

# Analytical and preparative HPLC of carbohydrates: inositols and oligosaccharides derived from cellulose and pectin

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New methods are given for the production of cellodextrins by the TFA-catalyzed hydrolysis of cellulose and for the subsequent analytical and preparative high performance liquid chromatography of these useful oligosaccharides. In addition, recent methods developed in this laboratory for the analytical and preparative HPLC of inositols and pectin oligosaccharides are reviewed.

## INTRODUCTION

Over the past 20 years, high performance liquid chromatography (HPLC) has developed into a powerful method for analysis and preparative isolation of carbohydrates. In this paper new methods are described which can allow separations that have traditionally been difficult or impossible to achieve: those involving the inositols, and the oligosaccharides that are derived from cellulose and pectic polysaccharides. Some information will be given here on stationary phases, instruments, and other equipment useful for specific HPLC analyses. If more details in these areas are required, the reader is referred to recent reviews (Hicks & Hotchkiss, 1992; Hicks, 1988).

## EXPERIMENTAL

All carbohydrates were purchased\* from Sigma Chemical Co., St Louis, Missouri, unless stated otherwise. All inositols, other than myo-inositol (Sigma), were gifts from Professors S.J. Angyal (University of New South Wales, Australia) and L. Anderson (University of Wisconsin, Madison). The following cellulose samples were obtained from the following companies:  $\alpha$ -cellulose, Sigma; CC-31 cellulose powder, Whatman Ltd.; Solka-Floc BW-300, James River Corporation; Avicell PH-101, FMC Corporation, Philadelphia. Pectic poly-

and oligosaccharides were prepared as described previously (Hotchkiss & Hicks, 1990; Hotchkiss *et al.*, 1991). The chromatographic instruments, both analytical and preparative, have been described in detail (Hicks & Sondey, 1987; Hicks & Hotchkiss, 1988; Hotchkiss *et al.*, 1991). AG 50W-X4, (20–30  $\mu$ m particle size), and Aminex Q-15S, (19–25  $\mu$ m particle size) cation exchange resins were purchased from BioRad Labs and converted into the  $\text{Ag}^+$  and  $\text{Ca}^{2+}$ -forms, respectively, and packed into preparative (2.0  $\times$  30.0 cm) stainless steel columns as described (Hicks & Sondey, 1987).

Cellulose samples were hydrolysed by the method of Wing and Freer (1984). A sample (10 g) of vacuum oven-dried cellulose was mixed with 150 ml trifluoroacetic acid (Sigma) in a 250 ml water-jacketed flask. The flask was sealed with a rubber septum and allowed to incubate at room temperature, with occasional shaking of the flask, in a fume hood until the sample had completely dissolved (usually 4 days). Ten ml of distilled water was added and the flask was re-sealed and stirred magnetically while it was heated for 24 h at 70°C. The reaction was cooled to room temperature, poured into a crystallizing dish and allowed to evaporate. The soluble cellodextrins in the syrupy mass were then carefully extracted with 6  $\times$  200 ml portions of distilled water, which were combined, deionized by passage through a column of Amberlite MB-3 indicator-grade mixed-bed ion exchange resin, and then evaporated at reduced pressure at 35°C to a volume of 200 ml. This sample was then lyophilized to yield 3.5 gm of a slightly yellow, hygroscopic powder. Attempts to evaporate the sample to dryness rather than using lyophilization led to an aggregated cellodextrin mixture that had much lower

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

solubility in water than did the lyophilized sample. Samples were purified by preparative HPLC as described below and then lyophilized to white, hygroscopic powders.

## RESULTS AND DISCUSSION

### Inositols

Inositols are widespread in nature and have important roles in cellular signal transduction processes (Berridge & Irvine, 1984). *Myo*-Inositol serves as a precursor of the uronic acid and pentose residues of plant cell-wall polysaccharides. It is converted into those sugar residues and incorporated into polysaccharides of growing seedlings (Sasaki & Taylor, 1986). Hence, supplying deuterium-labeled *myo*-inositol to germinating seeds, and following metabolism and subsequent incorporation into growing plant cell walls (using NMR spectroscopic or glc-ms methods) would enable one to study mechanisms of cell-wall biosynthesis, growth, and development. Deuterium-labeled *myo*-inositol can be prepared by  $^1\text{H}$ - $^2\text{H}$  exchange of its carbon-bound hydrogen atoms when it is reacted in deuterium oxide in the presence of Raney nickel catalyst (Koch & Stuart, 1977). Unfortunately, extensive epimerization also occurs during this reaction, leading to the production of various other isomeric inositols (Sasaki *et al.*, 1987). Although several methods for the separation of one or two isomeric inositols have been reported, there had been no report of the complete separation of all eight isomers which could theoretically be formed in this reaction until this report (Sasaki *et al.*, 1988). This method will now be briefly described as an example of the versatility and usefulness of HPLC separations on cation exchange resins in the calcium form. This technique, the mechanism of which has been reviewed (Hicks, 1988) is called ligand exchange chromatography, because polyols passing through the column form transient coordination complexes with the column-bound cation. Different polyols will have differing affinities for the cation and, therefore, be eluted at different times thus allowing chromatographic resolution. The ability of polyols to form coordination complexes depends on the geometrical arrangement of their hydroxyl groups (Angyal & Davies, 1971; Angyal, 1973, 1974; Goulding, 1975). For cyclic polyols such as the cyclitols, the following generalizations have been drawn: a set of three adjacent hydroxyl groups in an axial-equatorial-axial (a-e-a) arrangement provides the strongest complex with  $\text{Ca}^{2+}$  ions; a pair of adjacent hydroxyl groups in axial-equatorial (a-e) sequence forms a weak, but significant complex with  $\text{Ca}^{2+}$ ; sequences of adjacent hydroxyl groups with axial-axial (a-a) or equatorial-equatorial (e-e) arrangements provide very weak coordination complexes.

