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PREPARATIVE LIQUID CHROMATOGRAPHY OF CARBOHYDRATES: MONO- AND DI-SACCHARIDES, URONIC ACIDS, AND RELATED DERIVATIVES

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ABSTRACT

The general principles and practical aspects of preparative high-performance liquid chromatography (l.c.) of mono- and di-saccharides, sugars acids, lactones, and *N*-acetylated amino sugar derivatives are described. Milligram to gram quantities of these carbohydrates were isolated on semi-preparative (0.78 × 30 cm) or preparative (~2.0 × 30 cm) columns packed with aminopropyl silica gel or cation-exchange resins in the H^+ or Ca^{2+} form. Columns of aminopropyl silica gel provided better resolution of individual mono- and di-saccharides, but columns of cation-exchange resin had higher capacity and were more durable and economical to use. Preparative, cation-exchange columns were operated at flow rates of <5 mL/min and pressures of ~1–2 MPa, allowing them to be used on unmodified analytical l.c. systems. Details are given for the efficient packing, use, and care of these columns, and on the effects of column selectivity, packing technique, and sample size on chromatographic resolution. Isolation of naturally occurring sugars from biological sources on a laboratory-packed column is described.

INTRODUCTION

Liquid chromatography (l.c.) on bonded-phase silica gel and on high-performance ion-exchange resins has become a common and valuable method for the analysis of carbohydrates. In l.c., carbohydates are usually well separated under mild conditions, and detected by non-destructive techniques. These features make this method useful for the preparative isolation of pure carbohydrates. Although there have been several reports on the isolation of pure sugars[1–3] and simple sugar derivatives[4,5] by preparative l.c., no description of the practical aspects of this method, such as phase selection, and column packing, use, and care, has been published. We now demonstrate methods for practical, high-resolution preparative l.c. of mono- and di-saccharides, and of several simple derivatives such as the 2-

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acetamido-2-deoxy-D-hexoses, and the uronic acids and their lactones. Most of the columns used here require low flow-rates and back pressures, and are easily packed and regenerated. In many cases, the methods are easily adaptable to standard analytical l.c. equipment and can be used to isolate gram quantities of pure carbohydrates.

RESULTS AND DISCUSSION

General principles of preparative l.c. of carbohydrates. — Polar, water-soluble carbohydrates may be preparatively isolated on the same stationary phases as are commonly used for analytical l.c. separations, namely, the aminopropyl silica gels and the polystyrene-based ion-exchange resins. In the work described here, the goal was to isolate the largest amount of pure carbohydrate in the shortest length of time, while using the most economical and practical methods possible. Therefore, only high-resolution systems, which produce relatively pure samples after one pass through the column, were used. Low-resolution preparative l.c. systems may have higher sample capacities, but they usually produce only partially purified samples that must be repetitively recycled through the chromatograph[4,5]. Such methods are not practical for the routine purification of small to intermediate amounts of pure carbohydrates. In this study, the resolution between adjacent chromatographic peaks was calculated by the standard resolution equation

$$R = \Delta t/t_w, \qquad (1)$$

where Δt is the distance between peaks, and t_w is the average peak-width at baseline. Our goal was to develop separations in which all peaks of interest were separated by R values of 1 or greater. Under these conditions we found that each component could be isolated in >95% purity. The resolution between peaks on a given column was affected by the amount of sample that was injected; accordingly, when only a few mg of a sample were required, preparative l.c. was performed on an analytical-sized column. For larger samples, a preparative-sized column was used. Small-scale preparative chromatography on analytical-sized columns will be described first.

Preparative l.c. on analytical-type columns. — Although analytical columns (with inner diameters of $\leq\sim$0.5 cm) packed with high-performance aminopropyl silica gel are excellent for the separation of various carbohydrates, they should be used with great caution in preparative applications. Many reducing sugars can form covalent linkages to the amino groups of the stationary phase[6], causing loss of resolution. In addition, this silica-based stationary phase tends to dissolve in the mobile phase, producing voids in the column[6,7]. On the analytical scale, these problems can be avoided by proper selection[6] of solvent pH, by the use of low flow-rates and silica saturator columns[6], and by injecting very small samples into the column. Preparative chromatography, however, requires the repeated injection

of relatively large and impure samples. Because these delicate analytical columns are rapidly degraded under these conditions, their use in preparative chromatography is not recommended.

