AUTOMATED LIQUID CHROMATOGRAPHIC SYSTEM FOR ANALYSIS OF CARBOHYDRATE MIXTURES

H. D. SCOBELL, K. M. BROBST, and E. M. STEELE, A. E. Staley Manufacturing Company, Decatur, IL 62525

Cereal Chem. 54(4): 905–917

ABSTRACT

An automated liquid chromatographic (LC) system is described for quantitative analysis of carbohydrate mixtures. Dextrose, fructose, and related saccharides of commercial sweeteners are separated on cation-exchange resins with water as the eluent. The column systems described give rapid, high-resolution separations on a stable baseline—features which allow accurate and precise quantitation. All the carbohydrate components of a sweetener elute from the columns, permitting quantitation on a simple area normalization basis. No supporting analyses are required. Analysis time ranges from 5 to 30 min. The instrumentation described includes a custom-built automatic sample injector/system controller, coupled with a conventional LC and a computing integrator.

The separation of carbohydrate mixtures by high-performance liquid chromatography (LC) is rapidly becoming an accepted technique within the sweetener industry for the analysis of the carbohydrate composition of a wide variety of sweetener products. Early work by Brobst *et al.* (1) reported all aqueous separations of the mono- and oligosaccharides of several sweeteners on cation-exchange and polyacrylamide resins in 1 to 2 hr. Palmer and Brandes (2) also reported separations of dextrose and fructose on all aqueous cation-exchange resin columns. Recently, with the introduction of the chemically-bonded stationary phase carbohydrate-type columns with acetonitrile-water solvent systems, several authors have reported a wide variety of rapid separations. Conrad and Fallick (3) have utilized this system to analyze brewer's adjunct syrup, high-fructose syrup, wort, and others. Palmer (4) reported separations of mono-, di-, and oligosaccharides from various natural products. Linden and Lawhead (5) described a semi-automated system for the separation of dextrose, fructose, sucrose, and raffinose from beet sugar invert syrups using a chemically-bonded, stationary-phase column.

This paper describes a completely automated method, based on all aqueous column systems, that can be utilized in any area that requires rapid, accurate analysis of large numbers of sweetener samples with a minimum of operator attention.

MATERIALS AND METHODS

Automated LC Equipment

The following list of commercially available equipment is used in the automated system.

1. Waters Associates ALC-201 LC, equipped with differential refractometer and 1000-lb solvent delivery system.
2. Waters Associates 24 × 0.305 in. empty stainless-steel column assemblies, equipped with 10-μ end fittings. Shorter columns cut from this stock size.
3. Waters Associates column heating blocks 12 or 24 in. long.

4. Two Haake model FE circulating heating baths. One bath regulates detector temperature; the other regulates column temperature.

5. Spectra Physics System I computing integrator, equipped with calculation accessory and auxiliary power supply.

6. Houston Instrument Omniscribe dual pen recorder Model 5211-12, one pen at 100 mV full scale, the other at 1 V full scale.

7. Column packing material, Aminex 50W-X4, 20- to 30-μ particle size, Aminex Q15S, 19- to 25-μ, and Aminex A-5, 11- to 15-μ, all available from Bio-Rad Laboratories.

8. Technicon Sampler II sample turntable (modified).

9. Hamilton high-pressure sampling valve, Model 77503, equipped with 5- or 10-μl transfer shaft.

10. Cole Parmer Masterflex tubing pump (sample pump), Model 7545-13, equipped with silicon rubber tubing.

11. The custom-built system controller consists primarily of four major components that function as follows:

A. A Hecon presettable counter, catalog number A0423-138-1, determines the sample interval. Whenever either a momentary contact 'start' switch is depressed or the preset value is reached, this unit initiates a new sampling cycle by sending a pulse to component B.

B. This component is an Industrial Time, type RC-5, single-cycle, multicam timer equipped with a 5-min cycle gear. When initiated by component A, this timer controls the functions of the sample turntable, the sampling pump, and the high-pressure sampling valve. The first cam switch of the timer is wired to the diptube and turntable control functions of the Sampler II. The cam on this switch is adjusted to provide an initial 2-min 20-sec wash period followed by a 20-sec sampling period and finishes the cycle with an additional 2-min 20-sec wash period. The second cam switch of the timer is wired to the solenoid switching valves on the high-pressure sampling valve. The cam on this switch is adjusted in such a manner that the valve will initially remain in the wash/load position for 2 min 40 sec, then, as the sampler diptube withdraws from a sample cup, the valve switches to the inject position for 30 sec and finally returns to the wash/load position for the remaining 1 min 50 sec of the 5-min cycle. The third switch of the timer is wired to provide power to the timer and the sampling pump control unit for a 5-min interval.

