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LIQUID CHROMATOGRAPHY OF SACCHARIDES

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SUMMARY

The analysis of saccharides by liquid chromatography on an automated instrument is described. Conditions for the resolution and quantitation of fructose, glucose, sucrose, melibiose, raffinose, betaine and three kestose isomers as well as starch hydrolysates are given. Liquid chromatographic analysis equals the precision and accuracy of gas–liquid chromatographic analysis. Greater analysis flexibility and reduced sample preparation are important advantages over gas–liquid chromatographic analysis.

INTRODUCTION

The use of liquid chromatography (LC) for sugar analysis removes some of the limitations imposed by other methods of analysis. Traditional wet chemical methods nearly always incur interference from other saccharides in mixture. Enzymatic methods are extremely specific but the cost of reagents for large numbers of analyses is expensive. Derivatization for gas–liquid chromatography (GLC) is also expensive and time consuming, although quantitation is, for the most part, excellent. Analysis by paper chromatography is difficult to quantitate. High-pressure LC, on the other hand, offers rapid analysis of a large spectrum of saccharides and requires a minimum of sample preparation. As will be shown, accuracy of quantitation equals that of other currently used methods.

EXPERIMENTAL

The following equipment was used: Waters Ass. ALC 201 liquid chromatograph with differential refractometer and sample loop valve injector. Leeds and Northrup Speedomax W recorder with chart speed at 6 in./h, Varian Aerograph Model 481 electronic integrator, Technicon AutoAnalyzer Sampler II, Technicon AutoAnalyzer Proportioning Pump I and a Hoke double-acting pneumatic operator.

Two chromatographic systems were used. Initially, Bondapak AX/Corasil (37–50 μm particle size) packed in two connected columns, each 2 ft. × 1/8 in. O.D. with solvents composed of ethyl acetate, isopropanol and water in the proportions

described below. Improved resolution was obtained using acetonitrile–water elution solvents on prepacked micro Bondapak carbohydrate (10-μm particle size) columns of 1 ft. × 1/4 in. O.D. dimensions. Both packing materials were obtained from Waters Ass. (Framingham, Mass., U.S.A.).

Chromatographic standards were saccharides from commercial sources. Acetonitrile was nanograde quality supplied by Mallinkrodt (St. Louis, Mo., U.S.A.). Various juices and syrups analyzed were obtained from various stages of beet sugar manufacture. Samples were diluted to 10 mg/ml solids either with water or with the elution solvent.

Injection of samples was either with a 25-μl syringe (Precision Sampling, Baton Rouge, La., U.S.A.) or a 100-μl sample loop. An automatic sample injector was fabricated as diagramed in Fig. 1. Samples were pumped through the sample loop continuously while the previous sample was being separated on the column. After a timed interval, the length of which was slightly longer than the time required for elution of the saccharides in the sample, a second timing sequence began as follows: accuation of pneumatic operator to move valve to inject position (0 sec), return of pneumatic operator to move valve to normal position (20 sec), movement of sample line to water rinse and advancement of sample turntable one position (25 sec), movement of sample line to next sample (55 sec), and resumption of chromatographic timed interval (60 sec).
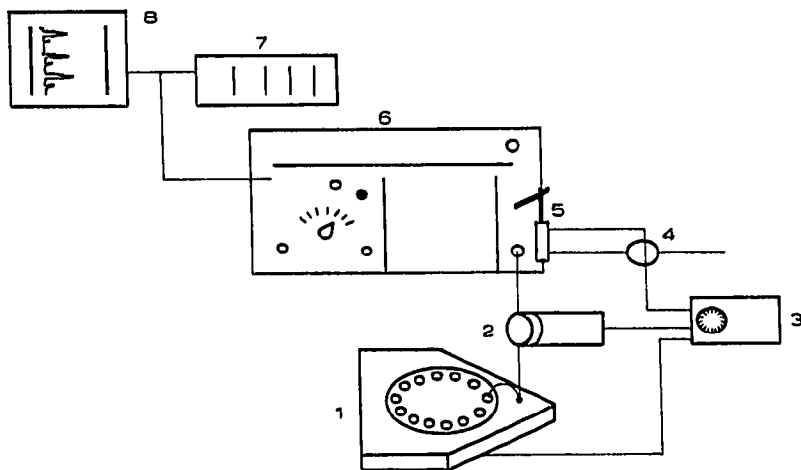

Fig. 1. Schematic diagram of the chromatographic system. 1 = Samples; 2 = pumps; 3 = timer; 4 = solenoid for control of air pressure; 5 = pneumatic operation and valve and loop injector assembly; 6 = liquid chromatograph; 7 = integrator; 8 = recorder.