Unlike aminopropyl silica columns, analytical columns packed with high-performance cation-exchange resins are quite useful for preparative applications. These resins are mechanically stable, can be eluted with simple mobile phases (usually water) at low flow rates, and can be easily regenerated and repacked[8–10] with a minimum of equipment. Carbohydrates are separated on cation-exchange resins by a combination of size-exclusion, ion-exclusion, and ligand-exchange mechanisms[11–13], and columns packed with these resins are commercially available. Many of these commercial analytical columns have a relatively large internal diameter (0.78 cm) which makes them ideal for small-scale preparative chromatography. The use of such a cation-exchange column in the H^+ form is shown in Figs. 1 and 2, for the separation of selected hexuronic acids and 2-acetamido-2-deoxy-D-hexoses. The use of this type of column for analytical separation of these compounds has previously been described[11]. Resolution was optimized by using the correct temperature for the particular compounds to be separated. The resulting resolution that was obtained determined the amount of sample that could be isolated by preparative injections. For the isomeric hexuronic acids (see Fig. 1), which were separated by an *R* value of only 1.2 when 0.1 mg was injected (see Fig. 1a), a maximum of only 3 mg of each acid could be injected before the resolution decrease to the minimally acceptable value of 1 (see Fig. 1b). For easier separations, such as that of glucuronic acid and 2-acetamido-2-deoxy-D-glucose, where $R = 3.4$ for a 0.1-mg injection (see Fig. 2a), up to 15 mg of each component could be injected and separated (see Fig. 2b). These two separations represented the approximate extremes of resolution values that were obtained in the separation of sugar acids and *N*-acetylated amino sugars, and could be used as an approximate guide in estimating the amounts of pure sample that may be obtained from a semi-preparative l.c. injection.

Preparative l.c. on large columns (2.0 × 30 cm) of cation-exchange resins. — For the isolation of larger quantities of pure carbohydrates, the use of columns with larger diameters was necessary. Such columns have not hitherto been extensively used, because of the commonly held belief that they require high flow-rates, are to be operated at extremely high pressures, and are prohibitively expensive; interestingly, most of these concerns were not substantiated in this study. In the work described herein, on columns packed with ion-exchange resins, back pressure ranged from only 0.7–1.4 MPa (~100 to 200 lb. in.$^{-2}$). This pressure was exhibited when preparative columns were operated at flow rates that would provide the same retention times that were observed for compounds on analytical columns with identical length. This flow rate was determined by the following equation

$$F_2 = F_1 [\text{i.d.}_2/\text{i.d.}_1]^2, \qquad (2)$$

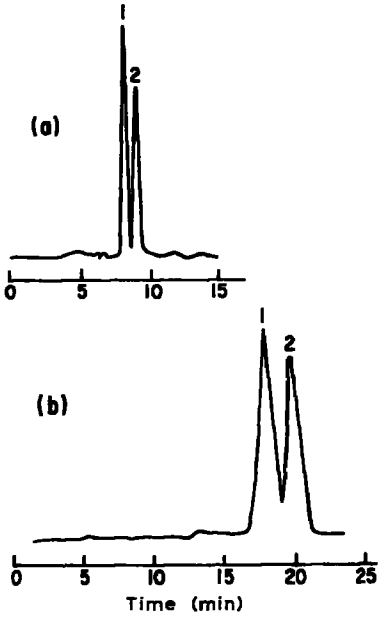

Fig. 1. Separation of glucuronic (1) and galacturonic (2) acids on the analytical and preparative scale, using an HPX-87H$^+$ column (0.78 × 30 cm). (a) Each component (0.1 mg) injected in 20 μL; (b) 3 mg of each component injected in 150 μL. Mobile phase: 35mM formic acid, at 25°, at either 0.6 mL/min in (a) or 0.3 mL/min in (b). U.v. detection at 225 nm and 2.0 AUFS.