C. The third component of the controller is a Hecon presettable counter, catalog number G0422-589-1. This counter's output is wired to the 'run' function of the computing integrator and provides initiation of the integrator at a preset time after the sample has been injected.

D. The fourth component is an Industrial Time, type MC-1 multicam recycling timer, equipped with a 10-sec cycle gear. This timer is the controller's clock and provides counting pulses to the presettable counters at 10-sec intervals. With the combination of this timer and the six-digit counters, one can preset sample interval and integration delay times from 5 min to 2,778 hr in increments of 10 sec. As a final note, the system controller is wired to the 'end of run' switch of the Sampler II in such a way that when the last sample has been run, power is removed

from the controller's clock and the recorder power outlet. Figure 1 illustrates the interconnections of the system controller with the remainder of the LC system.

12. Amberlite MB-1 mixed bed, ion-exchange resin.
13. Calcium chloride dihydrate.
14. Silver nitrate.
15. Lab Crest — 15 mm i.d. × 400 mm L. glass chromatographic column or equivalent.
16. Sugars for calibration. Dextrose and sucrose are available from the National Bureau of Standards, maltose from Applied Science Laboratories, Inc., trisaccharides from Pierce Chemical Company. Other oligosaccharides may be prepared by preparative paper chromatography (6).

Resin Preparation Procedure

The resin of choice is slurried in deionized distilled water in a ratio of 4:1, water:resin and is degassed for 1 hr with water aspirator vacuum. The resin is then transferred to a 15 mm i.d. × 400 mm L. glass column and a tenfold excess of a 2*N* solution of the appropriate ion is either pumped or allowed to percolate through the bed. After the resin has been converted to the appropriate form, it is washed with three bed volumes of water. Finally, the resin is reslurried in 2 liters of water, transferred to a 2-liter graduated cylinder, and checked for fines. If the resin is free of fines, a sharp boundary layer will soon form with no light

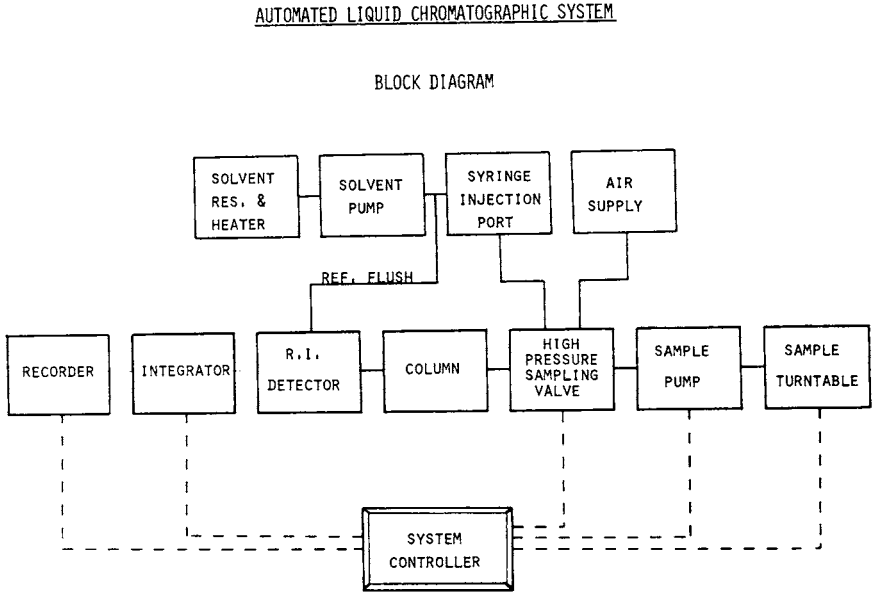

Fig. 1. Automated LC system block diagram.

scattering particles observable above the boundary layer. If fines are observed, allow the majority of the resin to settle; then decant the upper layer. Repeat until a clear boundary is obtained.

