High-performance liquid chromatography of plant-derived oligosaccharides on a new cation-exchange resin stationary phase: HPX-22H

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Cation-exchange resins, in a number of ionic forms, are commonly used stationary phases for high-performance liquid chromatography (HPLC). Carbohydrates and derivatives can be separated on these phases by a combination of mechanisms[1–3]. Most cation-exchange resins are prepared from sulfonated polystyrene that has been crosslinked with divinylbenzene. The amount of crosslinking determines both mechanical stability, and size-exclusion characteristics. Resins with 8% levels of divinylbenzene are relatively rigid and can withstand the highest pressure (*i.e.*, 3000–4000 p.s.i.) generated during HPLC. With these resins, however, only carbohydrates within a narrow size range (monosaccharide to trisaccharide) can be separated. Resins containing 4% divinylbenzene are less mechanically stable but the larger pore sizes allow for fractionation of larger oligosaccharides [degree of polymerization (DP) 1–6 for H^+-form resins]. Resins containing only 2% of crosslinking have not been used for HPLC because of the assumption that these soft gels cannot withstand the required back-pressures. However, earlier studies[1] involving low-pressure chromatographic separations showed that these resins have an excellent potential for separation of even larger oligosaccharides. There is currently a need for durable HPLC stationary phases that can separate a wider size range of both neutral and acidic oligosaccharides, such as those that are produced during the partial hydrolysis of plant-derived polysaccharides. For this reason we now report the useful chromatographic properties of the title stationary phase.

EXPERIMENTAL

Materials

Malto-oligosaccharides were prepared as previously described[4]. Cellodextrins were prepared by trifluoroacetic acid hydrolysis[5]. Polygalacturonic acid hydrolysates were generous gifts from Drs. Landis Doner and Peter Irwin. Chitin oligosaccharides were prepared as described elsewhere[6].

Liquid chromatography

A DuPont Model 8800 pump and heated column compartment, a Rheodyne (20 μl) injector, a Hewlett-Packard 3390 integrator, and an ultra-sensitive (detection

limit of less than 200 ng of monosaccharide) Erma ERC-7510 refractive index detector was used. The stainless-steel (30 cm × 1.0 cm I.D.) column was custom packed by Bio-Rad Labs. with 20–25 μm, spherical, sulfonated polystyrene beads (H^+ form), crosslinked with a total of 2% divinylbenzene (HPX-22H). The mobile phase was filtered (0.2-μm Nylon 66 filter) 0.005 *M* sulphuric acid. A Bio-Rad Labs. precolumn, filled with cation-exchange resin (H^+ form) was also used. Column flow-rate was adjusted carefully (see Results and discussion), in order to prevent damage to the stationary phase. Also, cessation of flow while the column was at elevated temperature caused an increase in backpressure during subsequent runs. Hence, during shutdown, it was important that there was always a slight flow through the column until it had reached room temperature.

All samples were treated with a strong cation-exchange resin (H^+ form), then dissolved in mobile phase, and filtered through nylon filters before injection. Because the stationary phase is not resistant to organic solvents, they were removed from samples by evaporation.

RESULTS AND DISCUSSION

General operating characteristics of the stationary phase

The 2% crosslinked stationary phase was very sensitive to the applied flow-rate. To prevent damage to the column, it had to be operated at low flow-rates and, most importantly, at very low back-pressures. At room temperature (23°C), only flow-rates of $\leq$0.2 ml/min were acceptable (Fig. 1). At the upper temperature limit of 85°C, the column could withstand flow-rates slightly over 0.5 ml/min. Under these conditions, pressures were only 15–300 p.s.i. When flow-rates exceeded these guidelines, the column packing compressed, leading to high back-pressures and to loss of resolution. When optimum flow-rates were not exceeded, however, no loss of chromatographic resolution was observed over one year of operation. When a column was slightly overpressurized, it could be rejuvenated by storing it for several days and then by running it in the opposite direction. If the stationary phase is more severely compressed repacking may be required.