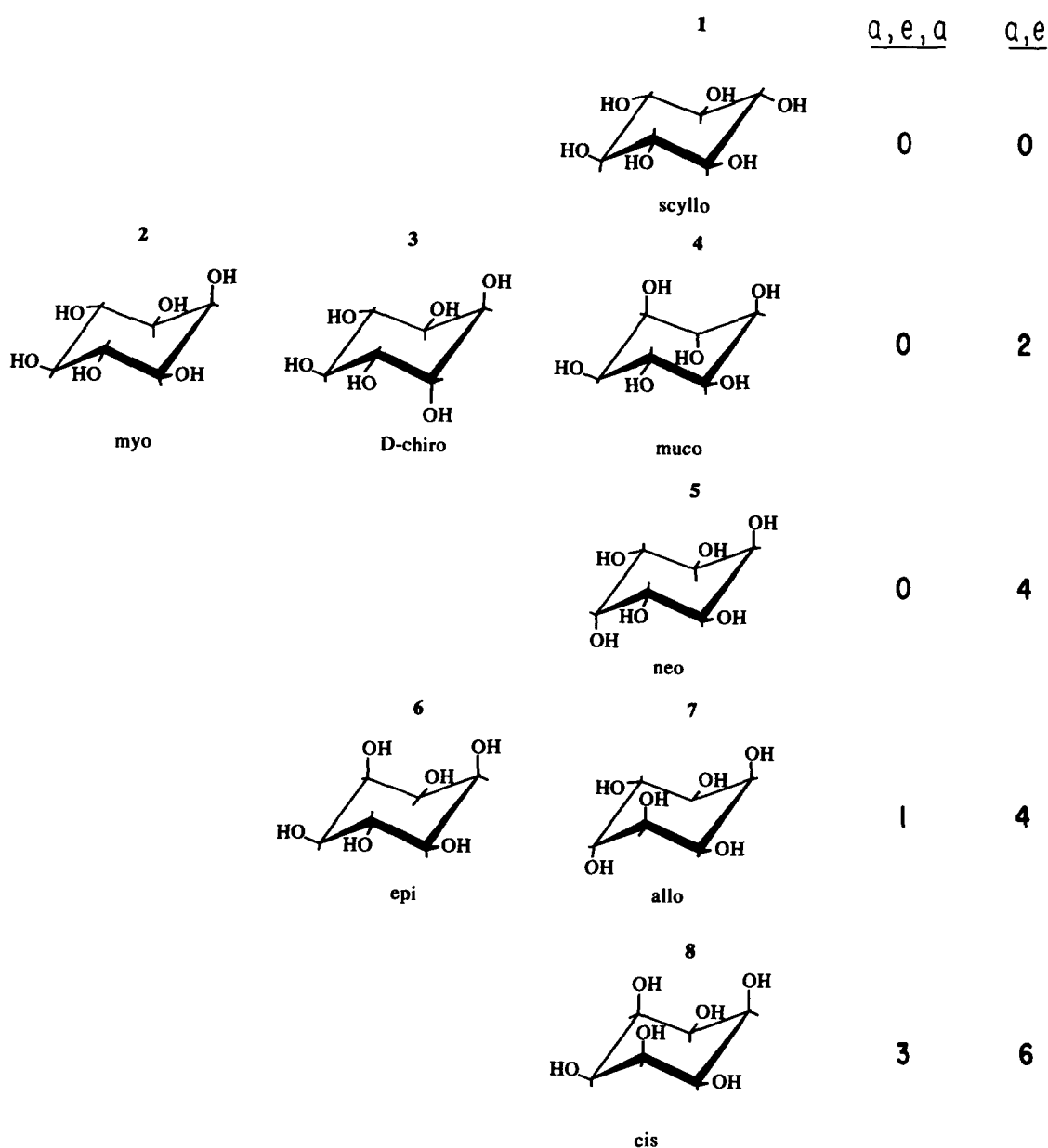
As shown in Fig. 1, *cis*-inositol has three potential a-e-a sites for forming strong complexes with  $\text{Ca}^{2+}$ . The *epi*- and *allo*-inositol isomers each have 1 a-e-a site or 4 a-e sites and therefore would be expected to form strong complexes, but weaker than those with *cis*-inositol. By continuing this analysis one can arrange the inositols in order of their theoretical ability to form complexes with  $\text{Ca}^{2+}$  ions (predicted strongest to weakest complexes): *cis* > *allo* = *epi* > *neo* > *myo* = *D-chiro* = *muco* > *scyllo*. This analysis predicts ligand exchange HPLC with  $\text{Ca}^{2+}$ , as the ligand would separate many of the inositols.

As shown in the chromatogram in Fig. 2, this theory is born out of practice. All inositols are readily separated at room temperature on this system. *Cis*-Inositol, predicted to have the strongest complexing ability with  $\text{Ca}^{2+}$ , elutes very late in the chromatogram (> 100 min) and is one of the most strongly retained polyols ever examined on this phase. Retention times of all isomers are temperature dependent and separation at elevated temperature (up to 85°) allows faster elution of all analytes, with corresponding decreases in resolution between earlier-eluting isomers. This is seen in the analytical chromatogram in Fig. 3, which verifies the existence of several different inositols in the products from the Raney nickel-catalysed reaction of *myo*-inositol.

Various feature of ligand exchange HPLC, including simple solvent (water), high capacity, and non-destructive detection, makes it ideal for preparative isolation of pure compounds. The use of a preparative column packed with cation-exchange resin will be discussed in the next section for the separation and isolation of oligosaccharides.