Column Packing Procedure

Two columns of the same length are joined with a tubing union. A suitable reservoir (at least 500 ml) is attached to the upper end and a capped 10-μ end fitting is attached to the lower end of the assembly. The assembly is clamped in a vertical position and filled to the bottom of the reservoir with distilled water. The resin is reslurried in 300 ml of water and transferred into the reservoir. The resin is allowed to settle for 3 hr. After settling is complete, the reservoir is removed and the assembly is connected to a high-pressure pump. After removing the lower end fitting cap, the flow from the pump is adjusted to a value that will be used during actual analysis. The assembly is pumped in a down-flow direction at this rate for 30 min. Next, the flow rate is doubled and pumping is continued for 3 additional hr.

The final step in the packing procedure is the capping of the lower column. To successfully cap a column, a 10-μ end fitting is prepared in the following manner. First, the flow to the assembly is shut off and the column pair is disconnected from the pump. The upper column is removed and laid aside. The union is removed and the resin from it is transferred to a 10-μ end fitting. Several drops of water are added to the resin to settle it into the fitting. The water is then removed from the resin by applying suction to the small end of the fitting. Finally, the end fitting is attached to the column and tightened. Although the column can be used in either direction, the end that is capped last is the preferred inlet.

Sample Preparation

A. Samples that contain protein, such as crude enzymatic starch hydrolyzates, are adjusted to pH 4 and placed in a steam bath for 10 min. The sample is then cooled and filtered.

B. Samples that contain salts or that are acidic or basic are diluted to either 15 or 30% dry solids to a total volume of 10 ml with water. One hundred to 200 mg of Amberlite MB-1 is added, and the solution is placed on a wrist-action shaker for 10 min.

C. If the sample is clean, *i.e.*, is neutral and contains neither protein nor salts, dilute as in Step B and omit deionization.

LC Operating Conditions

Typical operating conditions for the LC system are as follows:
Solvent: Deionized, distilled, degassed water.
Flow Rate: 0.3 to 1.2 ml/min.
Column Temperature: 85°C.
Detector Temperature: 45°C.
Detector Attenuation: 4 to 16×.
Sample Volume: 5 to 10 μl.
Sample Weight: 1.5 to 3.0 mg dry substance basis.

Preparation of Calibration Mixtures

Due to the expense of some standard carbohydrates, most calibration mixtures are made to contain 100 mg total carbohydrate in 1 ml water. The individual sugars are rapidly weighed to the nearest tenth of a milligram and then are dissolved in 1 ml water. When not in use, store at −20° C.

System Calibration

Calibration of the system is accomplished by injecting about 15 μl of the appropriate calibration mixture and instructing the computing integrator to perform a Method 1 automatic calibration sequence as outlined in the System I manual. At the end of the calibration sequence, usually consisting of three injections of the calibration mixture, the integrator computes and stores an area correction factor for each sugar in the mixture.

Evaluation of the Chromatogram

The chromatograms are automatically evaluated by the System I computing integrator. The results are printed at the end of each analysis as the per cent of each saccharide present on a dry substance basis.

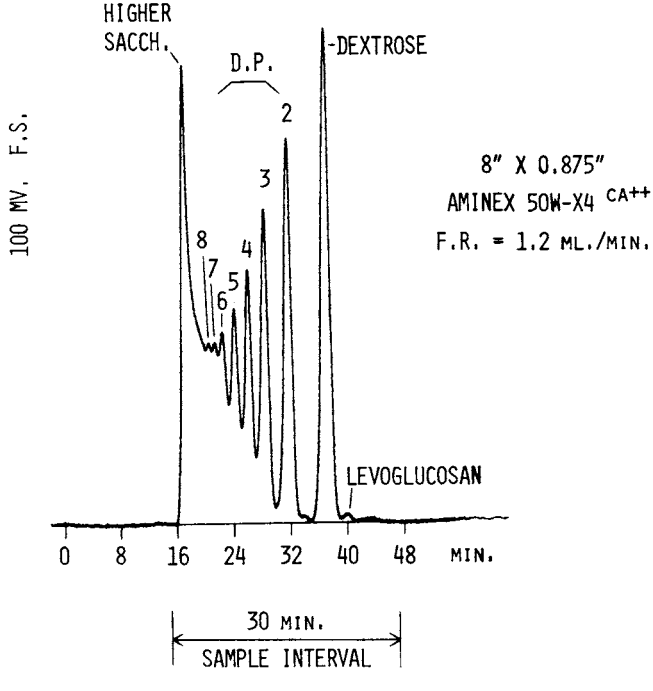

Fig. 2. Chromatogram of acid hydrolyzed corn syrup on Aminex 50W-X4 [Ca^{++}] at 85° C.