where i.d.$_2$ and i.d.$_1$ represent the inner diameters of the preparative and analytical column, respectively; F_1 is the flow rate of the analytical column; and F_2 is the calculated flow-rate of the preparative column. Because the analytical cation-

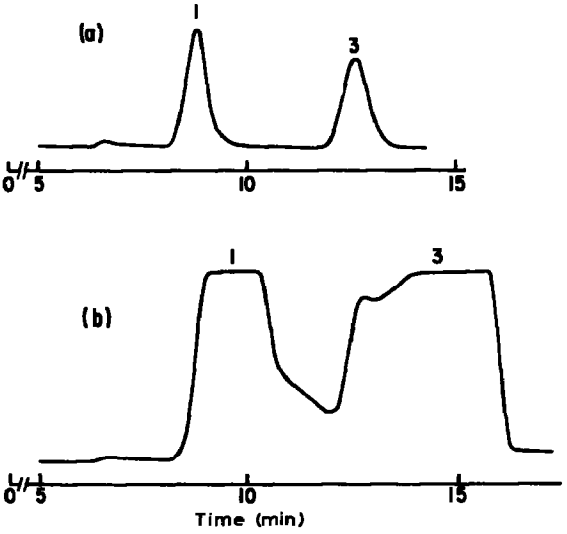

Fig. 2. Separation of glucuronic acid (1) and 2-acetamido-2-deoxy-D-glucose (3) on HPX-87 H$^+$. (a) Analytical separation, 0.1 mg of each carbohydrate in 20 μL; (b) preparative separation, 15 mg of each in 150 μL. Flow rate: 0.6 mL/min. Other conditions same as for Fig. 1.

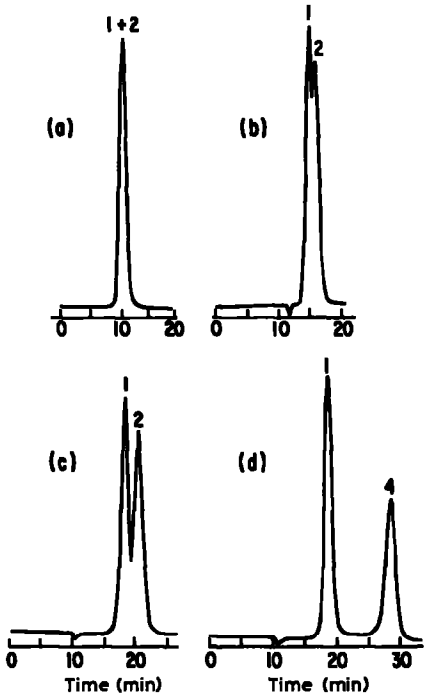

Fig. 3. Separation of sugar derivatives on laboratory-packed preparative l.c. columns (2.0 × 30 cm) (a) (1) Glucuronic and (2) galacturonic acids on gravity-packed AG-50W-X8 (H^+) resin, minus 400 mesh. (b) Same as (a) except resin was packed under pressure (21 MPa). (c) Same as b except AG-50W-X4 resin was used. (d) Same as (c) except compounds separated were D-glucuronic acid (1) and D-glucofuranurono-6,3-lactone (4). Conditions: 1 mg of each component injected in 50 μL; mobile phase: 0.01N H_2SO_4 at 4 mL/min in (a), and 2 mL/min in (b–d); refractive index detection at 16X. All columns were run at room temperature.

exchange columns were routinely run at flow rates between 0.3 and 1 mL/min, the preparative columns were always run at flow rates of less than 6 mL/min and usually at 2 mL/min or less. Under these conditions, retention times were nearly identical to those obtained on analytical columns, which were <30 min for all carbohydrates in this study. Based upon these facts, concerns over high back-pressures and flow-rates for these preparative columns are unjustified. The expense of the ion-exchange resins is related to their size and monodispersity, with small (10–15 μm), relatively monodisperse (±3 μm) resins costing the most. Although these resins were found ideal for packing into efficient, high-capacity columns, resins of lesser quality and expense could also be used in certain applications.