RESULTS AND DISCUSSION

In Fig. 2 are shown the capabilities of Bondapak-AX/Corasil on a mixture of sugars typical to the sugar industry. Water, ethyl acetate and isopropanol in the proportions of 25:50:35 yielded fair resolution of dextrose, sucrose and raffinose in about 25 min. The linearity response of the refractometer to increasing concentrations
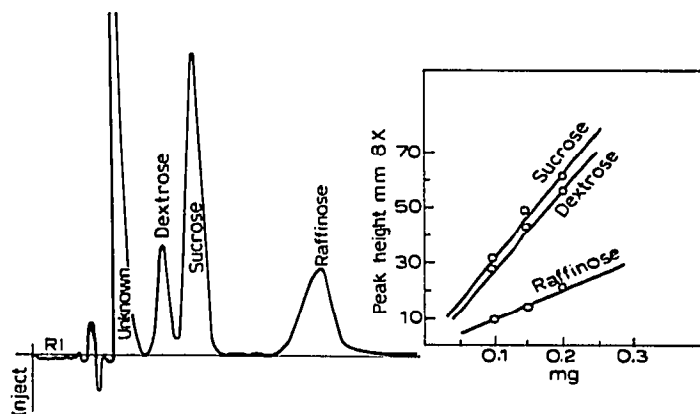
Fig. 2. Dextrose, sucrose and raffinose mixture chromatographed on Bondapak AX/Corasil column, 4 ft. × 1/8 in., in water–ethyl acetate–isopropanol (25:50:35). Insert: quantitation by peak height measurements of similar chromatograms.

of the sugars is quite good. Identical linearity and resolution have been obtained with the same packing material in about 15 min using a water–acetonitrile mixture in the proportion of 16:84.

In contrast, resolution from the micro Bondapak column is similar to that from gas–liquid chromatograms. In Fig. 3 are shown the resolution of glucose, fructose, sucrose and melibiose chromatographed on the micro Bondapak column and eluted in water–acetonitrile (25:75). Chromatogram interval was 11 min. Raf-
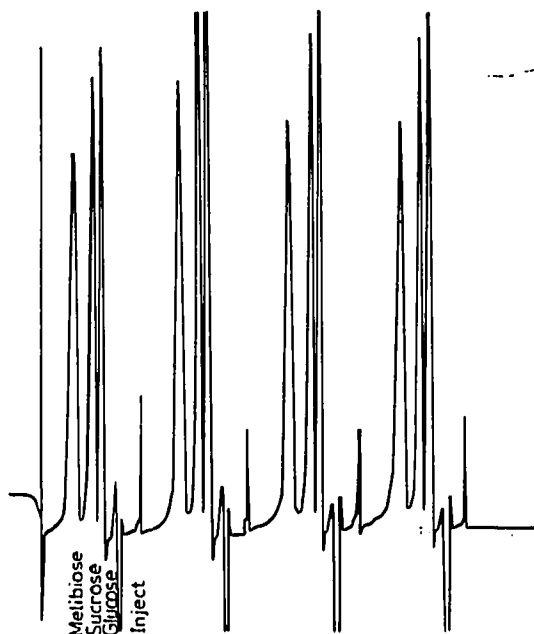

Fig. 3. Fructose, dextrose and sucrose chromatographed on a micro Bondapak column, 1 ft. × 4 in., in acetonitrile–water (75:25) at a flow-rate of 2.0 ml/min. Refractometer attenuation, 4 ×.