RESULTS AND DISCUSSION

The need for rapid, accurate, unattended analysis of sweetener products in process and quality control areas prompted the development of this automated LC system. The requirement of unattended operation was most easily met. The use of an autosampler, coupled with a system controller and a computing integrator, has reduced the operation of the system to loading samples and pressing a 'start' switch. To meet the requirements of speed and accuracy, however, required the development of a series of column systems that would not only provide rapid separation of the saccharides in a variety of products, but also permit an area normalization type of quantitation, which would require no supporting analyses. The first column system in the series is packed with Aminex 50W-X4 resin and is used to separate the oligosaccharides in low and medium conversion sweeteners.

Separation of Low and Medium Conversion Sweeteners on Aminex 50W-X4

Figure 2 illustrates the separation of the oligosaccharides in an acid-hydrolyzed corn syrup on the calcium form of Aminex 50W-X4. This column separates the saccharides primarily on a basis of size, with saccharides having a degree of polymerization (DP) greater than 8 being excluded from the resin matrix and eluting first. Saccharides of DP 8 and smaller elute in decreasing order of size, down to the monosaccharides. The monosaccharides elute in an

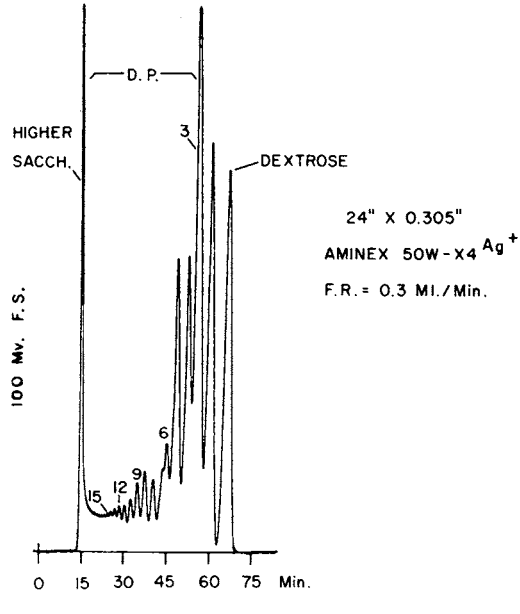

Fig. 3. Chromatogram of enzymatically hydrolyzed corn syrup on Aminex 50W-X4[Ag^+] at 85°C.

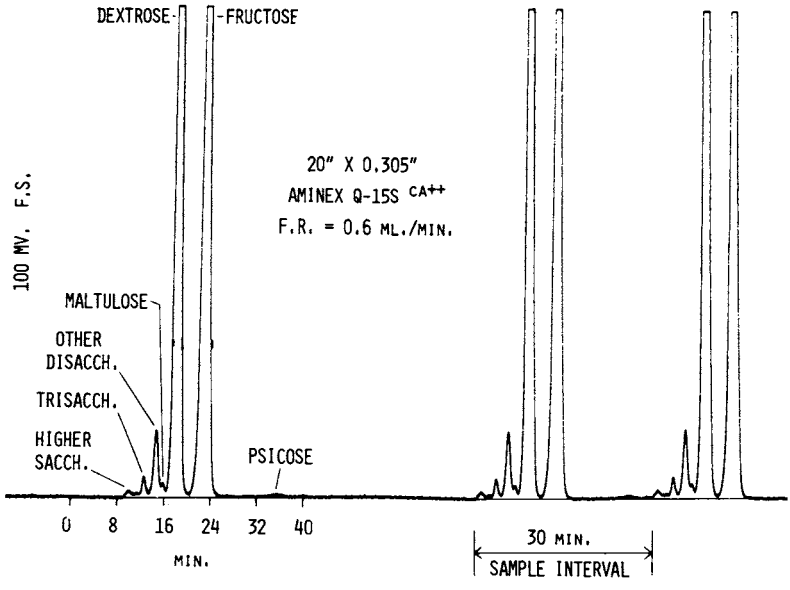

Fig. 4. Chromatogram of high fructose corn syrup on Aminex Q15S [Ca^{+2}] at 85°C.

