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Isolation of oligogalacturonic acids in gram quantities by preparative h.p.l.c.

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ABSTRACT

Oligogalacturonic acids up to a degree of polymerization of 7 (d.p. 7) were isolated in gram quantities by preparative h.p.l.c. from endo-polygalacturonase- and pectate lyase-depolymerized polygalacturonic acid. A Dynamax-60A NH_2 (21.4 × 250 mm) 1-aminopropyl silica gel column was used with an isocratic acetate buffer (ca. 0.9M, pH 5) mobile phase. Automated operation of the preparative h.p.l.c. system allowed for rapid, high-resolution separation and collection of oligogalacturonic acids that typically were 95–99% pure on a chromatographic peak area basis. The chromatographic system described represents an advance in oligogalacturonic acid isolation and purification methodology since it is faster, less labor intensive, and it provides higher isolation rates (over 300 mg/h of total oligosaccharides) than the traditionally used ambient pressure strong anion-exchange chromatography.

INTRODUCTION

Oligogalacturonic acids, or linear α-1,4-linked D-galacturonosyl-oligosaccharides, are formed by the depolymerization of polygalacturonic acid (PGA; pectic acid with negligible neutral sugar content). The average degree of polymerization (d.p.) is an important factor in the gelling properties of pectin[1], a valuable food ingredient. Certain size classes of oligogalacturonic acids have been reported to be physiologically active as elicitors of plant defense responses to pathogens[2–7]. Currently there is a need for significant quantities of highly purified oligogalacturonic acid standards in research areas such as these.

Since 1952 when Luh and Phaff[8] reported the first chromatographic separation of oligogalacturonic acids, several chromatographic techniques have been used on the analytical scale. Generally, high-performance liquid chromatography (h.p.l.c.) has provided the most efficient and highest resolution technique for oligogalacturonic acid separations[9,10]. Modes of oligogalacturonic acid separation by h.p.l.c. have included gel-filtration[11], strong anion-exchange[10,12], weak anion-exchange[12,13], ion-pair reversed-phase[12,14], and combined size- and ion-exclusion[15] methods. The maximum d.p. oligogal-

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

acturonic acids well resolved by these techniques were d.p. 8, 50, 25, 11, and 10, respectively.

Preparative-scale isolation of oligogalacturonic acids typically has involved the use of ambient-pressure strong anion-exchange resins including Dowex 1-X8[16], AG 1-X8[17], DEAE-Sephadex A-50[18], DEAE-Sephadex A-25[3], QAE-Sephadex (A-25-120)[5], and AG MP-1[19]. These methods are time consuming, labor intensive, and generally lack adequate resolution. Although the largest oligogalacturonic acid separated with these resins was d.p. 17, generally oligogalacturonic acids larger than d.p. 7 were poorly resolved. To date, no attempts to isolate oligogalacturonic acids by preparative h.p.l.c. have been reported.

In this report, we describe the first automated preparative h.p.l.c. separation and isolation of oligogalacturonic acids in gram quantities with a weak-base 1-aminopropyl silica gel anion-exchange stationary phase. The effects of alterations in sample load, mobile phase buffer molarity, and pH on oligogalacturonic acid separation were examined. Previously, this type of preparative h.p.l.c. column has been successfully used for the normal-phase separation and isolation of gram quantities of malto-oligosaccharides[20].

EXPERIMENTAL

Chromatography. — The automated preparative h.p.l.c. and analytical h.p.l.c. instrumentation used for separations of oligogalacturonic acids has been previously described[15,20]. Column effluent was monitored by a Waters 403 differential refractometer for isocratic separations, by a Gilson Holochrome variable wavelength u.v./vis. absorption detector for gradient separations, and by an ultra-sensitive Erma ERC-7510 refractive index detector for HPX-22H (Bio-Rad Laboratories) separations.

