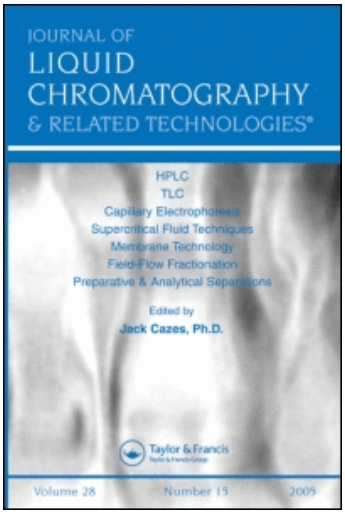

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC SYSTEMS TO SEPARATE AND QUANTIFY A MIXTURE OF NINE SUGARS AND FOUR POLYOLS

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ABSTRACT

Three different chromatographic systems were described to separate and quantify nine carbohydrates (stachyose, raffinose, maltose, lactose, sucrose, galactose, glucose, fructose and xylose) and four polyols (mannitol, sorbitol, xylitol and maltitol) from a mixture. The results were collected in a guide in order to provide the analyst with a quick reference to the proper column and adequate chromatographic conditions to perform the analyses of these compounds.

INTRODUCTION

The high performance liquid chromatography (HPLC) technique has marked a great improvement in the qualitative and quantitative analyses of carbohydrates mostly due to an improvement in the chromatographic columns employed.

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In the last few years there have been many contributions to the HPLC determination of sugars in foods (1-6) and in the biological fluids (7-8). In previous publications, we have applied this method to the analysis of sugars in foods (9-11) and to the separation of sugars and polyols of difficult resolution (12).

With our previous experience, we have attempted to unify criteria and define the conditions to carry out the separation and quantification of a complex mixture of sugars and polyols. As a result, a guide has been made in order to provide the analyst with a quick reference to the proper column and adequate chromatographic conditions to perform the separation of these sugars and polyols.

EXPERIMENTAL

High performance liquid chromatography was carried out in a chromatograph ALC/GPC(model 201) equipped with model 6000A pump, dual reciprocating piston heads,model U 6K septumless injector, model R401 differential refractometer detector optical deflection type,maintained at 30ºC. The detector signal was recorded on a M730 data module (Waters Associates, Milford, Mass. USA).

The following chromatographic systems were used:

A.- µBondapak/carbohydrate, with a guard column of Bondapak C_{18}/Porasil.
Mobile phase: acetonitrile/water (75/25), previously filtered through FH Millipore membrane (0.5µm) and degassed in ultrasonic bath. Flow rate 1 ml/min. 100 µl were injected. Attenuation x8.

B.- Sugar Pak I column, with a column temperature control accessory at 90ºC, precolumn filter.
Mobile phase: bidistilled water filtered through a Millipore HA (0.45 µm), degassed by immersion in an ultrasonic bath. Flow rate 0.4 ml/min. 25 µl or 10 µl were injected. Attenuation x8, x16.

C.- Sugar Pak I column, with a column temperature control accessory at 80ºC, precolumn filter. Mobile phase: acetonitrile/water (25/75), filtered through a Millipore membrane FH (0.5 µm). Flow rate 0.4 ml/min. 25 µl were injected. Attenuation x16.

Standard solutions.- Various amounts of stachyose, raffinose, maltose, lactose, sucrose, galactose, glucose, fructose, xylose, mannitol, sorbitol, xylitol and maltitol (Merck) were dissolved in the corresponding mobile phase, and filtered through the same membrane which was used to filter the mobile phase. The weights of the different components were generally maintained at a 1:1 ratio.

RESULTS AND DISCUSSION

It has been intended to define the best conditions to carry out the separation and quantification of a complex mixture of sugars and polyols. With this aim, we have employed two chromatographic columns, a µBondapak/Carbohydrate (Waters), which is of the chemically bonded phase type, with a particle size of 10 µ and the Sugar Pak I column (Waters), a microparticulate cation exchange bed, in the calcium form. Different mobile phases have been essayed, being selected finally that of acetonitrile/water (75/25), for the first column and bidistilled water or acetonitrile/water (25/75) for the second column.