When resins of the latter type were used, greater care had to be exercised in packing the column. An example of the effect of packing procedure on the performance of preparative columns packed with a non-l.c. grade resin, is shown in Fig. 3. A column (2.0 × 30 cm) packed by gravity sedimentation with AG-50W-X8 (H^+) resin (minus 400 mesh) did not separate a mixture of glucuronic and galacturonic acids (see Fig. 3a) under any of the conditions tested. When the same resin

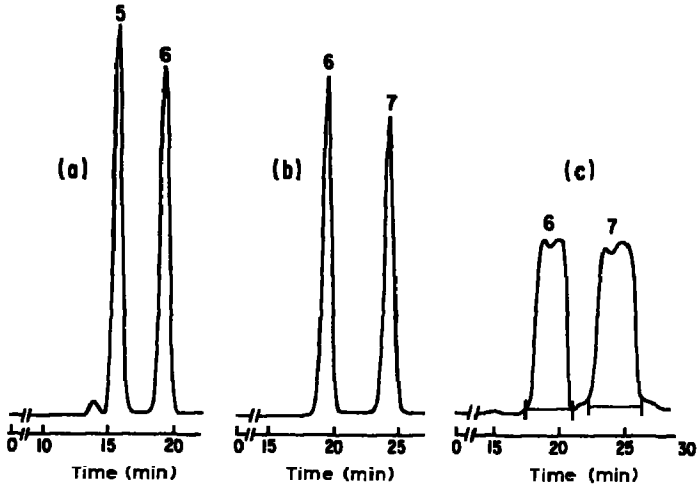

Fig. 4. Separation of maltose (5), D-glucose (6) and D-fructose (7) on a l.c. column (2.0 × 30 cm) laboratory-packed with Aminex Q-15S (Ca^{2+}). (a, b) 1-mg injections of sugars in 200 μL, refractive index detection, 32X. (c) 100 mg of each sugar injected in 1 mL, refractive index detection at 128 NL (non-linear). The irregularly shaped peaks are caused by overloading the detector and monitoring on the NL scale, and do not indicate peak heterogeneity. Isolated fractions are indicated by cross bars. Other conditions: column held at 85° and eluted at 2 mL/min (2 MPa) with H_2O.

was packed under high pressure (21 MPa or ~3000 lb. $in.^{-2}$), the resulting column gave a modest separation ($R = 0.5$). Further improvement in separation of the pair of isomers was seen when a resin with a lower degree of cross-linking was used. Thus, in Fig. 3c, the uronic acids were separated by a R value of 0.9, when the column was packed (under high pressure) with AG-50W-X4 (H^+) resin (minus 400 mesh). This resin, with a content of 4% of divinylbenzene, has larger pore sizes than the 8% type. These 4% resins had greater selectivity for lower-molecular-

TABLE I

CHROMATOGRAPHIC PARAMETERS FOR SELECTED MONO- AND DI-SACCHARIDES ON A LABORATORY-PACKED PREPARATIVE AMINEX Q-15S LIQUID-CHROMATOGRAPHY COLUMN[a]

Sugar[b]	T_r *(min)*	K'[c]	*N*[d]	*R*[e]
Lactulose	18.5	0.69	1940	
				1.3
Lactose	16.8	0.54	2370	
				—
Maltose	16.0	0.47	2090	
				2.8
D-Glucose	20.0	0.83	3250	
				3.2
D-Fructose	24.8	1.27	3940	

[a]2.0 × 30 cm column, Ca^{2+} form resin, held at 85°, eluted with H_2O at 2.0 mL/min. [b]200 μL (2.4 mg) of each sugar was injected. [c]Theoretical phase-capacity factor, assuming an unretained peak is eluted at 10.9 min. [d]Calculated number of theoretical plates. [e]Resolution between adjacent peaks.

weight solutes, but were less mechanically stable than the 8% type. Thus, they were packed at values ≤7 MPa, and operated at flow rates of less than about 5 mL/min.