(a)

Melibiose
Sucrose
Glucose
Fructose
Water

Inject

(b)

Sucrose

Glucose
Fructose
Water
Inject

(c)

Sucrose

Glucose

Fructose

Water

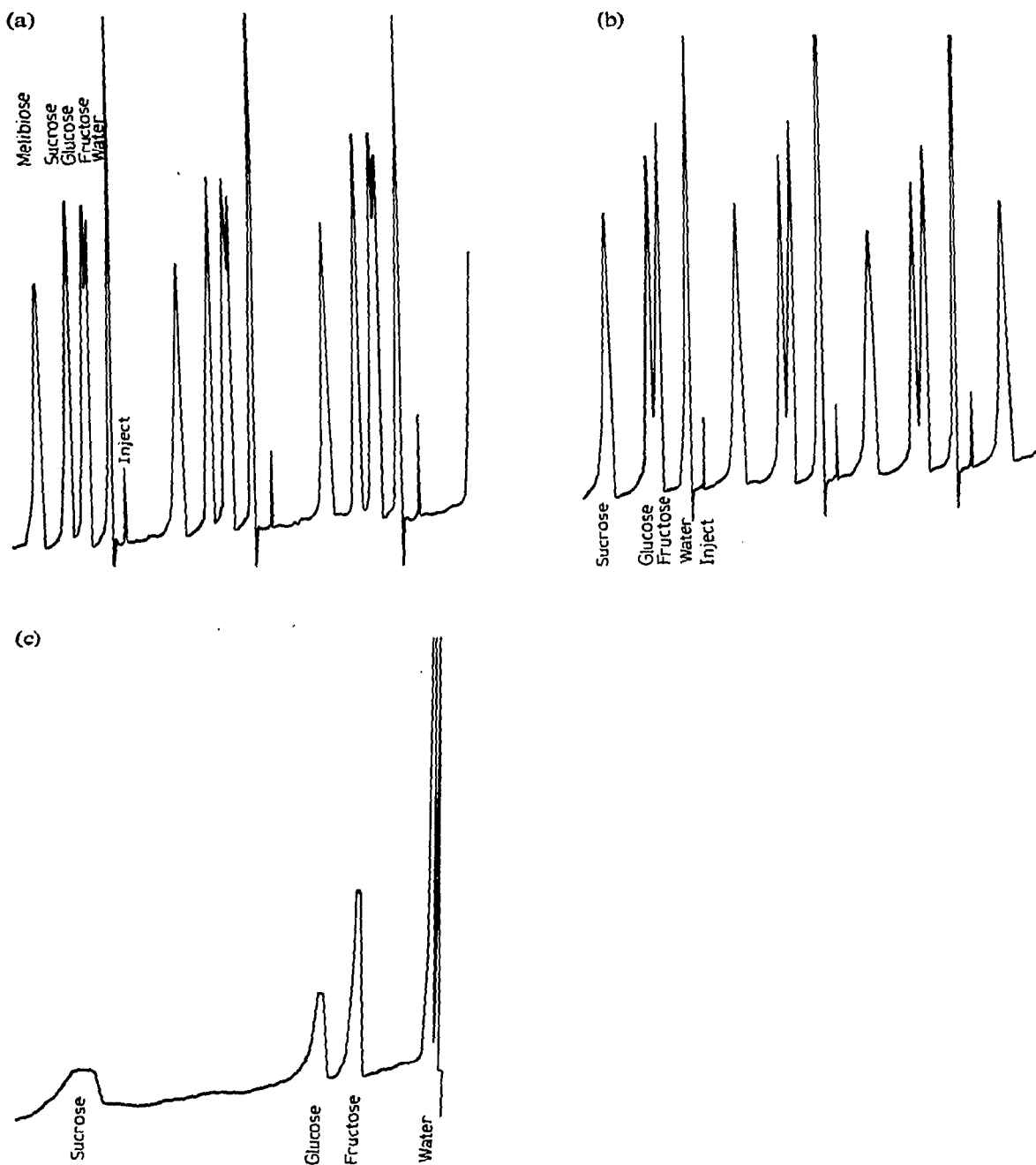Fig. 4. Fructose, dextrose and sucrose chromatographed on a micro Bondapak column, 1 ft. × 1/4 in., in an acetonitrile–water ratio of (a) 80:20, (b) 85:15, (c) 90:10 at a flow-rate of 2.0 ml/min. Refractometer attenuation, 8×.

finose would have eluted at 10 min had it been included in the mixture, and the chromatogram interval would have been 13 min. Areas determined by digital integration had a coefficient of variance of 1.44 for the invert sugar to melibiase ratio and 0.37 for the sucrose to melibiase ratio for the four determinations.

The changes in the elution of fructose, glucose, sucrose and melibiose in 80, 85 and 90% acetonitrile are illustrated in Fig. 4.