Dynamax-60A, NH_2 (Rainin Instrument Co.) stationary phases were used for the analytical- and preparative-scale separation of oligogalacturonic acids by weak-base anion-exchange h.p.l.c. Prior to preparative-scale (21.4 × 250 mm column) separations of oligogalacturonic acids, analytical-scale columns (4.6 × 250 mm) were used to optimize mobile-phase conditions. Two mobile phases performed optimally in analytical-scale separations. These were (A) 0.8M sodium acetate buffer, pH 5 (acetic acid was diluted in water, titrated with 50% NaOH to pH 5, and the volume adjusted to produce 0.8M), 1 mL/min, and (B) 0.1–0.4M potassium phosphate buffer, pH 5.9 (85:15 KH_2PO_4–K_2HPO_4) 25-min linear gradient, 1 mL/min. An isocratic 0.9M sodium acetate, pH 5, 10 mL/min mobile phase was used for preparative-scale oligogalacturonic acid separations. Capacity factors (k') and resolution values (R) were calculated using the formulas[21]

where t_R = the peak retention time, t_0 = the void elution time, Δt = the difference in the retention times for two peaks, and w = the peak width. In our previous experience, resolution values of at least $R = 1$ allowed for the collection of peaks with greater than 90% purity. It is advisable when using large sample loads with preparative-scale separations, to match the buffer concentration and pH of the sample with that of the mobile phase in order to obtain the best resolution. For acidic carbohydrates this should include adjusting the final pH of the sample to the appropriate value after dissolving the sample in the mobile phase.

To evaluate the chromatographic purity of oligogalacturonic acid fractions isolated by preparative h.p.l.c., an additional analytical-scale stationary phase was used, the HPX-22H cation-exchange column (10 × 300 mm). The HPX-22H column was operated at 85° with a 0.01N H_2SO_4 mobile phase at a flow rate of 0.4 mL/min[15].

Isolation of oligogalacturonic acids. — Large-scale (gram quantities) preparation of an oligogalacturonic acid mixture (d.p. ≤ 6), which was used for analytical- and preparative-h.p.l.c. separations, was prepared by endopolygalacturonase hydrolysis of PGA (Sigma, citrus) as previously reported[19]. Unsaturated (4,5-unsaturated function at the nonreducing end) oligogalacturonic acids (d.p. ≤ 7) were prepared by pectate lyase cleavage of PGA (1% PGA in mM $CaCO_3$, 11.5mM TRIS free base, pH 8.5, 15 units of pectate lyase (gift from Dr. Ching-Hsing Liao, ERRC, ARS, USDA), 30°, reaction agitation at 200 rpm). Pectate lyase cleavage was stopped by lowering the pH with an excess amount of Amberlite IR-120 [H^+] resin. Following filtration to remove the resin, the sample was evaporated at 35° under reduced pressure to a minimal volume (≤ 50 mL) and lyophilized.

Routinely 375 mg (1.5 mL of 250 mg/mL) of oligogalacturonic acids were automatically injected on the preparative Dynamax-60A NH_2 column. Like fractions were collected by an "intelligent" peak-sensing fraction collector and pooled. The oligogalacturonic acids were precipitated by the addition of an equivalent amount of barium acetate and a 75% volume of acetone. The barium salt of the oligogalacturonic acids was collected by centrifugation (20 min, 10,000 rpm) and then converted into the free acid form by mixing in a minimal volume of H_2O with sufficient Amberlite IR-120 [H^+] ion-exchange resin to completely dissolve the precipitate. The resin was removed by filtration, and the filtrate was reduced to a minimal volume (≤ 50 mL) by evaporation at 35° under reduced pressure prior to lyophilization. The HPX-22H h.p.l.c. system was used to monitor the removal of acetate buffer from the oligogalacturonic acid fractions as well as the final purity of the product. The purity of isolated oligogalacturonic acids also was determined by the carbazole assay[22] using D-galacturonic acid monohydrate (Sigma) as a standard.