### Oligosaccharides from the hydrolysis of cellulose

Much interest exists in the partial hydrolytic products of cellulose, known as the celloextrins. They are useful model compounds for studies on the structure of cellulose and on the breakdown of cellulose-containing biomass, to fermentable carbohydrates via chemical or enzymatic methods. In addition, these non-digestible oligosaccharides may have potential uses as non-caloric bulking agents in food products. Wolfson and Dacons (1952) were the first to prepare the celloextrins from DP 3 through 7 from the crystalline acetates of each parent oligosaccharide, produced by the acetolysis of cellulose. Later, Miller *et al.* (1960) hydrolysed cellulose in fuming hydrochloric acid and prepared quantities of the celloextrins by chromatographic resolution on stearic acid-treated mixtures of charcoal and Celite. This method has been used extensively over the last 30 years. Occasionally, sulfuric acid has also been used for hydrolysis, with acceptable results (Voloach *et al.*, 1984). Fengel and Wegener (1979) and Wing and Freer (1984) used trifluoroacetic acid-water mixtures for hydrolysis

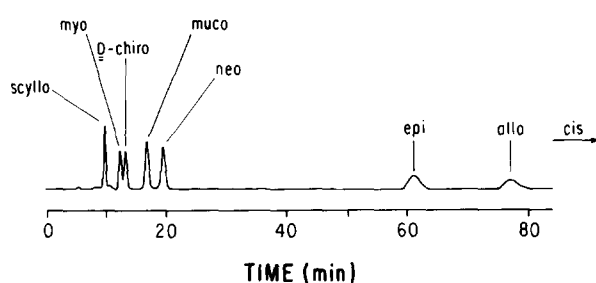


**Fig. 1.** Stereochemical features of the inositols, including numbers of adjacent axial-equatorial-axial (a-e-a) and axial-equatorial (a-e) hydroxyl groups.

of cellulose. Unlike hydrochloric and sulfuric acids, trifluoroacetic acid was easily removed from the hydrolysis mixture by simple evaporation. The mineral acids are generally more difficult to remove; they are neutralized by base and the resulting salts must then be removed from the oligosaccharide fractions.

Those cellodextrins produced by trifluoroacetic acid hydrolysis (Wing & Freer, 1984) had DP values from 2 to at least 6. No attempt was made to separate these into pure fractions; they were fermented directly into ethanol by special strains of yeast. In addition to their separation on charcoal-Celite columns mentioned above, cellodextrins have been isolated by size exclusion chromatography techniques (Hamacher *et al.*, 1985;

Kennedy *et al.*, 1985, and references therein). These methods can be scaled up to produce gram quantities of oligosaccharides but may require chromatographic run times of 24 h or more. Since size exclusion chromatography is a relatively low resolution method, the oligosaccharides isolated are not of high purity (Hamacher *et al.*, 1985). Ladisch and Tsao (1978) were the first to separate cellodextrins on the analytical scale by use of HPLC. They used 4% cross-linked Aminex resins in the calcium form as the stationary phase and pure water as the eluent. Under these conditions, cellodextrins up to DP 6 or 7 could be resolved. Later, Bonn *et al.* (1984) showed that cation exchange resins in the  $\text{Ag}^+$ -form, similar to those used to analyze maltodextrin mixtures

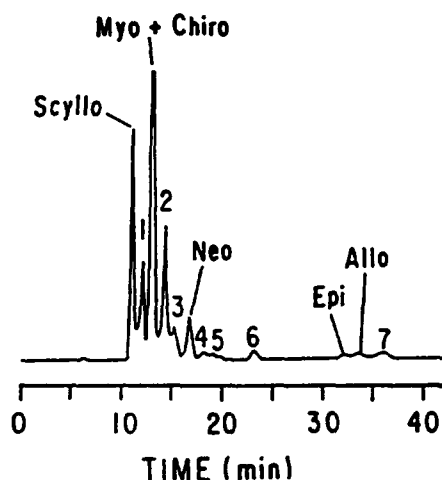


**Fig. 2.** Separation of seven inositol isomers by HPLC on an HPX-87C ( $\text{Ca}^{2+}$ -form) column, eluted with water at 0.65 ml/min at 24°C. Refractive index detection (Sasaki *et al.*, 1988).

(Scobell *et al.*, 1977; Scobell & Brobst, 1981), could resolve cellodextrins up to DP 7 or 8. These simple systems appeared to have excellent potential for scale-up to preparative levels, except that originally it was thought (Ladisch & Tsao, 1978) that such cation exchange resins were too mechanically weak to pack into wider diameter, preparative columns. The use of trifluoroacetic acid hydrolysis is now described for the first time, followed by preparative, HPLC on calcium and silver-form cation exchange resins, to prepare milligram to gram quantities of cellodextrins (DP 2–8) from cellulosic materials.

Four different types of 'cellulose' samples were hydrolysed by the trifluoroacetic acid procedure (Wing & Freer, 1984). Each of these cellulosic materials have been used previously by various investigators for preparation of cellodextrins. After hydrolysis, extraction, and deionization, as described in the Materials and Methods section, the samples were each analyzed by HPLC.

Figure 4(A–D) reveals that measurable quantities of DP 2–7 cellodextrins were formed in the reactions and that most samples contained measurable quantities of



**Fig. 3.** Separation of the products from myo-inositol boiled with Raney nickel in water. HPLC as in Fig. 2 except that flow rate was 0.6 ml/min and temperature was 85°C (Sasaki *et al.*, 1988).

minor peaks that can be attributed to xylose and oligosaccharides containing that sugar. Since Whatman CC 31 contained only small amounts of xylose, this substrate was used for all subsequent hydrolyses.