The examples in Fig. 3a–c are of extremely difficult separations where the compounds of interest had very similar elution-volumes. Accordingly, with the column conditions in Fig. 3c, only 10 to 15 mg of each compound could be separated effectively. However, for many routine separations in which greater selectivity was displayed among the compounds of interest, much larger amounts could be injected. Thus, in Fig. 3d, D-glucuronic acid and D-glucofuranurono-6,3-lactone were separated on the preparative column with an R value of 4.0, when 1 mg of each component was injected. Up to 100 mg of each of these components could be completely separated on this column with $R > 1$.

Cation exchange resins in the Ca^{2+} form are frequently used for the analytical l.c. separation of neutral mono- and di-saccharides. We examined one such l.c.-grade resin, Aminex Q-15S (Ca^{2+}), for preparative purposes. This resin has a nearly optimum particle size (22 ±3 μm) and was easily slurry-packed at 21 MPa into preparative columns. All neutral sugar separations were run at 85° in order to prevent the separation of sugar anomers, and to increase the rate of diffusion of mobile phase and solute through the resin. As shown in Fig. 4 (a and b), the preparative column separated the common sugars maltose (5), D-glucose (6), and D-fructose (7) with resolution similar to that seen on analytical columns. As in the previous columns, flow rates and back pressures were nominal. When 2.4-mg samples of common carbohydrates were injected, the column parameters shown in Table I were calculated. The theoretical-plate values were very similar to those reported for analytical columns packed with similar resins[8], and, on the average,

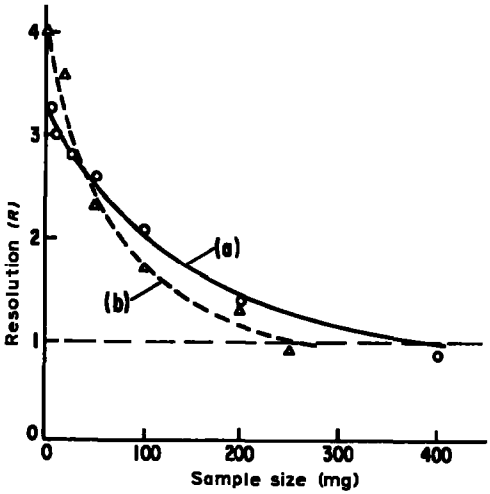

Fig. 5. Effect of the size of injected sample (D-glucose and D-fructose mixture) on chromatographic resolution (R) during preparative chromatography on (a) the Aminex Q-15S and (b) the Zorbax NH_2 columns. See text for further description.

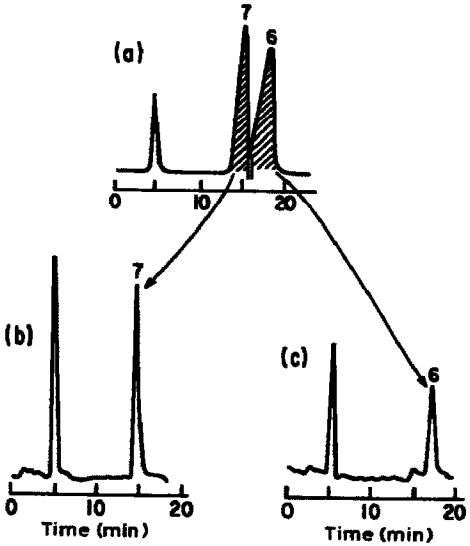

Fig. 6. Separation of D-glucose (6) and D-fructose (7) on a Zorbax NH_2 preparative column. (a) 100 mg of each sugar injected in 2 mL. Refractive index detection at 64X. (b) and (c) injection of 2-mL aliquots of fractions collected in (a). RI detection at 16X. Other conditions: mobile phase: 77:23 acetonitrile–water; flow rate: 15 mL/min (7 MPa).

they were 1.3 to 2.5 times those calculated for the column, shown in Fig. 3, packed with non-l.c. grade resins (data not shown). Retention times and resolution values given in Table I are also comparable to those calculated for analytical l.c. separations.