RESULTS AND DISCUSSION

Analytical-scale separations of oligogalacturonic acids up to d.p. 12 were produced with the Dynamax-60A NH_2 stationary phase and a pH 5.9 phosphate buffer gradient (Fig. 1a). The slope of the phosphate buffer gradient was observed to be

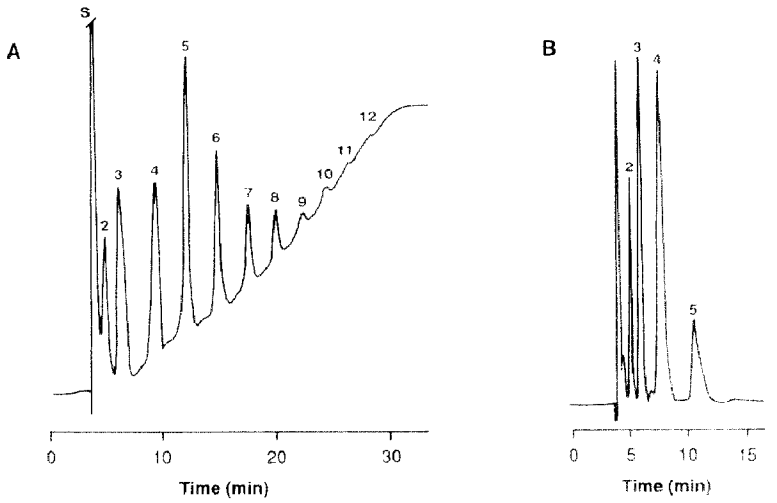

Fig. 1. Analytical-scale separation of oligogalacturonic acids on a Dynamax-60A NH_2 (4.6 × 250 mm) column. The oligogalacturonic acid d.p. value is indicated above each peak (S = solvent). (a) Gradient separation of an endo-polygalacturonase hydrolysate of PGA (2 mg sample injected). Chromatographic conditions included 0.1–0.4M phosphate buffer in 25 min, pH 5.9, 1 mL/min mobile phase, u.v./vis. 220 nm absorption detection. (b) Isocratic separation of a mixture of oligogalacturonic acids (d.p. ≤ 6, 1 mg sample injected). Chromatographic conditions included 0.8M acetate buffer, pH 5, 1 mL/min mobile phase, refractive index detection.

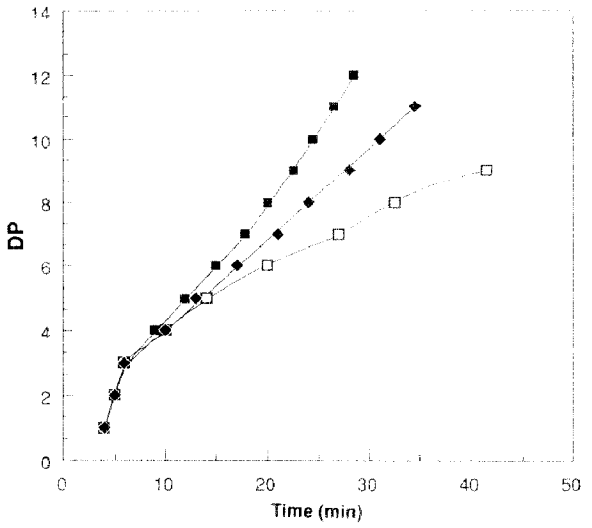

Fig. 2. The effect of phosphate buffer gradient steepness on oligogalacturonic acid retention time: 0.1–0.2M (□), 0.1–0.3M (◆), and 0.1–0.4M (■)(chromatogram shown in Fig. 1a). Other conditions the same as in Fig. 1a.

directly related to the maximum d.p. that could be detected (Fig. 2). While phosphate buffer had stronger buffering capacity and lower absorption at 220 nm than acetate buffer, it was difficult to remove from collected oligogalacturonic acid fractions, which prohibited its use as a mobile phase for preparative h.p.l.c.