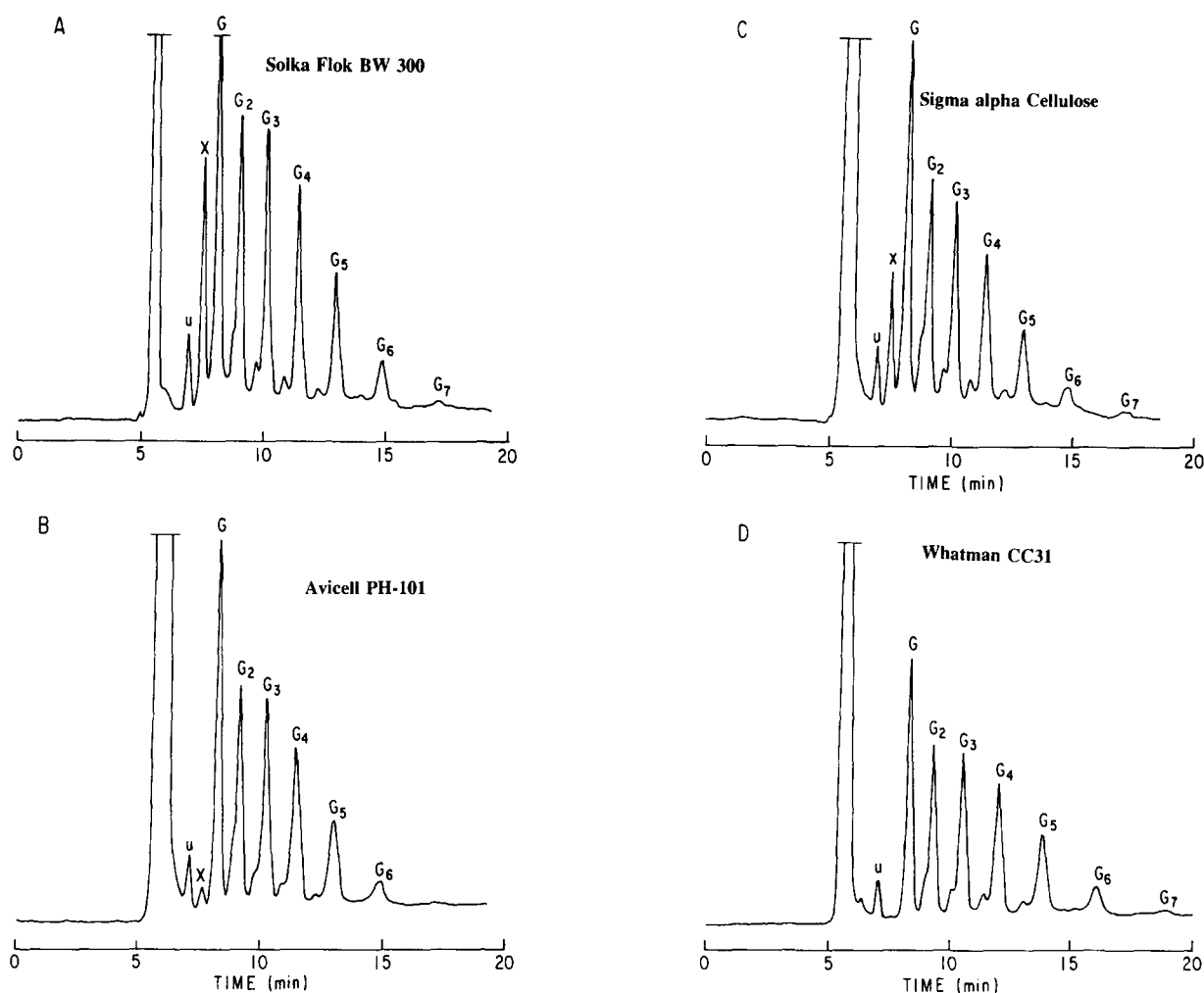
While the aminopropyl silica gel (APS) columns used in Fig. 4 provided good analysis of the hydrolysates, the cellodextrins were very sparingly soluble in the acetonitrile-containing mobile phase. Preparative HPLC on such columns is therefore not advisable. Preparative HPLC of cellodextrins on cation exchange columns, with aqueous mobile phases are more appropriate. Hence, the separation of a 12 mg sample of the cellodextrin mixture on a preparative (2.0 × 30 cm) column packed with AG-50W-X4  $\text{Ag}^+$ -form was readily achieved using water as a mobile phase and holding the column at 85°C (Fig. 5(A)).

Separation of oligosaccharides up to DP 10 was achieved in approximately 1 h. Injection of 125 mg (in 1 ml of injected volume) was also achieved in the same time frame without overloading the column (Fig. 5B). Unlike early studies suggested (Ladisch & Tsao, 1978), HPLC columns with wide (2.0 cm) diameters provided excellent resolution (better, in fact than narrow-bore columns, data not shown) of oligosaccharides in reasonable times with surprisingly low back-pressures (10–100 psi). The column was also very durable; it operated for over 3 years without any change in efficiency.

Because the cellodextrin mixture analyzed in Fig. 5 contained significant amounts of glucose and cellobiose (which were not of interest in this study), a two step preparative HPLC procedure was used to purify the DP-3–8 fractions: glucose and cellobiose were first quickly removed by repeated injections of the cellodextrin mixture onto a preparative (2.0 × 30 cm) stainless steel column packed with Aminex Q-15S,  $\text{Ca}^{2+}$ -form (Hicks & Sondey, 1987) (8% cross-linked) cation exchange resin. This step, processed repetitively and automatically with a microprocessor-controlled solvent delivery system, injector, and 'peak-sensing' fraction collector allowed overlapping injections of 125 mg samples every 20 min (Fig. 6(A)). The DP-3 and higher oligosaccharides were collected and lyophilized. The resulting sample, depleted in mono- and di-saccharides, was then injected on the  $\text{Ag}^+$ -form, AG 50W-X4 column shown in Fig. 6(B). Because glucose and cellobiose had been removed, 125 mg injections of DP-3 and larger cellodextrins could be injected every 28 min. Cellodextrins isolated from 29 of these automated injections were analyzed for purity by HPLC (Fig. 7).