The preparative Aminex column was used for large-scale isolation of simple sugars. A sample containing 100 mg each of D-glucose and D-fructose was easily separated (see Fig. 4c), and the fractions collected (as indicated) contained the expected component in excess of 98% purity, based on peak area. The effect of column loading (sample size) on the resolution of D-glucose and D-fructose is shown in Fig. 5a. Up to 200 mg of each component (a total of 400 mg) could be injected, before resolution dropped below a value of 1. Even under those conditions, the resolution value was 0.85, and samples collected from the column, as in Fig. 4c, contained D-glucose and D-fructose of high purity, 94 and 89%, respectively. Although it has been shown[14,15] that sample volume, as well as sample mass, can affect peak shape and resolution in preparative l.c., we did not notice a significant effect under the conditions used here. For instance, a given amount of sample injected in a 0.5- or a 1.0-mL volume, gave similar resolution values, provided that the overall concentration of carbohydrate in the sample was ≤400 mg/mL.

Preparative l.c. on columns (2.2 × 25 cm) of aminopropyl silica gel. — As previously discussed, aminopropyl silica columns are easily degraded by reactive reducing sugars, column contaminants, and aqueous mobile phases. None-the-less, several preparative-sized columns packed with this stationary phase are commercially available. The flow rates (15–20 mL/min) and back pressures (7–10

MPa) for these columns limit their use to only modified analytical or dedicated preparative l.c. instruments. The use of such a column for the preparative separation of D-fructose from D-glucose is demonstrated in Fig. 6a, in which 100 mg of each sugar was injected. The resulting fractions (Fig. 6b and c) contained the respective sugars in >97% purity. The capacity of the column for loading (see Fig. 5b) was significantly less than that of the Aminex column and, after several weeks of use, the capacity fell further. Because of their low capacity, high initial cost, low durability, and the necessity for high flow-rates of acetonitrile–water mobile phases, the use of these columns for mono- and di-saccharide isolation was impractical. These columns, however, were excellent for isolation of oligosaccharides.

Applications. — The Aminex column was used for a variety of applications, including the isolation of pure 1-(^{13}C)lactulose from a reaction mixture that contained significant proportions of four other sugars (see Fig. 7a). Samples containing 130 mg (in 0.5 mL) of the crude reaction-mixture were injected, and over 90 mg of pure lactulose (see Fig. 7b) was isolated in less than 40 minutes. In repeated injections, samples were carefully overlapped, resulting in three injections per hour. Fractions isolated from the column were relatively concentrated and required minimal evaporation to yield dry, colorless syrups, which were easily crystallized[16]. Isolation of simple sugars from plant extracts was also readily accomplished. In Fig. 8a, over 50 mg of pure sucrose (peak 12) was isolated from an injection of deionized orange-juice concentrate. In Fig. 8b, over 40 mg of pure D-fructose (peak

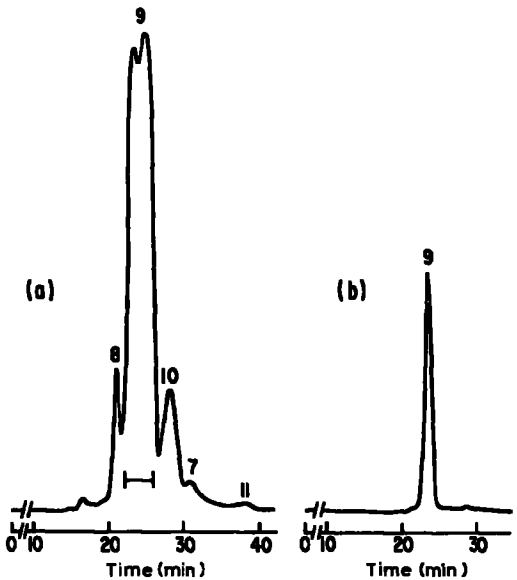

Fig. 7. Isolation of pure 1-(^{13}C)lactulose from crude synthetic product by preparative l.c. on column described in Fig. 4. (a) Injection of crude lactulose preparation. Cross bar indicates collected fraction. (b) Injection of 100 μL of fraction collected in (a). Column temperature: 85°; mobile phase: H_2O at 1.5 mL/min. Refractive index detection at 128 NL in (a) and 16X in (b). Peaks: 7, fructose; 8, lactose; 9, lactulose; 10, galactose; and 11, tagatose.