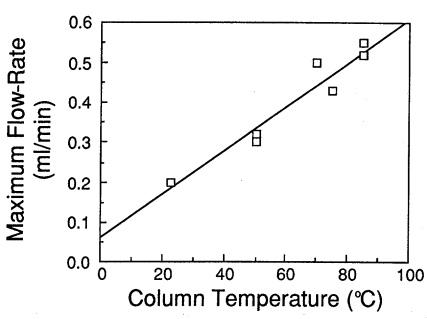

Fig. 1. Graphical representation of the maximum flow-rates allowable on an HPX-22H column at specified temperatures.

Typical separation of oligosaccharides

Fig. 2 demonstrates the utility of the 2% crosslinked stationary phase for some plant-derived homo-oligosaccharides. In Fig. 2a, malto-oligosaccharides from DP 1 to over DP 13 are separated in about 40 min. Similar separations[7] have been achieved on 4%, Ag^{+}-form resins but that system required more time (60 min) and was not useful for acidic oligosaccharides. Malto-oligosaccharides can also be separated on aminopropyl-bonded phases[8], but that system requires acetonitrile–water eluents, is not suited for acidic oligosaccharide analysis, and is not very durable.

The cello-oligosaccharides (Fig. 2b), were also easily resolved on this column. Separation is not practical on aminopropyl-bonded silica gel since these oligosaccharides are relatively insoluble in the required mobile phases (acetonitrile–water mixtures). The present system allowed for the high-resolution separation of larger cello-oligosaccharides (up to DP 10) than was previously possible on the commonly-used Ca^{2+}-form, 4% cation-exchange resin stationary phases[9].

Because ionic molecules are well tolerated on this phase, it was possible to separate the oligogalacturonic acids (Fig. 2c) prepared from the acidic polysaccharide, polygalacturonic acid with DP values up to about 10 in 35 min. This type of simple, rapid, and complete separation has hitherto not been reported. Separation

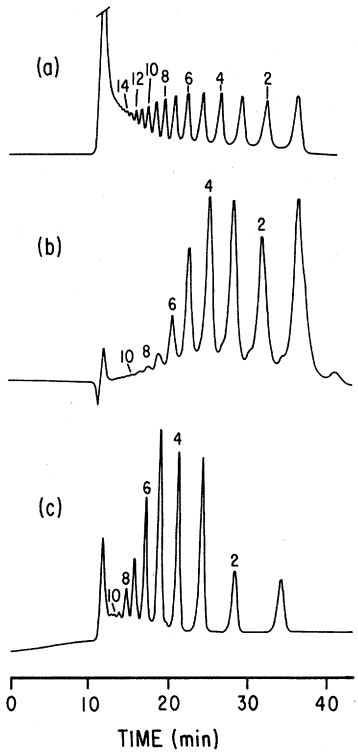

Fig. 2. Separation of plant-derived oligosaccharides on an HPX-22H column. Column was operated at 85°C and at 0.5 ml/min. (a) Malto-oligosaccharides, (b) cello-oligosaccharides, and (c) oligosaccharides from polygalacturonic acid. 300 μg of each mixture injected. Refractive index detection at 1 ×.

of unsaturated oligogalacturonic acids up to a DP value of 8 by anion-exchange HPLC has been described[10], but this system required a buffered mobile phase, did not provide baseline separations and did not permit elution of the larger oligogalacturonic acids. Some acidic oligo- and polysaccharides undergo gellation during contact with H^+-form resins. Samples containing such carbohydrates should be pretreated with H^+ resins, and then filtered, prior to injection.

Chitin oligosaccharides were only partly resolved on this system. Under these conditions (85°C and about pH 2), some partial de-N-acetyation of the oligomers might have occurred. In fact, any readily hydrolyzable carbohydrate substituent or labile glycosidic linkage may become cleaved during chromatography at elevated temperatures on this column. Proper precautions, such as the use of lower column temperatures, should therefore be taken during such analyses.