The weights of the combined samples and estimated percent purity as measured by percent peak area on a refractive index detector are given in Table 1.

Further purification of each cellodextrin can be easily achieved by passing through the  $\text{Ag}^+$ -form column another time. However, for many purposes, these samples can be used as shown since purity of all DP



**Fig. 4.** HPLC analyses of hydrolysed commercial cellulose samples. (A) Solka Flok BW 300; (B) Avicell PH-101; (C) Sigma  $\alpha$ -cellulose; (D) Whatman CC31. All samples analyzed on an analytical IBM amino column (aminopropyl silica gel) eluted at 0.5 ml/min with acetonitrile/water, 65/35. Refractive index detection. X = xylose; G = glucose; G<sub>2</sub> = cellobiose, etc.

levels, except for DP 6 and 8, appear to be in the 90% range. After the samples were collected they were lyophilized and stored at low temperature ( $-20^{\circ}\text{C}$ ). Care should be taken to prevent aqueous samples of the pure oligosaccharides larger than cellobiose from sitting for a prolonged time. Under these conditions, the cello-dextrins (especially above DP-4) spontaneously aggregate and precipitate from solution. After precipitation, it is not possible to re-solubilize them in aqueous solution. Additionally, it has been found that freshly lyophilized samples are initially quite soluble in water, but upon storage, even at low temperature, samples become progressively less soluble. Previous investigators (Wolfrom & Dacons, 1952; Voloch *et al.*, 1984; Hamacher *et al.*, 1985) used ethanol to precipitate cello-dextrins from aqueous solution. In this study, ethanol-precipitated cello-dextrins were difficult to re-solubilize and hence collected HPLC fractions were lyophilized to produce white, fluffy solids.

One important aspect of the present method needs to be emphasized. Fractions collected from the  $\text{Ag}^{+}$ -form

column occasionally contain  $\text{Ag}^{+}$  ion, that upon storage can become reduced, leading to reddish-brown or black, oxidized cello-dextrin mixtures. For this reason, it is suggested that samples be passed through a short column of a de-ashing ion exchange resin, such as Amberlite MB-3 resin, prior to lyophilization, and stored in opaque vials. This problem can also be greatly reduced by ensuring that all samples are completely de-ashed prior to injection onto the HPLC column. This will prevent displacement of silver ion from the resin by other cations present as contaminants in the sample.

#### Pectic oligosaccharides

Oligosaccharides released during the partial hydrolysis of pectins are of intense interest to scientists studying plant growth, development, and pathology since they appear to act as signal or regulatory molecules in a variety of those processes. The simplest oligosaccharides of this type are the oligogalacturonic acids, homo-oligosaccharides composed of  $\alpha$ -1,4-linked D-galac-

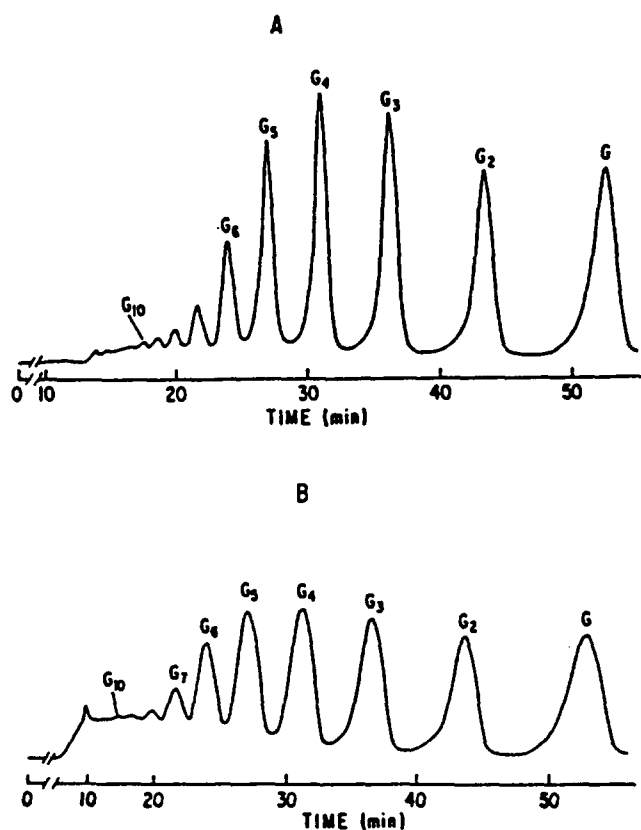


Fig. 5. Preparative HPLC separation of Whatman CC31-derived celloextrin mixture on a  $2 \times 30$  cm column, packed with AG 50W-X4 ( $\text{Ag}^+$ -form) cation exchange resin, eluted at 1.1 ml/min with water at  $85^\circ\text{C}$ . Pressure = 15 psi. (A) 12 mg injection. (B) 125 mg injection.