Due to its volatility, acetate was a more attractive choice for a mobile phase since it could be evaporated (as acetic acid) from oligogalacturonic acid fractions following collection from a preparative h.p.l.c. column. With an isocratic, 0.8M, pH 5, acetate buffer mobile phase, oligogalacturonic acids up to d.p. 5 were separated on an analytical-scale Dynamax-60A NH_2 column in less than 12 min (Fig. 1b). Voragen *et al.*[12] used a 0.11M, pH 7.5, acetate buffer as a mobile phase for separations of unsaturated oligogalacturonic acids up to d.p. 5 in 15 min by weak anion-exchange h.p.l.c. (LiChrosorb 10 NH_2). We observed that conditions including higher buffer concentration and lower pH were optimal for the Dynamax-60A NH_2 stationary phase. Voragen *et al.*[12] demonstrated that buffer concentration and pH were inversely proportional to capacity factor values for oligogalacturonic acids. Our observations confirm these findings for the Dynamax-60A NH_2 column in that retention of oligogalacturonic acids decreased as buffer molarity and pH increased.

The chromatographic performance of the preparative Dynamax-60A NH_2 column was similar to that of the analytical Dynamax-60A NH_2 column for the separation of oligogalacturonic acids by weak anion-exchange h.p.l.c. On the preparative-scale, capacity factors (and retention times) were inversely proportional to buffer molarity (Fig. 3a). The curves agreed with those we observed on the analytical-scale with this stationary phase and also with those curves reported by Voragen *et al.*[12] Peak resolution decreased with increasing buffer molarity and larger sample loads (Figs. 3b, 3c). In this analysis, resolution values were more than adequate for preparative-scale isolations of up to 125-mg injections with a 0.6M, pH 5, acetate buffer mobile phase. It should be noted that if the sample pH was less than pH 5, oligogalacturonic acids were retained too long with a 0.5M or 0.6M acetate buffer (pH 5) mobile phase, resulting in band broadening and decreased resolution. Therefore, all samples were adjusted to pH 5 prior to injection.

The preceeding column performance data was collected from separations performed on a prototype model of the Dynamax-60A NH_2 preparative column, which was no longer used after it exhibited decreased performance for oligogalacturonic acid separations. Subsequently, a new commercially available model of the Dynamax-60A NH_2 preparative column was used for automated injections and collection of oligogalacturonic acids. The new preparative column performed optimally with a 0.9M, pH 5, acetate buffer mobile phase for the majority of the runs. After operating this preparative column for ca. 35 h, during which 14 g of oligogalacturonic acids were injected, the mobile phase buffer concentration was reduced to 0.85M in order to maintain identical retention times. Although column aging necessitated the reduction in mobile phase buffer concentration, the column was operated for an additional 25 h without any significant loss in column performance. While we feel that these mobile-phase parameters are representative of the optimal conditions for this column, they are not absolute, and it is anticipated that minor variations may exist from column to column.