Decreasing the water content of the elution solvent slowed the respective elution of each, and thereby improved resolution of the invert sugars. In Table I are listed the elution times of the saccharides at 75, 80, 85 and 90% acetonitrile. With decreasing water in the solvent, peak broadening occurs on slower eluting compounds, *e.g.* melibiose or raffinose, and the accuracy of the integration is thereby decreased. A compromise must be drawn in the selection of solvent composition in accordance to the compounds of most interest.

TABLE I

ELUTION TIME (min) OF SACCHARIDES IN SOLVENTS VARYING IN ACETONITRILE COMPOSITION AT A FLOW-RATE OF 2.0 ml/min ON A MICRO BONDAPAK COLUMN

Sugar	% Acetonitrile in solvent			
	75	80	85	90
Fructose	3.7	4.7	6.2	13.8
Glucose	3.7	5.3	7.5	17.8
Sucrose	4.7	7.2	13.4	50.0
Melibiose	6.2	10.9		
1-Kestose	9.0			
Neokestose	9.7			
6-Kestose	10.1			
Raffinose	11.2			

Raffinose and kestoses may be studied in solvents containing 75% acetonitrile and 25% water. The elution times of raffinose and three kestose isomers are also listed in Table I. Complete separation of the kestose isomers is possible by conversion of the chromatograph to the recycle mode as shown in Fig. 5.

A number of disaccharides which might serve as an internal standard were chromatographed. It was found that melibiose would elute directly between sucrose and raffinose in either solvent system. When the samples were diluted for analysis, an aliquot of standard melibiose solution was accurately pipetted into the sample. Quantitation of each saccharide was achieved by comparison of the integration ratio of melibiose and the saccharide under question in both a standard solution and in the unknown sample.

Fig. 6 shows the chromatographic separation in 77% acetonitrile and 23% water of alternate injections of machine syrup and standard prepared in the manner just described. The first refractometer response after injection is water followed by sucrose and melibiose. Invert sugars and raffinose are not seen because of their minor compositions in relation to the refractometer attenuation. The area ratios of sucrose to melibiose, obtained by digital integration, from ten determinations of the standard
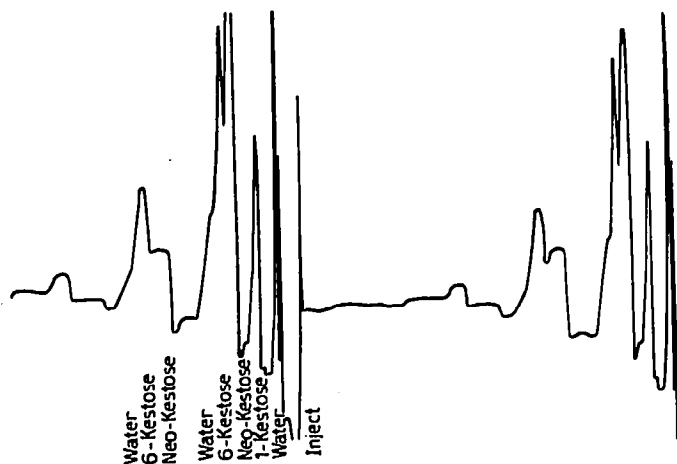
Fig. 5. Three kestose isomers chromatographed on a micro Bondapak column, 1 ft. × 1/4 in., in acetonitrile–water (75:25) at a flow-rate of 2.0 ml/min in the recycle mode. Refractometer attenuation, 4×.
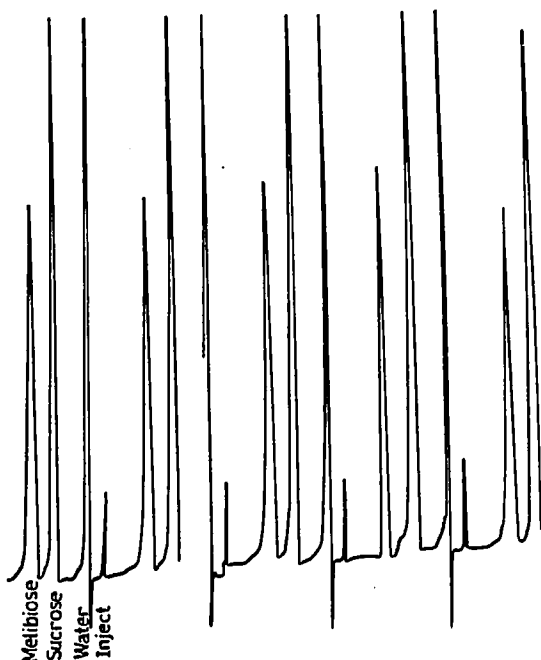

Fig. 6. Chromatographic separation of sucrose and melibiose alternatively in a standard mixture and in machine syrup to which melibiose had been added on a micro Bondapak column, 1 ft. × 1/4 in., in acetonitrile–water (77:23) at a flow-rate of 2.0 ml/min. Refractometer attenuation, 8×.

had a coefficient of variance equal to 1.0 and that from ten machine syrup determinations equalled 1.8. The sucrose content agreed with that obtained by GLC, the current standard method for true sucrose analysis.