Table 1. Celloextrins isolated from 29 preparative injections (in 13.5 h)

DP value	Weight (mg)	% Purity (based on peak area)
3	662	99
4	809	97
5	504	96
6	372	81
7	93	93
8	68	85
Total	2498	

turonic acid residues. These are thought to regulate phenylalanine ammonia lyase activity (De Lorenzo *et al.*, 1987), proteinase inhibitor production (Bishop *et al.*, 1984), lignification (Robertsen, 1986), and phytoalexin elicitation (Nothnagel *et al.*, 1983; Jin & West, 1984; Davis *et al.*, 1986) in specific plant systems. In each of these responses, a specific DP range of oligogalacturonic acids was associated with optimal biological activity. Obviously, analytical and preparative HPLC systems for separation or isolation of these compounds would be of great interest. When the present studies began in this area, no HPLC methods were available to separate oligogalacturonic acids with DP values greater than about 8 or 9 (see Hicks (1988) for a review). Having used the sulfonated, polystyrene-divinyl benzene-based cation exchange resins for the analytical and preparative separation of neutral oligosaccharides such as maltodextrins (Hicks & Sondey,

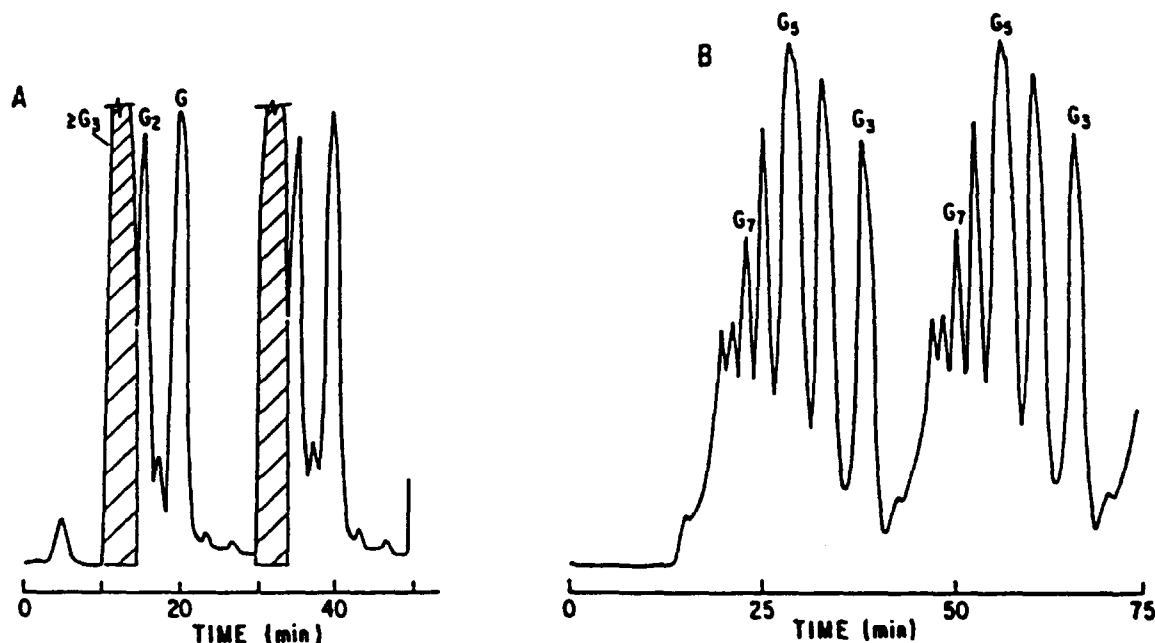


Fig. 6. Two-step isolation procedure for Whatman CC-31-derived celloextrins. (A) Quick removal of glucose and cellobiose on a  $2 \times 30$  cm column, packed with Aminex Q-15 S ( $\text{Ca}^{2+}$ -form) cation exchange resin, eluted at 2.0 ml/min with water at  $85^\circ\text{C}$ . Pressure = 100 psi. Cross-hatched peaks (DP-3 and greater) were collected and reinjected in (B). (B) Two consecutive preparative HPLC injections of 125 mg samples collected in (A). Conditions same as in Fig. 5(B).

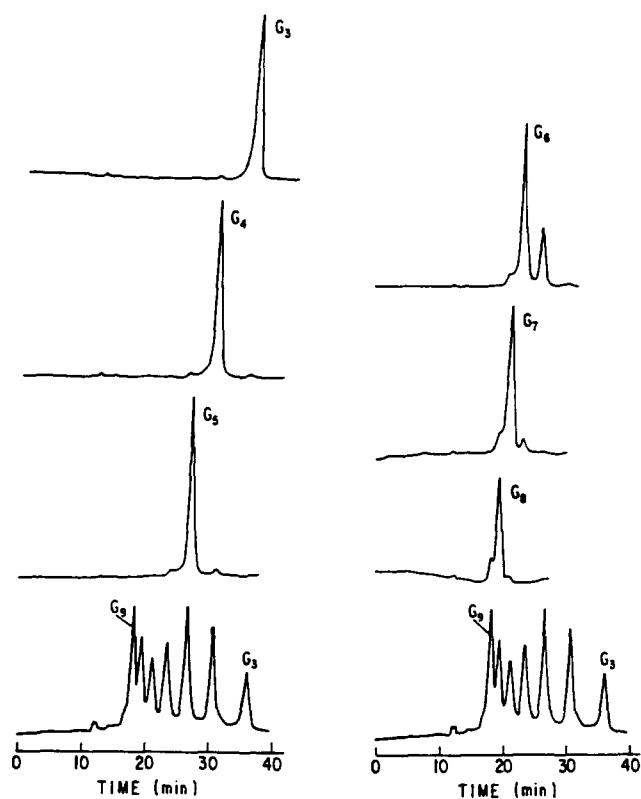


Fig. 7. HPLC analysis of fractions isolated in Fig. 6(B). Conditions same as in Fig. 5(A).