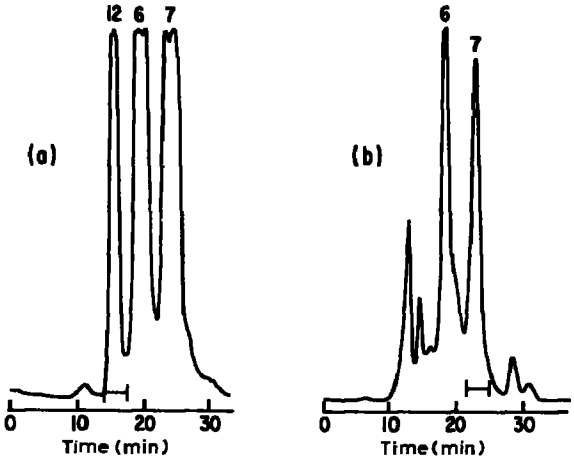

Fig. 8. Isolation of sugars from plant extracts by preparative l.c. Same column and conditions as described in Fig. 7. (a) Isolation of sucrose (12) from orange juice. (b) Isolation of D-fructose (7) from pineapple-leaf extract. Peak 6: D-glucose. Injection sizes were 0.5 mL, containing 200 mg of total solids. Cross bars indicate fractions that were isolated.

7) was isolated from de-ionized extract from pineapple. These methods were used to isolate pure sugars from fruit juices for stable-isotope ratio analysis, a technique that can determine the biological source of sugars and thereby detect adulteration of natural fruit-juices with inexpensive sugars from corn or sugar beet[17]. Other applications included the separation and isolation of epimeric ^{13}C-labelled sugars produced by Kiliani–Fischer synthesis[18], and the purification of other synthetic monosaccharides[19], and disaccharides[3].

We have found that under the conditions described in which samples were de-ionized and filtered prior to injection, these cation-exchange columns could be operated for several years with only occasional need for regeneration or repacking. Even the small pre-columns lasted for several months without noticable increase in back pressure. It is also noteworthy that virtually 100% of all carbohydrate injected into the column was always recovered from the column effluent. This is in sharp contrast to the values of 50–90% recovery that have been reported[20,21] for normal- and reversed-phase columns. Fractions that were evaporated from the effluent of the aminopropyl column contained observable quantities of a white precipitate presumed to be silica that dissolved from the column. No introduction of foreign material into samples was observed with any of the cation-exchange resin phases.

Finally, there are several factors that should be considered when choosing the type of cation-exchange resin (l.c.- or non-l.c.-grade) to use for a given separation. For applications where there is high selectivity between the carbohydrates to be separated, clearly the less-efficient non-l.c.-grade resins are a reasonable choice. For more difficult separations, the l.c.-grade resins may be required. However, unlike most silica-based, l.c. packings, which are considered to be expendable

materials, these resins are re-usable for many years, and this fact lessens the impact of their greater initial cost.

EXPERIMENTAL

General. — Carbohydrate standards were purchased from Sigma Chemical Co.* L.c.-grade acetonitrile was purchased from Burdick and Jackson. Water used for l.c. was doubly distilled and filtered prior to use. 1-(^{13}C) Lactulose was prepared by base-catalyzed isomerization[16] of 1-(^{13}C)lactose, provided by Dr. Peter Klein. Pure orange juice and other fruit juices were centrifuged and filtered (Nylon 66; 0.2 μm filters) to remove insoluble solids, and then de-ionized prior to l.c., by passage of 25 mL of the filtrate through 50 mL of Amberlite MB-3 resin (Rohm and Haas). The solutions were lyophilized, and then made up in distilled water ($\leq$500 mg/mL) for injection.