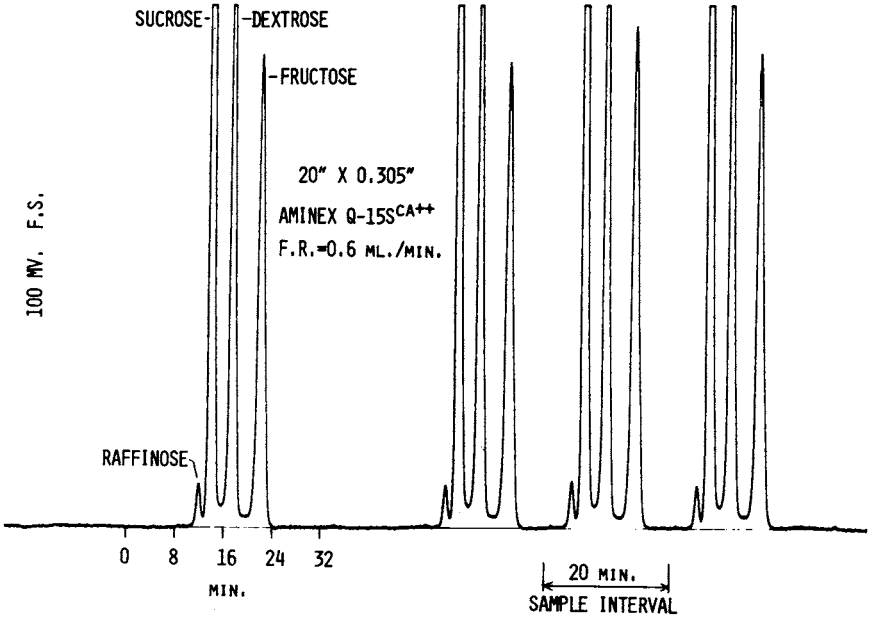

Fig. 5. Chromatogram of medium invert sugar syrup on Aminex Q 15S[Ca^{+2}] at 85°C.

order that depends on the strength of the complex they form with the bound calcium ion, as is illustrated by the separation of dextrose and levoglucosan. This resin, when converted to the calcium form, is too soft to pack into long narrow bore columns and operate at high flow rates. Therefore, we use the short, large diameter column to reduce the linear flow rate, yet maintain relatively short analysis time. By programming the autosampler for overlap injections, *i.e.*, the injection of another sample before the last has finished, we are able to analyze a series of low conversion sweeteners at a rate of one sample per 30 min.

Recently, while investigating different metallic forms of Aminex 50W-X4, we found silver ion has two beneficial features. Figure 3 shows the separation of the oligosaccharides in an enzymatically hydrolyzed corn syrup on Aminex 50W-X4 in the silver form. In the silver form, the exclusion limit of the resin is extended from DP 8 to about DP 20 with good resolution being maintained up to DP 15. Since the resin has a high relative affinity for silver ion, more shrinkage occurs than with calcium, which results in a more dense resin particle. This allows packing in conventional dimension columns and operation at relatively high flow rates. This column has been operated with flow rates up to 1 ml/min without damage. However, with analyses times less than 30 min, the resolution of the saccharides above DP 10 is reduced considerably. This column's primary use is in the analysis of very low conversion dextrins, where the majority of the saccharides are in the DP 6 to 20 region.

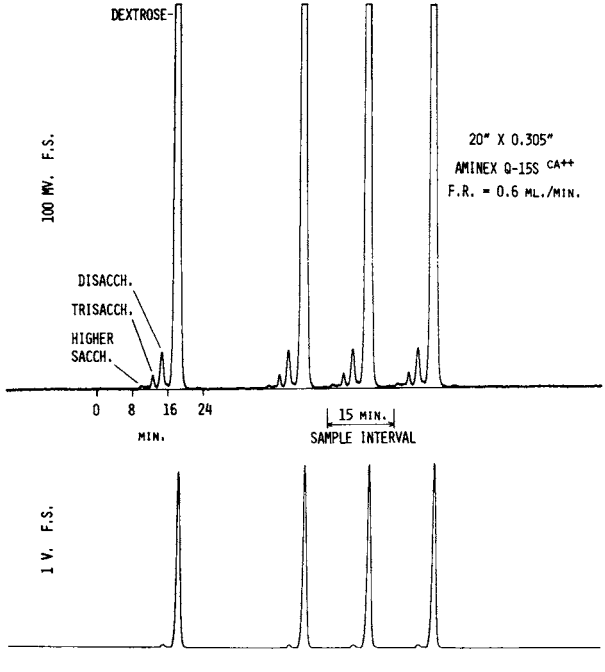

Fig. 6. Chromatogram of high dextrose syrup on Aminex Q15S [Ca^{+2}] at 85°C.