1987), this technology was applied to oligogalacturonic separations. Chromatography of oligogalacturonic acids on commercial, 8% cross-linked  $H^+$ -form cation exchange resins only resulted in separation of galacturonic acid from a broad peak that contained all the higher oligomers. However, it is well known that reducing the cross-linkage of such resins results in enhanced separations of larger oligomers. The commercially available 4% cross-linked resins therefore allowed slightly better separation, but this was still not adequate (data not shown). Discussions with a manufacturer of HPLC columns led to the development of a new 2% cross-linked cation exchange resin (called HPX-22H by the manufacturer) which provided (Hicks & Hotchkiss, 1988) excellent separation of oligogalacturonic acids (Fig. 8(C)) and the neutral oligosaccharides derived from amylose and cellulose as well (Fig. 8(A) and (B), respectively).

Although this system provided a simple and effective method for separating oligogalacturonic acids, the 2% cross-linked resin was quite fragile and the column had to be treated with great care in order to avoid compression of the packed bed. For that reason and because oligogalacturonic acids  $> DP-10$  were not resolved, the use of high performance anion exchange chromatography was examined, coupled to pulsed amperometric detection (HPAEC-PAD) for separation of oligogalacturonic acids. This method (Hotchkiss & Hicks, 1990) allowed

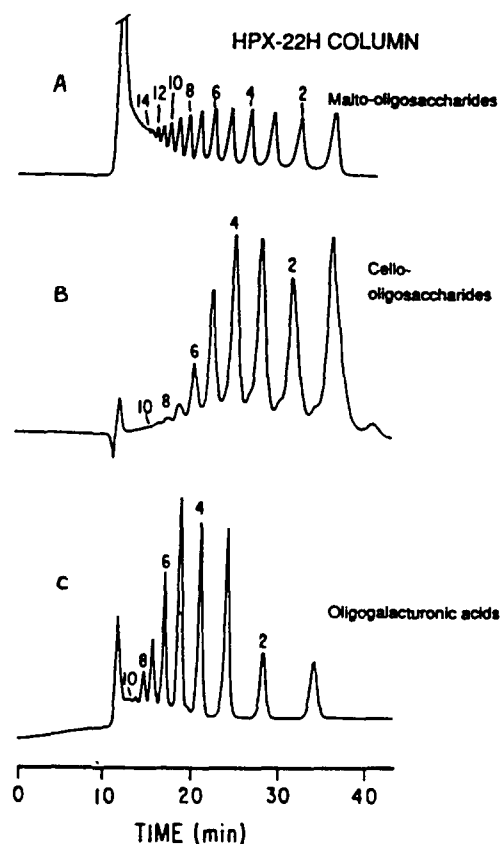


Fig. 8. Separation of plant-derived oligosaccharides on an HPX-22H column eluted with 0.005 M sulfuric acid at 0.5 ml/min and at 85°C. Refractive index detection. From Hicks and Hotchkiss (1988).

the unprecedented separation of oligogalacturonic acids up to a DP value of approximately 50 (Fig. 9).

The column for this system is relatively durable under the conditions applied (gradient elutions with sodium acetate or potassium oxalate buffers at pH values of 5–8) and it provided excellent selectivity and reproducible retention times. Pulsed amperometric detection was sensitive (sub-nanomole detection levels), but unfortunately did not give a predictable detector response for each oligosaccharide on either a weight or molar basis.

HPAEC-PAD systems have been developed predominantly for analytical, rather than preparative applications. Preparative-sized HPAEC columns are currently being used, which should allow isolation of milligram levels of pure oligogalacturonic acids. For isolation of gram scale quantities of oligogalacturonic acids up to DP-7, the use of a preparative-size aminopropyl silica gel column has recently been reported, in the anion exchange mode (Hotchkiss *et al.*, 1991), to efficiently isolate both normal and unsaturated oligogalacturonic acids produced by the action of endopolygalacturonase and pectate lyase enzymes on polygalacturonic acid, respectively. Figure 10 shows a preparative injection on this system and subsequent chromatographic analysis of the resulting fractions.

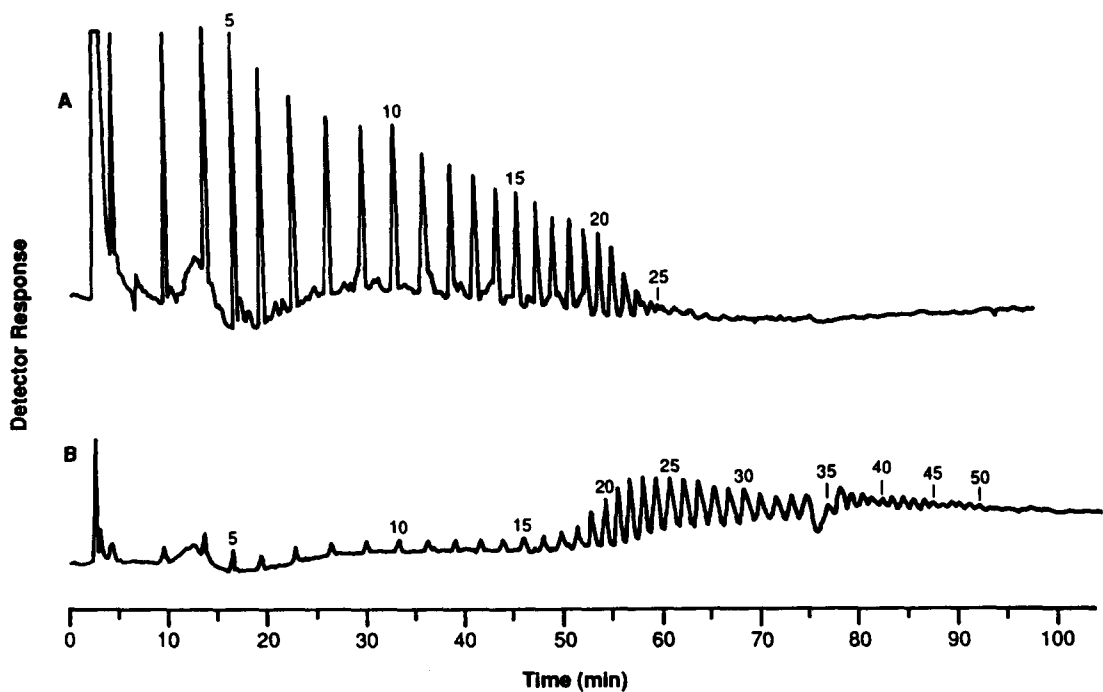


Fig. 9. Oxalate buffer gradient (pH 7.8) HPAEC separation of oligogalacturonic acids. Pulsed amperometric detection. Numbers above peaks refer to DP values (Hotchkiss & Hicks, 1990).