Tables 1-9 collect the chromatographic constants, as well as the correlation coefficients between the height of each one of the peaks and the concentration of the corresponding sugar or polyol in the standard solution. With this data we arrived at the standard curve by means of the least squares method, using a Digital PDP 11-55.

Tables 1 and 2, show the chromatographic constants obtained for a mixture of fructose, glucose, sucrose, maltose, lactose, raffinose, and stachyose, and a mixture of xylose and glucose, in system A (μBondapak/ Carbohydrate and acetonitrile/water (75/25) as the mobile phase).

In Table 1 there is no indication of the resolution nor the number of the theoretical plates of lactose and maltose since the separation between these two peaks is less than half the peak height and the measurement of W (base width) could not be properly made, this happened also in Tables 5 and 6. Despite this drawback, the separation achieved is sufficient to carry out the quantification of both sugars since, in the concentration range we have used, the detector gave a linear response between peak height and weight of these compounds. With this type of column it is not possible to separate glucose fron galactose, carbohydrate which may be present in dairy products.

With regard to polyols, it has been observed that xylitol elutes with fructose, sorbitol and mannitol with glucose, and maltitol with maltose. Accordingly the separation of polyols with this system can be carried out only in the case that these sugars are not present. The elution order would be: xylitol, sorbitol or mannitol and maltitol.

Tables 3 and 4 represent chromatographic constants obtained with a mixture of stachyose, raffinose, sucrose, glucose, galactose, fructose, mannitol and sorbitol, and glucose, maltitol and fructose respectively, using system B (Sugar Pak column and bidistilled water as mobile phase). With this chromatographic system sucrose and maltose did not separate.

In those cases in which the separation between two components is not complete their concentration in the

TABLE 1.-

Chromatographic constants of Waters μBondapak /Carbohydrate high performance liquid chromatographic column , using acetonitrile/water (75/25) as mobile phase and a flow rate of 1 ml/min.

	Rt_{Gl}	Fructose	Glucose	Sucrose	Maltose	Lactose	Raffinose	Stachyose	k'	N	equation a	y=a+bx b	r	μg
		Separation factor (α)												
Fructose	0.885	—	1.330	2.088	2.636	2.862	4.452	8.836	0.646	1648	0.100	0.108	0.999	17 - 67
Glucose	1.000	1.234	—	1.570	1.983	2.153	3.348	6.645	0.859	1637	0.200	0.072	0.999	21 - 83
Sucrose	1.263	3.881	2.549	—	1.262	1.371	2.132	4.231	1.349	2206	0.225	0.079	0.999	25 - 99
Maltose	1.453	—	—	—	—	1.086	1.689	3.352	1.703	—	0.100	0.048	0.999	25 - 102
Lactose	1.532	—	—	—	—	—	1.555	3.087	1.849	—	-0.025	0.045	0.999	29 - 116
Raffinose	2.084	9.167	7.936	5.825	4.067	2.992	—	1.985	2.876	2284	0.100	0.043	0.999	33 - 131
Stachyose	3.608	14.408	13.388	11.773	9.262	8.551	6.550	—	5.708	2464	0.250	0.021	0.992	41 - 164
		Resolution (R)												

Rt_{Gl}= relative retention time to glucose; Separation factor (α)=k'_2/k'_1; W= peak width; Resolution (R) = $V_2-V_1/\frac{1}{4}(W_2+W_1)$; V= retention volume; V_0= void volume; Capacity factor (k') = $V-V_0/V_0$; Number of theoretical plates (N)= $16\ (V/W)^2$; r= correlation coefficient.

