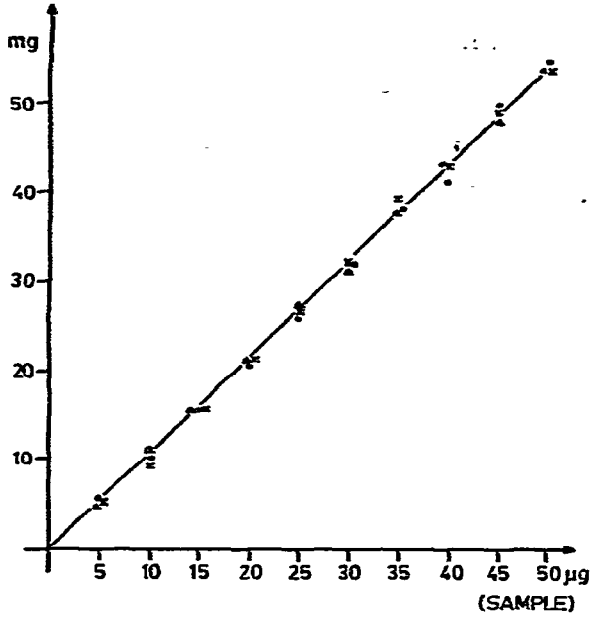

Fig. 8. Calibration graph for determination of the dependence of UV absorption (220 nm) on amount of material applied (micrograms). Magnitude of UV absorption given as peak area, weighed in milligrams. Compounds: ×, 10; ○, 11; △, 15.

curate method for the analysis of these carbohydrate derivatives. The separation of complex reaction mixtures and the qualitative and quantitative analysis of the composition of polysaccharides encourage the application of this method to further studies in this field.

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