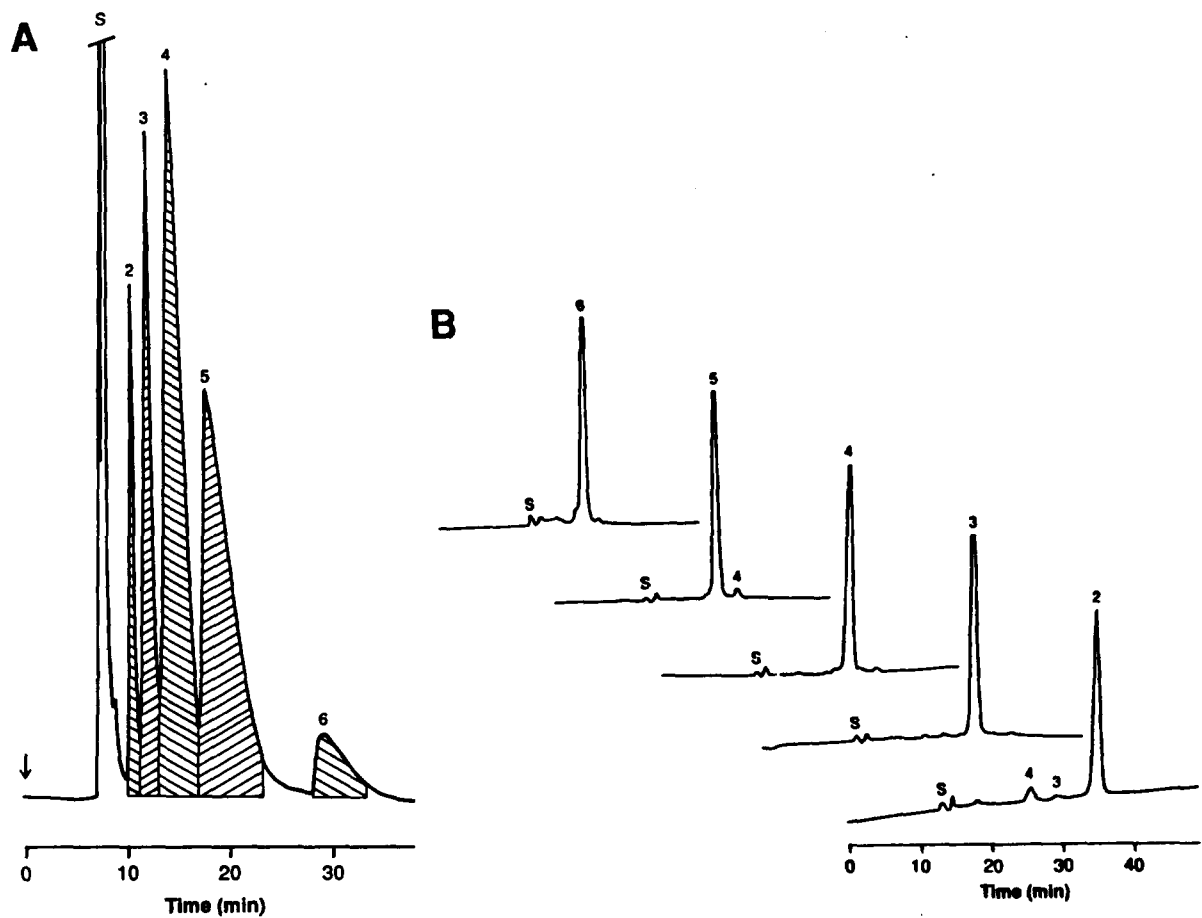


Fig. 10. (A) Preparative HPLC injection (375 mg) of DP-2-6 oligogalacturonic acid mixture on a Dynamax-60A NH<sub>2</sub> (21.4 x 250 mm) column eluted with 0.9 M acetate buffer, pH 5, at 10 ml/min. Refractive index detection. (B) Analysis of collected fractions on an HPX-22H column described in Fig. 8 (Hotchkiss *et al.*, 1991).



## CONCLUSIONS

HPLC is a very useful technique for chemical and biochemical studies on carbohydrates. Compounds as structurally similar as the inositols can now be separated readily on the analytical and preparative scale with the methods described here. Simple oligogalacturonic acids from the partial hydrolysis of polygalacturonic acid can now also be separated up to DP-50 using new HPAEC techniques. Pure oligogalacturonic acids up to a DP value of seven can be isolated on preparative size (2.5 × 30 cm) HPLC columns. The cellodextrins, useful compounds for basic and applied studies on biomass conversion processes, can be isolated in pure form up to a DP level of at least six by the rapid, high capacity and high resolution HPLC techniques given here. Large diameter (2.5 cm) preparative HPLC columns packed with aminopropyl silica gel and used in a weak, anion exchange mode, have high sample capacities for acidic oligosaccharides. For separation of carbohydrates like the cellodextrins, however, the cation-exchange HPLC columns are recommended because of their durability, inexpensive operation and simple requirements for flow rate, mobile phase and back-pressure. In addition, separations on cation exchange resins can be scaled up as large as necessary for preparative purposes.

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