TABLE 2.-

Chromatographic constants of Waters μBondapak/Carbohydrate high performance liquid chromatographic column, using acetonitrile/water (75/25) as mobile phase and a flow rate of 1ml/min .

	Rt_{Gl}	Xylose	Glucose	k´	N	equation a	y=a+bx b	r	μg
		Separation factor (α)							
Xylose	0.814	—	1.527	0.780	956	-0.225	0.057	0.999	25 - 100
Glucose	1.000	1.653	—	1.188	1106	-0.100	0.087	0.999	21 - 83
		Resolution (R)							

Rt_{Gl}= relative retention time to glucose; Separation factor (α)=k_2/k_1; W= peak width; Resolution (R)= $V_2-V_1/\frac{1}{2}(W_2+W_1)$; V= retention volume; V_0= void volume; Capacity factor (k´)= $V-V_0/V_0$; Number of theoretical plates (N)= 16 $(V/W)^2$; r= correlation coefficient.

TABLE 3.-

Chromatographic constants of Waters Sugar Pak I high performance liquid chromatographic column at 90°C, using bidistilled water as mobile phase and a flow rate of 0.4 ml/min.

	Rt_{Gl}	Stachyose	Raffinose	Sucrose	Glucose	Galactose	Fructose	Mannitol	Sorbitol	k'	N	equation a	y=a+bx b	r	µg
						Separation factor (α)									
Stachyose	0.623	—	1.623	2.589	4.527	5.546	6.377	8.903	11.391	0.207	1573	−0.250	0.168	0.992	18 - 70
Raffinose	0.689	0.993	—	1.595	2.789	3.417	3.929	5.485	7.018	0.336	1512	−0.200	0.166	0.995	17 - 68
Sucrose	0.793	2.429	1.397	—	1.748	2.142	2.463	3.438	4.399	0.536	1702	−0.201	0.170	0.994	17 - 68
Glucose	1.000	5.172	4.038	2.595	—	1.225	1.409	1.967	2.517	0.937	2326	−0.151	0.140	0.997	18 - 71
Galactose	1.109	6.558	5.368	3.898	1.291	—	1.150	1.605	2.054	1.148	2704	−0.100	0.142	0.997	18 - 72
Fructose	1.197	6.882	5.805	4.474	2.111	0.938	—	1.396	1.786	1.320	2116	0.049	0.135	0.998	17 - 70
Mannitol	1.468	10.455	9.175	7.689	5.148	3.902	2.664	—	1.279	1.843	3535	−0.101	0.116	0.997	18 - 72
Sorbitol	1.733	12.622	11.346	9.905	7.482	6.299	4.933	2.508	—	2.358	3757	−0.175	0.117	0.998	18 - 71
				Resolution (R)											

Rt_{Gl} = relative retention time to glucose; Separation factor (α)=k'_2/k'_1; W= peak width; Resolution (R)= $V_2-V_1/\frac{1}{2}(W_2+W_1)$; V= retention volume; V_0= void volume; Capacity factor (k')= $V-V_0/V_0$; Number of theoretical plates (N)= 16 $(V/W)^2$; r= correlation coefficient.

TABLE 4.-

Chromatographic constants of Waters Sugar Pak I high performance liquid chromatographic column at 90°C, using bidistilled water as mobile phase and a flow rate of 0.4 ml/min.

							equation y= a+ bx			
	Rt_{Gl}	Glucose	Maltitol	Fructose	k´	N	a	b	r	µg
		Separation factor (α)								
Glucose	1.000	---	1.137	1.406	0.936	1833	-0.075	0.135	0.999	19-74
Maltitol	1.066	0.673	---	1.237	1.064	1675	0.000	0.116	0.999	18-73
Fructose	1.197	2.147	1.343	---	1.316	2839	-0.125	0.133	0.999	19-75
		Resolution (R)								

Rt_{Gl} = relative retention time to glucose; Separation factor (α)= k'_2/k'_1; W= peak width; Resolution (R)= $V_2-V_1/\frac{1}{2}(W_2+W_1)$; V= retention volume; V_0= void volume; Capacity factor (k')= $V-V_0/V_0$; Number of theoretical plates (N)= $16(V/W)^2$; r= correlation coefficient.

mixture plays a critical role in their separation and quantification. Such are the cases of sucrose and lactose (table 5), and maltitol and galactose (table 6). In these two cases it has been observed that a separation that allows quantification of both components requires a weight ratio of 2/1.