In Fig. 7 are shown two chromatograms of the same saccharide mixture containing fructose, glucose and sucrose as well as betaine. Both separations were conducted at the same flow-rate (1.2 ml/min) in elution solvent of 85% acetonitrile and 15% water. The solvent for Fig. 7A is at pH 8.5, that of the acetonitrile water mixture. The elutions of glucose and betaine coincide at pH 8.5. The pH of the solvent in Fig. 7B was adjusted to 6.0 using acetic acid. Below pH 8.0, the pK value for betaine, the elution of betaine occurs directly between glucose and sucrose.
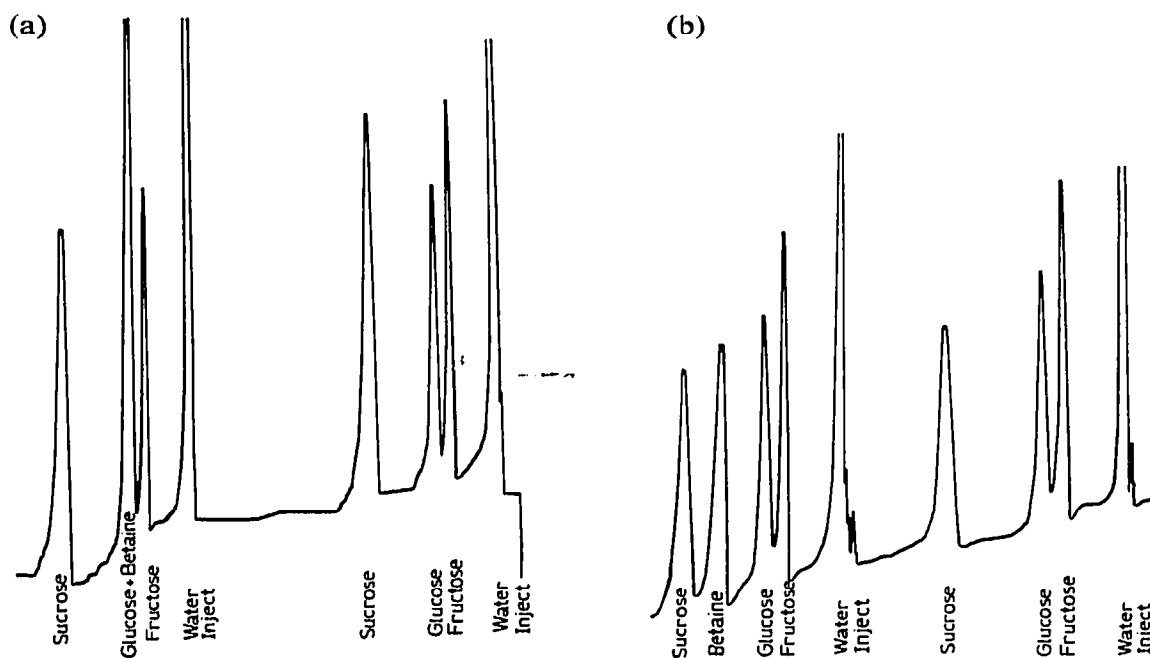

Fig. 7. Fructose, glucose, betaine and sucrose chromatographed on a micro Bondapak column, 1 ft. × 1/4 in., in acetonitrile–water (85:15) at (a) pH 8.5 and (b) pH 6.0 at a flow-rate of 2.0 ml/min. Refractometer attenuation, 4×.

The chromatograms in Fig. 8 illustrate separation obtainable with the micro Bondapak column of substances in a starch hydrolysis sample.

Glucose, maltose, maltotriose, maltotetraose and maltopentaose are cleanly separated. Without the capability to program increased flow-rates, larger oligosaccharides are not practically analyzed, but would be cleanly separated for preparative work.