Separation of Highly Converted Sweeteners on Aminex Q15S

Since the type of quantitation used in this method requires the accurate detection of all saccharides in a sample, a different column is used for the analysis of highly converted sweeteners. These products consist primarily of monosaccharides with small amounts of higher oligosaccharides usually being present. Figure 4 illustrates the separation of a high fructose corn syrup on Aminex Q15S in the calcium form. This resin contains twice as much crosslinking as the 4% crosslinked Aminex 50W-X4 and, consequently, excludes saccharides larger than DP 4. This feature allows all the low-level saccharides larger than DP 4 to elute as a single well-defined peak that is much easier to quantitate than a series of much smaller peaks. The column also produces an excellent separation of dextrose from fructose and is capable of separating maltulose, isomerized maltose, from maltose. Psicose, the C-3 epimer of fructose, elutes considerably later than the other monosaccharides, but with overlap injections, the analysis can be carried out at a rate of one sample per 30 min. The separations to the right in Fig. 2 indicate automated operation with overlap injections. By using the transfer shaft type sampling valve and all aqueous columns, we are able to obtain separations on a relatively flat baseline. No baseline upsets are observed, due to either injection pressure spikes or solvent/sample dissimilarities. We have found that good baseline stability is of

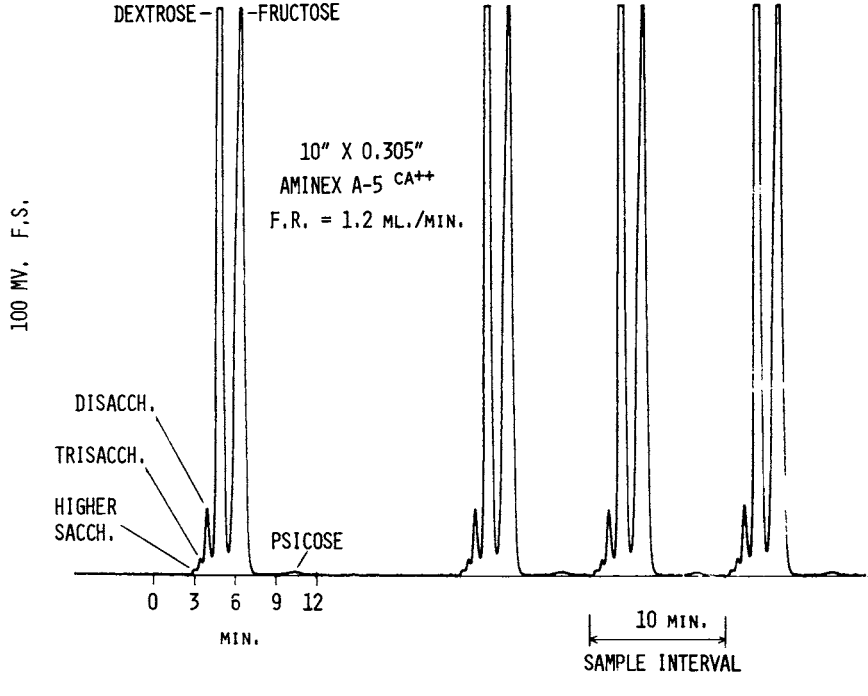

Fig. 7. Chromatogram of high fructose syrup on Aminex A-5 [Ca^{+2}] at 85°C.

prime importance in optimizing the precision of the method.

Another sweetener product that can be analyzed with the Aminex Q15S column is the invert sugar syrups. Shown in Fig. 5 is the separation of the saccharides from a medium invert syrup produced from beet sugar. Fructose, dextrose, sucrose, and raffinose can be separated in these products at a rate of one sample every 20 min. We have found that this analysis is possible only if the resin packing of the column is completely converted to the calcium form. If some of the packing remains in the hydrogen form, inversion of the sucrose in a sample will occur. This is the main reason why excessive amounts of salts are used in preparing the resin and why we pretreat acidic samples.

An important use of the Aminex Q15S column is the analysis of very high dextrose syrups. Figure 6 shows the separation of the saccharides present in this type of product. This sample contains over 97% dextrose and is difficult to analyze by conventional methods such as optical rotation, glc, or automated colorimetric procedures. Any error in the dry substance determination required by these techniques is directly reflected in the amount of dextrose determined. Reproducibility at the 97% level is seldom better than ±0.5%. With the total recovery feature of the LC system, we are able to determine dextrose at the 97% level with accuracy and precision both better than 0.2% in less than 15 min. The lower pen tracing shown in Fig. 6 is used in this analysis to monitor excessive peak height since the detector saturates at about 0.5 V.