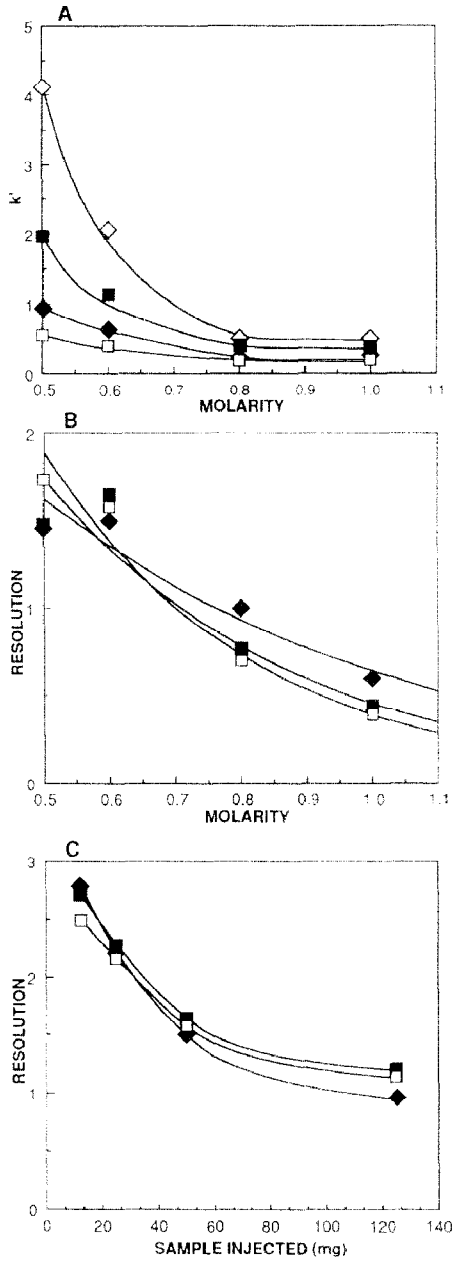

Fig. 3. Evaluation of parameters for the separation of oligogalacturonic acids on a preparative Dynamax-60A NH_2 (21.4 × 250 mm) column. An isocratic acetate buffered (pH 5) mobile phase at 10 mL/min flow rate and refractive index detection was used. (a) The effect of acetate buffer molarity on oligogalacturonic acid capacity factors: d.p. 2 (□), d.p. 3 (◆), d.p. 4 (■), and d.p. 5 (◇). (b) The effect of acetate buffer molarity on the resolution of oligogalacturonic acids: d.p. 2–3 (□), d.p. 3–4 (◆), and d.p. 4–5 (■). (c) The effect of sample load on the resolution of oligogalacturonic acids: d.p. 2–3 (□), d.p. 3–4 (◆), and d.p. 4–5 (■). A 50-mg sample load was used in (a) and (b), and a 0.6M acetate-buffered mobile phase was used in (c).

The commercially available Dynamax-60A NH_2 preparative column allowed for automated 375-mg injections (35–45 min frequency) of up to d.p. 6 oligogalacturonic acids derived from endopolygalacturonase hydrolysis (Fig. 4a) and up to d.p. 7 of pectate lyase-generated, unsaturated oligogalacturonic acids (Fig. 5a). The peaks were sufficiently resolved so that highly purified oligogalacturonic acid fractions were collected. The chromatographic purity of oligogalacturonic acids isolated from pooled fractions collected after a series of automated injections on a preparative Dynamax-60A NH_2 column was demonstrated following injection on an analytical HPX-22H h.p.l.c. column (Figs. 4b, 5b). The d.p. value of isolated oligogalacturonic acids lacking a 4,5-unsaturated function at the nonreducing end was determined by observing coelution with oligogalacturonic acid standards isolated by preparative ambient-pressure anion-exchange (AG MP-1) chromatography, with molecular weights confirmed by