Table 7 show the separation obtained with the polyols maltitol, mannitol and sorbitol. The xylitol elutes with sorbitol. Using acetonitrile/water (25/75) at 80ºC with this same column, an acceptable separation of the same polyols was achieved (table 8), as well as a good separation of fructose, glucose and galactose (table 9).

It should be pointed out that maltitol shows a retention volume very close to that of fructose and, consequently, the separation of these two components would be difficult under these conditions.

In previous publication (13) we have indicated that the use of acetonitrile/water with a Sugar Pak column caused a pressure rise after 8-10 hours use. It was necessary to backflush the column overnight with bidistilled water to restore the normal operating conditions. This phenomenon has not appeared during the separation of monosaccharides and polyols.

As previously exposed, a combination of the various chromatographic systems here described allows for the separation and quantification of nine different carbohydrates and four polyols in a mixture. The elution sequence was as follows:

A system:

Xylose,|Fructose and Xylitol,| Galactose, Glucose, Sorbitol and Mannitol,| Sucrose,| Maltose and Maltitol,| Lactose,| Raffinose,| Stachyose.

B system:

Stachyose,| Raffinose,| Sucrose and Maltose,| Lactose,| Glucose,| Maltitol,| Galactose,| Fructose,| Mannitol,| Sorbitol and Xylitol.

TABLE 5.-

Chromatographic constants of Waters Sugar Pak I high performance liquid chromatographic column at 90°C using bidistilled water as mobile phase and a flow rate of 0.4 ml/min.

	Rt	Sucrose	Lactose	k'	equation y=a+bx a	b	r	µg
		Separation factor (α)						
Sucrose	8.87	—	1.133	0.570	-0.075	0.096	0.999	21 - 85
Lactose	9.30	—	—	0.646	-0.050	0.129	0.999	11 - 46
		Resolution (R)						

Rt= retention time; Separation factor (α)= k'_2/k'_1; W= peak width; V= retention volume; V_0= void volume; Capacity factor (k')= $V-V_0/V_0$; Number of theoretical plates (N)= $16(V/W)^2$;. r= correlation coefficient.

TABLE 6.-

Chromatographic constants of Waters Sugar Pak I high performance liquid chromatographic column at 90°C, using bidistilled water as mobile phase and a flow rate of 0.4 ml/min.

	Rt	Maltitol	Galactose	k'	equation y=a+bx a	b	r	µg
		Separation factor (α)						
Maltitol	11.96	---	1.053	1.080	-0.010	0.097	0.997	16 - 63
Galactose	12.29	---	---	1.137	-0.100	0.206	0.999	8 - 31
		Resolution (R)						

Rt_{Gl}= retention time; Separation factor (α)= k_2'/k_1'; W= peak width; V= retention volume; V_0= void volume; Capacity factor (k')= $V-V_0/V_0$; Number of theoretical plates (N)= $16(V/W)^2$.; r= correlation coefficient.