Cation-exchange resins AG-50W-X8 and AG-50W-X4 (both minus 400 mesh size and in the H^+ form), the pre-packed HPX-87H^+ column, and empty preparative l.c. columns (2.0 × 30 cm) were purchased from Bio-Rad Laboratories. The HPX-87H^+ column was packed with 9 μm spheres of 8% crosslinked cation-exchange resin. The Aminex Q-15S resin was kindly provided by Mr. Mike Gray, Bio Laboratories. The Zorbax NH_2 preparative column was purchased from the Dupont Company, Analytical Instruments Division. Empty pre-columns (0.46 × 5 cm), a preparative-sized (175 mL) slurry reservoir, and a slurry packing apparatus (air-driven liquid pump) were purchased from Alltech/Applied Science. Additional fittings for coupling the preparative slurry reservoir to the pump and to the empty column were purchased from Parker Hannifin Corporation. Stainless-steel in-line filters, with replaceable 0.2-μm filters, were purchased from the Rheodyne Corporation.

Methods for packing preparative columns and precolumns. — All resins were conditioned for packing by an initial treatment to remove fines. The amount of resin to be used (usually 150–200 mL of moist resin) was slurried in a total of 500 mL of H_2O and allowed to settle for ~15 min. The layer of water (above the resin) that contained fines was carefully decanted; this process was repeated several times until no more fines were noticed. When required, the resins were converted into the Ca^{2+} form, as described by Ladisch *et al.*[8]. Precolumns were packed by attaching a 25-mL, slurry-packing reservoir, filled with a 50% slurry of AG-50W-X8 in H_2O, to the inlet end of the precolumn. The other end of the reservoir was attached to the l.c. pump, and distilled water at 6 mL/min was pumped through for 5 min. After removing the reservoir, a column-end fitting was immediately attached.

*Reference to a brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

Several methods were used to pack preparative (2.0 × 30 cm) columns. For the AG-50W-X8 and Aminex Q-15S resins, the following was used. The stainless-steel column was mounted vertically beneath the packing apparatus, and filled with water, with the bottom fitting temporarily plugged. The top of the column, without end fitting, was attached to the packing reservoir. The reservoir was filled with 150–200 mL of resin, well mixed and slurried in a minimum amount of water, and then quickly attached to the packing pump by way of "quick connect" fittings. The plug was removed from the bottom of the column, and the packing pump was set to deliver 21 MPa (~3000 lb. in.$^{-2}$), using H_2O as the packing solvent. When the pressure in the system had reached the set value (~2–3 min), the pump was stopped, the pressure reached zero, and the column was carefully removed from the reservoir. An end fitting was immediately placed on the column, to prevent the resin from extruding from the tightly packed bed. The AG-50W-X4 resins were packed in a similar manner except that the pressure was allowed to reach only 7 MPa during the packing procedure. If a column-packing pump is not available, columns can be packed by attaching a standard l.c. pump to the solvent end of the packing reservoir and pumping at the maximum flow-rate (≤10 mL/min). This procedure works relatively well for Aminex resins, but only marginally so for the non l.c.-grade AG-50W type.

Chromatographic methods. — The isocratic chromatographic system consisted of a Dupont Model 8800 pump and a heated column compartment, a Rheodyne fixed-loop injector, and either a Waters Model 403 preparative differential refractometer or a Gilson Holochrome u.v. detector. The gradient system was similar to the isocratic system except that solvent was delivered with a Gilson Gradient Automatic Preparative Chromatograph, equipped with two Model 303 pumps and controlled with an Apple IIe microprocessor. Chromatograms were recorded on a Houston Instruments recorder. All solvents were de-gassed by constant purging with helium.

Several important precautions were always followed. First, an in-line filter (stainless steel, 0.2 μm, Rheodyne) was placed between the column and detector to trap resin fines that escaped from the column bed. Secondly, fractions were collected by using an appropriate delay-time to compensate for the length of tubing between the detector cell and the tubing outlet. Recording integrators, which do not operate in "real time", should not be used to monitor column effluents for preparative collections. Finally, columns should be monitored with universal-type detectors, such as refractive index monitors, because they give a realistic view of the total mass being eluted from the column.

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