Sugar beet syrups frequently contain oligo- and polysaccharides containing galactose or galactose derivatives. The chromatographic separation of these on the micro Bondapak column is possible, as shown in Table II. Separations are not clean in all cases, but the potential exists for resolution and quantitation by solvent composition changes.
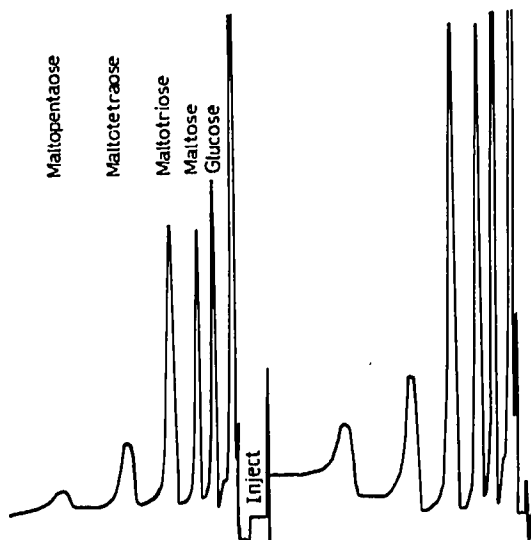
Fig. 8. Dextrose, maltose, maltotriose, maltotetraose and maltopentaose chromatographed on a micro Bondapak column, 1 ft. × 1/4 in., in acetonitrile–water (75:25) at a flow-rate of 1.8 ml/min. Refractometer attenuation, 4×.

TABLE II

ELUTION TIME OF GALACTOSE, GALACTOSE DERIVATIVES AND GALACTOSE CON-
TAINING OLIGOSACCHARIDES IN ACETONITRILE–WATER (90:10) AT A FLOW-RATE
OF 2.0 ml/min ON A MICRO BONDAPAK COLUMN

Sugar	Elution time (min)
Galactose	10.1
Galactosamine	12.5
Galacturonic acid	13.1
Ducitol	19.0
Melibiose	25.0
Galactinol	35.5
Raffinose	37.5
N-Acetylgalactosamine	37.5

CONCLUSIONS

The quantitation of a very wide range of saccharides is possible using recently developed stationary phase LC column packing materials. With the aid of newer pumping systems having the capability of flow gradients and mixture gradients, it should be possible to separate any given mixture of saccharides.

Nearly all saccharides occur in α and β anomeric forms. Quantitation of mono- and disaccharides by GLC has been hampered in that α and β anomers are readily resolved[1]. Quantitation of saccharides by high-pressure LC is not complicated by these factors, since the conformational forms are not separated.

The automatic injection system described here has improved the precision of

the results obtained on this instrument. However, the necessity for sample dilution with the elution solvent, in order to avoid peak broadening which occurs when samples are introduced to the column in water, poses other problems. Acetonitrile slowly evaporates from a series of samples prepared and waiting analysis in the automatic sampler turntable. In the course of a day, the ratio of melibiose to sucrose in a simple sample has decreased by 1 %. Whether this change is the effect of evaporation or of prolonged exposure of the saccharides to acetonitrile, the problem can be overcome and dilution accuracy improved by use of the proportioning pump to simultaneously dilute, mix and inject the sample. Thick syrups may then be maintained in the sampler turntable in a high-osmotic, spoilage-resistant state. Automatic dilution would be uniform and manual dilution error would be eliminated.

The problems and expense of organic solvents for elution make water elution systems attractive. Recently, the separation of sucrose, glucose and fructose on Aminex Q-150-S has been described[2]. This method shows promise for the chromatographic analysis of oligosaccharides because these compounds are eluted before monosaccharides. However, glucose and fructose could not be perfectly resolved by this method.

One micro Bondapak column has been used daily for four months for the analysis of carbohydrates in acetonitrile–water mixtures. The only obvious deterioration of the column is the requirement as time passed for solvents with lower water content in order to obtain similar separations. For example, as compared with the data in Table I, a new micro Bondapak column with acetonitrile–water (75:25) partially resolved fructose (5.5 min) and glucose (6.0 min) at a flow-rate of 2.2 ml/min.

REFERENCES

1 G. G. Birch, *J. Food Technol.*, 8 (1973) 229.
2 J. K. Palmer and W. B. Brandes, *J. Agr. Food Chem.*, 22 (1974) 709.