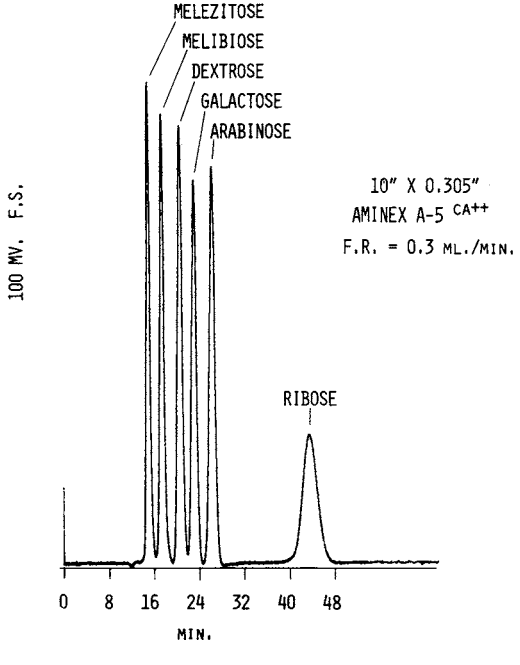

Fig. 8. Chromatogram of complex carbohydrate mixture on Aminex A-5 [Ca^{+2}] at 85° C.

Separations on Aminex A-5

For process control analysis, with its prime requirement being speed, we developed yet another column system. To increase the speed of analysis and still maintain acceptable resolution, we use shorter columns and pack them with a better grade of resin, Aminex A-5. This resin, again with 8% crosslinking, performs slightly better than Aminex Q15S due to its smaller particle size and particle size range of 11 to 15 μ. Figure 7 illustrates the separation of a high-fructose corn syrup on a 10×0.305 in. Aminex A-5 column in the calcium form. With this column, we can separate the saccharides of a high fructose syrup, including psicose, in 10 min, and invert syrups in 6 min and high-dextrose syrups in 5 min.

Figure 8 illustrates yet another application of the Aminex A-5 column. By reducing the flow rate from 1.2 to 0.3 ml/min, we are able to perform high resolution separations on the same column that we ordinarily use for high-speed work. In this mode of operation, the Aminex A-5 column can perform such difficult separations as dextrose from galactose, stachyose from raffinose, xylose from fructose, and dextrose from xylose in less than 30 min on an overlap basis.

Quantitative Aspects of the Method

The area normalization method of quantitation used by the computing integrator is possible since in the all aqueous column system, all the carbohydrate material injected into the column is recovered. The advantages of this method of quantitation are: no dry substance determination is required, no internal standard is needed, and, due to the excellent linearity of the area

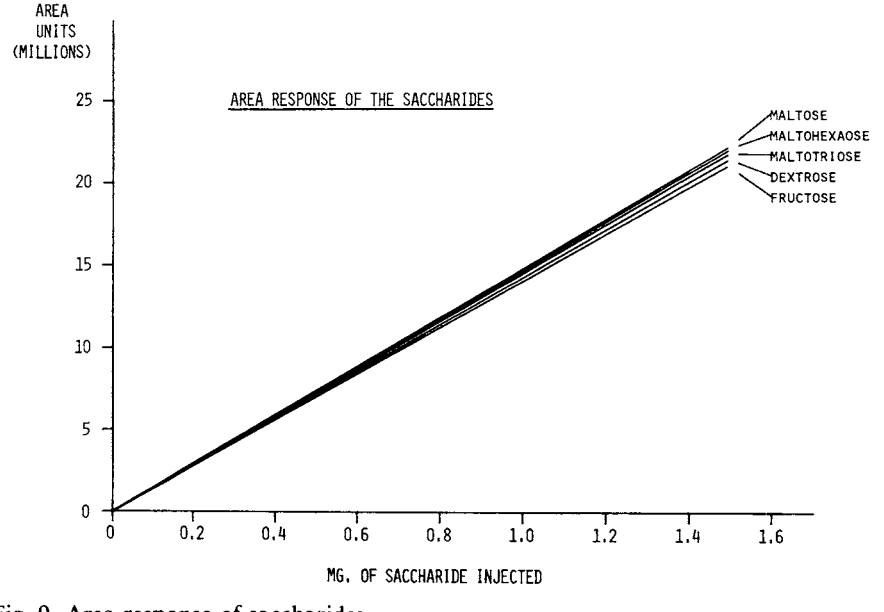

Fig. 9. Area response of saccharides.