TABLE 7.-

Chromatographic constants of Waters Sugar Pak I high performance liquid chromatographic column at 90°C, using bidistilled water as mobile phase and a flow rate of 0.4 ml/min.

							equation y= a+ bx			
	Rt_s	Maltitol	Mannitol	Sorbitol	k'	N	a	b	r	µg
		Separation factor (α)								
Maltitol	0.618	---	1.722	2.196	1.070	2095	-0.000	0.098	0.998	17-69
Mannitol	0.849	4.045	---	1.275	1.843	3179	-0.550	0.122	0.995	17-69
Sorbitol	1.000	6.400	2.405	---	2.350	3738	-0.150	0.103	0.998	18-73
		Resolution (R)								

Rt_s = relative retention time to sorbitol; Separation factor (α) = k'_2/k'_1; W= peak width Resolution (R) = $V_2-V_1/\frac{1}{2}(W_2+W_1)$; V= retention volume; V_0= void volume; Capacity factor (k') = $V-V_0/V_0$; Number of theoretical plates (N) = $16(V/W)^2$; r= correlation coefficient.

TABLE 8.-

Chromatographic constants of Waters Sugar Pak I high performance liquid chromatographic column at 80°C, using acetonitrile/water (25/75) as mobile phase and a flow rate of 0.4ml/min.

								equation y= a+ bx			
	Rt_s	Maltitol	Mannitol	Xylitol	Sorbitol	k'	N	a	b	r	µg
				Separation factor (α)							
Maltitol	0.583	---	1.552	1.877	2.095	1.875	1291	-0.009	0.027	0.993	39-156
Mannitol	0.793	3.105	---	1.210	1.350	2.910	2035	0.077	0.030	0.992	37-149
Xylitol	0.917	4.656	1.664	---	1.116	3.520	2186	0.118	0.027	0.993	36-146
Sorbitol	1.000	5.310	2.546	0.972	---	3.928	1891	0.071	0.028	0.993	38-150
				Resolution (R)							

Rt_s = relative retention time to sorbitol; Separation factor (α)= k'_2/k'_1; W= peak width; Resolution (R)= $V_2-V_1/\frac{1}{2}(W_2+W_1)$; V= retention volume; V_0= void volume; Capacity factor (k')= $V-V_0$; Number of theoretical plates (N)= 16 $(V/W)^2$; **r**= correlation coefficient.

TABLE 9.-

Chromatographic constants of Waters Sugar Pak I high performance liquid chromatographic column at 80°C, using acetonitrile/water (25/75) as mobile phase and a flow rate of 0.4ml/min.

							equation y= a+ bx			
	Rt_{Gl}	Glucose	Galactose	Fructose	k'	N	a	b	r	µg
		Separation factor (α)								
Glucose	1.000	---	1.289	1.501	1.161	1075	0.100	0.045	0.997	36-145
Galactose	1.155	1.342	---	1.164	1.497	1773	0.125	0.045	0.996	37-149
Fructose	1.269	2.101	0.931	---	1.743	1432	-0.025	0.043	0.998	36-146
			Resolution (R)							

Rt_{Gl} = relative retention time to glucose; Separation factor (α) = k'_2/k'_1; W= peak width; Resolution (R) = $V_2-V_1/\frac{1}{2}(W_2+W_1)$; V= retention volume; V_0= void volume; Capacity factor (k') = $V-V_0$; Number of theoretical plates (N) = $16\,(V/W)^2$; r= correlation coefficient.

TABLE 10.-

Most appropriate chromatographic systems to separate any mixture of carbohydrates and polyols

	STACHYOSE	RAFFINOSE	MALTOSE	LACTOSE	SUCROSE	GALACTOSE	GLUCOSE	FRUCTOSE	XYLOSE	MANNITOL	SORBITOL	XYLITOL	MALTITOL
STACHYOSE	—												
RAFFINOSE	A,B	—											
MALTOSE	A,B	A,B	—										
LACTOSE	A,B	A,B	A	—									
SUCROSE	A,B	A,B	A	A,B_1	—								
GALACTOSE	A,B	A,B	A,B	A,B	A,B	—							
GLUCOSE	A,B	A,B	A,B	A,B	A,B	B,C	—						
FRUCTOSE	A,B	A,B	A,B	A,B	A,B	A,B,C	A,B,C	—					
XYLOSE	A	A	A	A	A	A	A	A	—				
MANNITOL	A,B	A,B	A,B	A,B	A,B	B,C	B,C	A,B,C	A	—			
SORBITOL	A,B	A,B	A,B	A,B	A,B	B,C	B,C	A,B,C	A	B,C	—		
XYLITOL	A,B	A,B	A,B	A,B	A,B	A,B,C	A,B,C	B,C	A	A,B,C	A,C	—	
MALTITOL	A,B	A,B	B	A,B	A,B	A,B_1,C	A,B,C	A,B	A	A,B,C	A,B,C	A,B,C	—