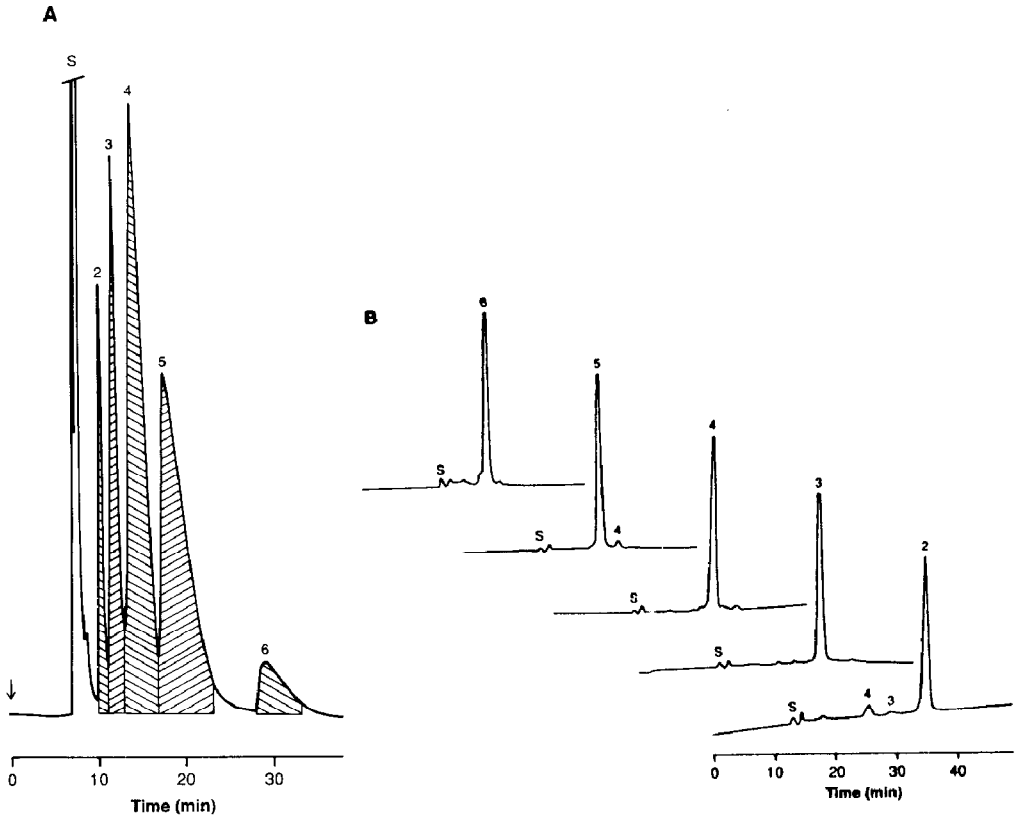

Fig. 4. (a) Automated, preparative-scale separation of oligogalacturonic acids (375-mg sample injected) with d.p. values indicated above the peaks (S = solvent). Chromatographic conditions included a Dynamax-60A NH_2 (21.4 × 250 mm) column, an isocratic 0.9M acetate buffer, pH 5, 10 mL/min mobile phase, refractive index detection. (b) Chromatographic purity of oligogalacturonic acids isolated by automated, preparative h.p.l.c. Pooled fractions collected from the preparative h.p.l.c. column were injected on a HPX-22H (10 × 300 mm) analytical column. The oligogalacturonic acid d.p. value is indicated above each peak (S = solvent). Chromatographic conditions included an 85°, 0.01N H_2SO_4, 0.4 mL/min mobile phase, refractive index detection.

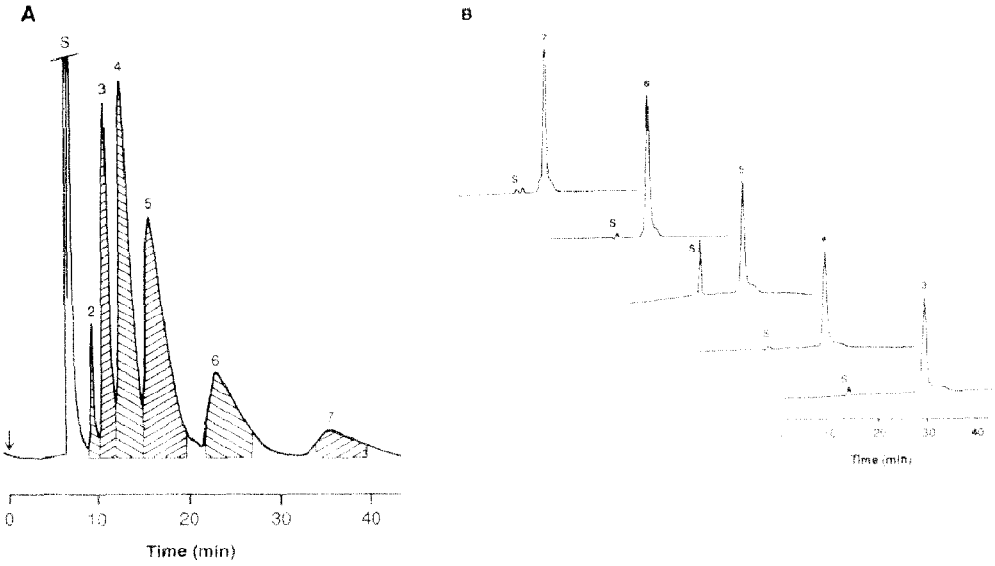

Fig. 5. (a) Automated, preparative-scale separation of unsaturated oligogalacturonic acids (375-mg sample injected). Conditions were the same as in Fig. 4a, except that over the course of several runs, the isocratic acetate buffer concentration varied from 0.85M to 0.9M to maintain identical retention times as column aging occurred. (b) Chromatographic purity of unsaturated oligogalacturonic acids isolated by automated, preparative h.p.l.c. Conditions were the same as in Fig. 4b.