Each oligosaccharide series had a distinct retention time profile (Fig. 3) and as expected for size-exclusion separations, a gradual decrease in retention time was seen for each ascending oligosaccharide. The cello- and malto-oligosaccharides have similar retention times for each specific corresponding oligomer. The oligogalacturonic acids have shorter retention times due to additional ion-exclusion effects[3]. The chitin oligosaccharides, which contain relatively non-polar acetyl groups, experience matrix adsorption effects[3] and are retained longer than the other corresponding neutral oligosaccharides.

This stationary phase is unique in that it allowed the separation of relatively large neutral and acidic homo-oligosaccharides in a short time with an aqueous, isocratic mobile phase. We used a mineral acid to acidify the mobile phase but it is possible[11] to use volatile acids such as acetic or formic acids. An additional benefit of this stationary phase was that all the injected sample, including high-molecular-weight oligosaccharides, was eluted. This feature is particularly useful for analyzing the pattern and extent of hydrolysis of polysaccharides by specific enzymes. Analysis of fragments can give information about the type of enzyme (endo *vs.* exo) used, and an analysis of the eluted peak areas can provide simultaneous information about the amount of remaining polymer and the generated fragments. Some compositional

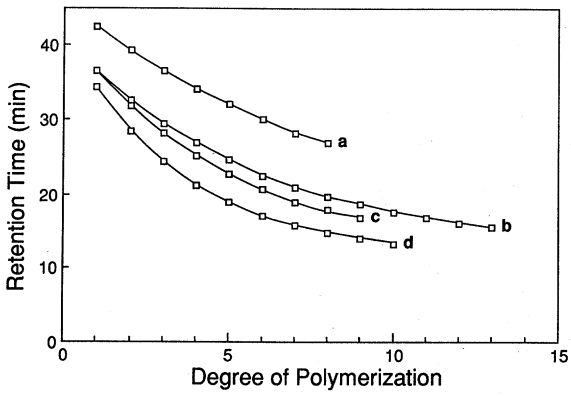

Fig. 3. Relationship between the degree of polymerization of common oligosaccharides and their retention times on an HPX-22H column operated at 85°C and at 0.5 ml/min. (a) Chito-oligosaccharides, (b) malto-oligosaccharides, (c) cello-oligosaccharides, and (d) oligogalacturonic acids.

TABLE I

RETENTION TIMES FOR COMMON CARBOHYDRATES ON AN HPX-22H HPLC COLUMN

The column was operated at 0.5 ml/min and at 85°C. 20 μg of each carbohydrate was injected.

Carbohydrate	*Retention time (min)*	*Carbohydrate*	*Retention time (min)*
Galacturonic acid	34.5	Dulcitol	38.5
Glucuronic acid	36.0	Sorbitol	38.6
Glucose	36.4	Arabinose	39.1
Fructose*	37.0	Rhamnose	39.1
Inositol	37.1	Ribose	39.5
Galactose	37.5	Fucose	40.9
Mannose	37.6	1,6-Anhydro-β-D-glucopyranoside*	41.0
Xylose	37.7	2-Acetamido-2-deoxy-D-glucose	42.5
Mannitol	38.4	2-Acetamido-2-deoxy-D-galactose	44.8

* Calculated retention times from chromatograms produced with column operation at 0.4 ml/min and at 85°C.

analysis of a polysaccharide can also be performed. An analysis of the retention times of various monosaccharides (Table I) shows, however, that the common monosaccharides are not well separated. Accordingly, the primary strength of the phase lies in its ability to separate oligosaccharides, not monosaccharides. Other systems have been described for the latter analysis[12].

In summary, 2% crosslinked strong cation-exchange resins are unique HPLC stationary phases which, when used cautiously, are extremely useful for the simultaneous analysis of poly-, oligo- and monosaccharides, such as those encountered in partial hydrolysates of polysaccharides.

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