A.- μBondapak/carbohydrate column, mobile phase acetonitrile/water (75/25); B.- Sugar Pak I column, mobile phase bidistilled water; C.- Sugar Pak I column, mobile phase acetonitrile/water (25/75); B_1.- Critical separation in B system.

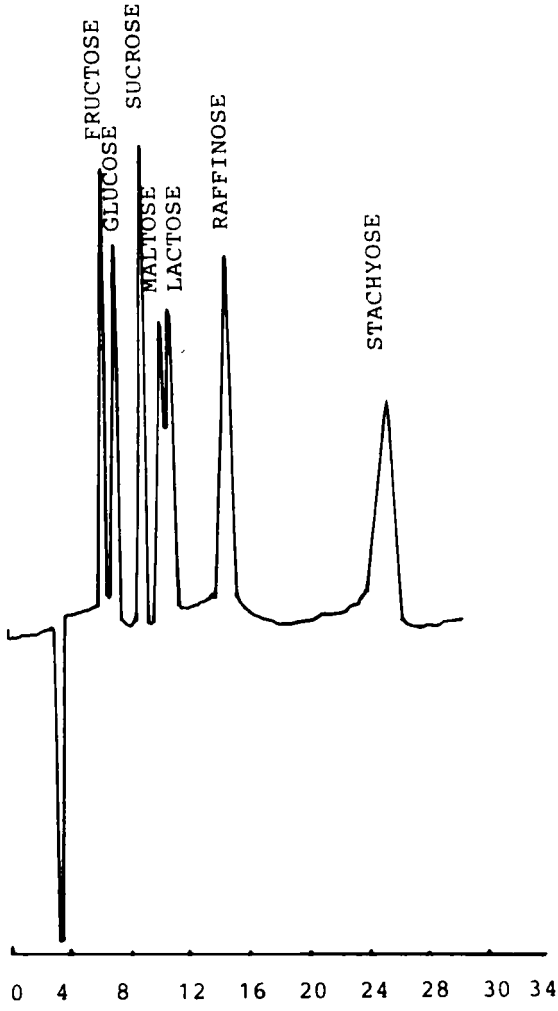

Fig. 1 : µBondapak /Carbohydrate column.
Mobile phase: acetonitrile/water 75/25
Flow rate: 1ml/min.