response of the saccharides, a quantitative sample injection is not necessary. Figure 9 is a plot of the area response of several representative saccharides that were chromatographed on a 20×0.305 in. Aminex Q15S [Ca^{++}] column. We have found that, even though peak shapes differ considerably from excluded peaks such as maltohexaose, to long-residing peaks such as fructose, only slight differences are observed in their area response.

The precision of the method, as mentioned earlier, is limited primarily by the stability of the baseline. With a reasonably well-maintained system, the within-day standard deviation of any given peak can be expected to be less than ±0.1 and over the period of several days to be less than ±0.2. Table I lists the results of a precision study of our most difficult analysis, the determination of very high levels of dextrose in the presence of very small amounts of oligosaccharides.

The accuracy of this determination, for all the components in a sample, can be better than ±0.1% absolute, providing that adequate care is exercised in selecting the standard sugars, preparing the calibration mixtures, and matching the

TABLE I
Within-Day and Day-to-Day Precision Study on Saccharides

Date	Saccharides, %							
	Dextrose		Disaccharides		Trisaccharides		Higher Saccharides	
	$\bar{x}$	S	$\bar{x}$	S	$\bar{x}$	S	$\bar{x}$	S
9-25-75	97.08	.04	2.14	.02	0.56	.01	0.22	.03
9-26-75	97.00	.04	2.18	.03	0.59	.01	0.22	.01
10-1-75	97.03	.07	2.16	.02	0.57	.02	0.23	.02
Grand mean	97.04		2.16		0.57		0.22	
Standard deviation	0.11		0.03		0.02		0.02	

TABLE II
Accuracy: Standard Addition of Fructose to High Dextrose Syrup

Fructose Addition %		Fructose %	Dextrose %	Maltose %	Maltotriose %	Higher Saccharides %
25.18	Determined:	24.98	72.68	1.60	0.50	0.25
	Actual:	25.18	72.51	1.63	0.45	0.23
	% Recovery	99.21	100.23	98.16	111.11	108.70
50.21	Determined:	50.14	48.06	1.20	0.38	0.17
	Actual:	50.21	48.25	1.09	0.31	0.15
	% Recovery	99.86	99.61	110.09	122.58	113.33
75.10	Determined:	75.11	23.91	0.65	0.22	0.09
	Actual:	75.10	24.14	0.54	0.15	0.07
	% Recovery	100.01	99.05	120.37	146.66	128.57

composition of the standard solution as nearly as possible to that of the sample. We have found, however, that accuracy on the order of ±0.2% absolute can be obtained for a wide variety of sample compositions by using a standard in which all the components are at the same level. Table II lists the results of such a study in which we produced a wide variety of sample compositions by adding known amounts of fructose to a synthetic high dextrose syrup.

In conclusion, we have found this system to be well suited for the rapid analysis of the entire spectrum of sweeteners produced by acid or enzymatic conversion of corn starch.

Literature Cited

1. BROBST, K. M., SCOBELL, H. D., and STEELE, E. M. Analysis of carbohydrate mixtures by liquid chromatography. Proc. of the 39th Annual Meeting of the Amer. Soc. Brew. Chem.: (1973).
2. PALMER, J. K., and BRANDES, W. B. Determination of sucrose, glucose, and fructose by liquid chromatography. J. Agr. Food Chem. 22: 709 (1974).
3. CONRAD, E. D., and FALLICK, G. J. High pressure liquid chromatography (HPLC) and its application in the brewing laboratory. Brewer's Dig. 49(D): 72 (1974).
4. PALMER, J. K. A versatile system for sugar analysis *via* liquid chromatography. Anal. Lett. 8(3): 215 (1975).
5. LINDEN, J. C., and LAWHEAD, C. L. Liquid chromatography of saccharides. J. Chromatogr. 105: 125 (1975).
6. COMMERFORD, J. D., VAN DUZEE, G. T., and SCALLET, B. L. Macro paper chromatography of corn starch hydrolyzates. Cereal Chem. 40: 482 (1953).

[Received November 3, 1976. Accepted March 25, 1977]