f.a.b.-m.s.[19] The basis for d.p. value assignment of unsaturated oligogalacturonic acid fractions was that these oligosaccharides eluted from the HPX-22H column as a homopolymer series that had 0.4–0.8 min longer retention times than oligogalacturonic acids lacking the unsaturated function. Oligogalacturonic acid fractions were observed to be least 95% pure in all cases except d.p. 2 (90% pure), with fraction purity based on peak area from HPX-22H chromatograms (Table 1). The carbazole assay supported the high purity of the isolated oligogalacturonic acids. The shoulders observed on unsaturated oligogalacturonic acid peaks (Fig. 5b) could not be significantly reduced following reinjection on the preparative Dynamax-60A NH_2 column.

Gram quantities of oligogalacturonic acids were isolated at rates exceeding 130 mg/h on an individual oligosaccharide isolated basis and over 300 mg/h on a total oligosaccharides isolated basis (Table I). Oligogalacturonic acid isolation by preparative h.p.l.c. was much more efficient than that possible with ambient-pressure strong anion-exchange chromatography, where the maximum reported isolation rate for baseline-resolved oligogalacturonic acids was ca. 27 mg/h (ca. 96 mg/h total oligosaccharides isolated for six oligosaccharides)[19]. Therefore, for the isolation of oligogalacturonic acids, the preparative h.p.l.c. system described herein has the following advantages compared to ambient-pressure chromatography: higher isolation rates, shorter run times (35–40 min *vs.* 6.5 h or longer), elimination of laborious procedures (manual sample loading, addition of step gradient buffers, and collection of a large number of fractions which are subsequently pooled after sampling with a secondary

TABLE I

Oligogalacturonic acid isolation efficiency

d.p.	*Prep. h.p.l.c. peak resolution*	*isol. rate (mg/h)*	*% purity (peak area)*
(A) "Normal"[a]			
2	1.5	12.8	90
3	1.1	37.7	99
4	0.8	136.9	99
5	2.0	121.0	98
6		14.6	99
(B) Unsaturated[b]			
3	0.9	33.9	95
4	0.9	80.1	95
5	1.3	82.5	97
6	1.5	38.6	97
7		10.9	98

[a] Oligogalacturonic acids lacking a 4,5-unsaturated function at the nonreducing end.
[b] Oligogalacturonic acids with a 4,5-unsaturated function at the nonreducing end.

colorimetric method), and the ability to make multiple injections without having to regenerate or repack the column. This preparative h.p.l.c. system is also flexible enough to separate higher d.p. oligogalacturonic acids by increasing the mobile phase buffer molarity.

The 1-aminopropyl silica gel stationary phase had less capacity for oligogalacturonic acids separated by weak anion-exchange than it did for malto-oligosaccharides separated by normal-phase partition chromatography. Isolation rates for malto-oligosaccharides by normal-phase preparative 1-aminopropyl silica gel h.p.l.c. were observed up to ca. 190 mg/h on an individual oligosaccharide isolated basis or ca. 600 mg/h on a total oligosaccharides isolated basis (five oligosaccharides)[20]. Additionally, the 1-aminopropyl silica gel column lifetime was shorter in the weak anion-exchange mode compared to the normal-phase mode. This reduction in column lifetime may have been caused by mobile-phase buffers leaching more silica from the stationary phase than normally occurs with an acetonitrile–water mobile phase used in the normal-phase mode. Column lifetimes were extended through the use of axial compression end fittings that reduced stationary phase voids formed from the leaching of silica gel by mobile-phase buffers.

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