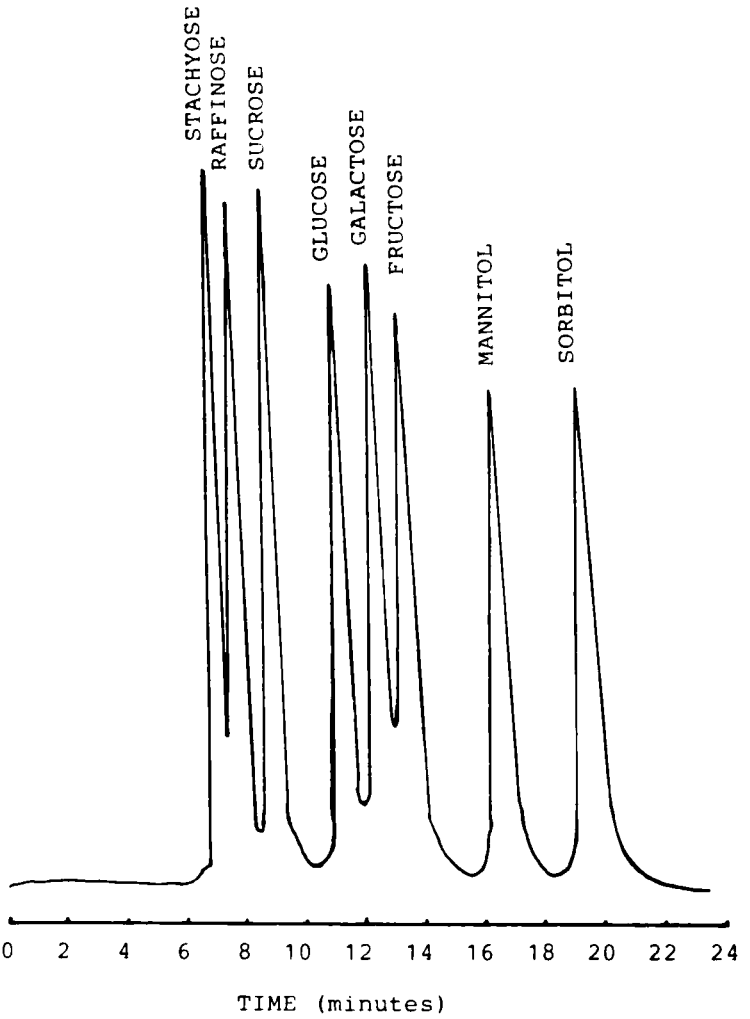

Fig. 2 : Sugar Pak I column at 90°C

Mobile phase: bidistilled water

Flow rate: 0.4 ml/min.

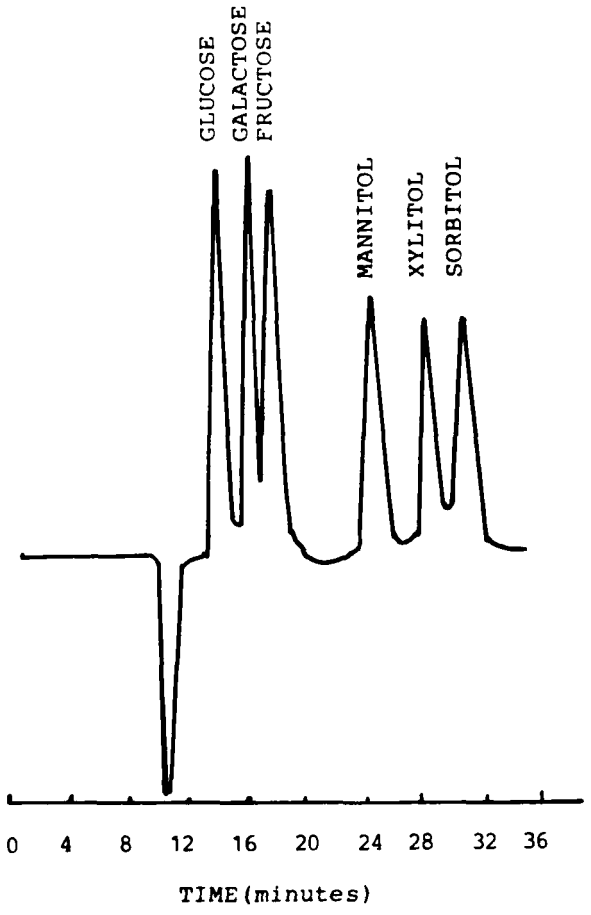

Fig. 3: Sugar Pak I column at 80°C

Mobile phase: acetonitrile/water, 25/75

Flow rate: 0.4 ml/min.

C system:
Glucose,| Galactose,| Maltitol and Fructose,| Mannitol,| Xylitol,| Sorbitol.

This is graphically collected in Table 10, a double entry table which permits the selection of most appropriate conditions to separate any mixture of the carbohydrates and polyols here studied under the conditions of analysis employed. Some of the separations achieved have been illustrated in Fig.1-3.

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