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# Isolation of oligogalacturonic acids up to DP 20 by preparative high-performance anion-exchange chromatography and pulsed amperometric detection☆

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**Abstract**

Milligram quantities of oligogalacturonic acids up to a degree of polymerization (DP) of 20 were purified by high-performance anion-exchange chromatography utilizing a preparative-scale (21-mm i.d.) CarboPac PA1 column and a nonlinear potassium acetate (pH 7.5) gradient. Detection was accomplished by pulsed amperometry without post-column addition of hydroxide. Pulsed amperometry at near-neutral pH is an excellent detection method for preparative separations of carbohydrates because it avoids base-catalyzed degradation reactions that can occur at high pH. This method was simpler, faster, had higher sample loading capacity and allowed for the isolation of higher DP oligogalacturonic acids than other methods reported previously. With this improved method, multi-milligram quantities of valuable oligogalacturonic acids (up to DP 20) can be readily isolated. 

*Keywords:* Oligogalacturonic acid; Degree of polymerization; DP 20; High-performance anion-exchange chromatography; Pulsed amperometric detection; Preparative HPAEC–PAD; CarboPac PA1 column

## 1. Introduction

Oligogalacturonic acids (α-D-Gal*p*A-[(1→4)-α-D-Gal*p*A]$_n$-(1→4)-D-Gal*p*A) are derived from the depolymerization of the pectic backbone polysaccharide, polygalacturonic acid (PGA). These oligosaccharides are important in plant growth and development, plant–microbe interactions and fruit ripening.[1–4] For each of these physiological responses, a specific oligogalacturonic acid size class has been shown to possess highest biological activity with the most bioactive being those with a degree of polymerization (DP) ≥ 9.[3] There is interest in isolating large quantities of oligogalacturonic acids to identify and characterize receptors for these oligosaccharides in plants,[4] for use as substrates for pectin-modifying enzymes, and as standards for various physical–chemical, biochemical and chromatographic analyses of pectin structure and its gellation. Pure standard oligogalacturonic acids above DP 3 are not commercially available, but several stationary phases have been used for preparative-scale separations of oligogalacturonic acids up to DP 7. We isolated

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gram quantities (137 mg/h) of oligogalacturonic acids up to DP 7 by preparative HPLC using a weak anion-exchange aminopropylsilica gel column (21 mm i.d.).[5] This column had a very high sample loading capacity, and the method was more rapid and efficient than the ambient pressure strong anion-exchange chromatography methods used previously.[5] However, gradient elution caused dramatic baseline drift of the refractive index and UV–Vis ($A_{220}$) detectors used to monitor the aminopropylsilica gel column effluent, which limited isolation of higher DP oligogalacturonic acids. Multi-milligram (more than 10) quantities of DP 10–15 oligogalacturonic acids were purified by a two-step procedure utilizing Q-Sepharose chromatography, followed by semipreparative CarboPac PA1 (9 mm i.d. column) high-performance anion-exchange chromatography and pulsed amperometric detection (HPAEC–PAD) of the Q-Sepharose fractions.[6] The semipreparative CarboPac PA1 column was also used to isolate oligogalacturonic acids up to DP 5, unsaturated oligogalacturonic acids up to DP 4 and a methyl-esterified DP 4 oligogalacturonic acid all from a low-molecular weight fraction of a citrus pectin hydrolysate.[7] Rhamnogalacturonan (RG) oligosaccharides were isolated from semipreparative[7–9] and preparative[10] CarboPac PA1 columns. Unsaturated RG oligosaccharides were isolated with a preparative CarboPac PA100 column,[11] a tryraminated DP 13 oligogalacturonic acid was isolated from a semipreparative CarboPac PA1 column,[12] biotin hydrazides of oligogalacturonic acids (DP 8–16) were isolated from an analytical CarboPac PA100 column (4 mm i.d.),[13] and partially methyl-esterified oligogalacturonic acids (up to DP 10) were isolated from an analytical CarboPac PA1 column.[14] It is clear that the CarboPac column has unequaled selectivity to separate structurally similar oligosaccharides.[15] Therefore, preparative methods that utilize this column characteristically are high resolution and can potentially be used to isolate oligogalacturonic acids up to DP 50.[16] However, the HPAEC–PAD method and the pellicular design of the CarboPac stationary phase are optimized for high sensitivity analytical-scale separations that have low sample loading capacity. Therefore, HPAEC–PAD methods must be modified so that they can approach the oligogalacturonic acid yield capacity obtained by preparative HPLC.[5]

## 2. Materials and methods

*Preparation of a DP 10–20 oligogalacturonic acid mixture.*—Polygalacturonic acid (1%, pH 4.4) was autoclaved and subsequently fractionated by lowering the pH to 2 as described previously.[16] The pH 2 soluble fraction was retained and neutralized by the addition of KOH. Sodium acetate (50 mM) and an equal volume of 45% ethanol (22.5% final concentration) were added to this fraction prior to refrigeration (4 °C) overnight. The resulting precipitate was collected by centrifugation (16,300*g*, 30 min), evaporated under reduced pressure to remove residual ethanol and lyophilized. The resulting mixture was enriched in DP 10–20 oligogalacturonic acids as confirmed by analytical HPAEC–PAD using a nonlinear 0.025–0.5 M potassium oxalate buffer (pH 6) mobile phase gradient reported earlier.[17]

*Preparative HPAEC–PAD.*—Manual injections (1 mL) of a 50 mg/mL DP 10–20 enriched oligogalacturonic acid mixture solution were made on a 21 × 250 mm CarboPac PA1 column (Dionex, Inc). A Dionex 4500i HPLC was used that included a Pulsed Electrochemical Detector (PED; 3 mm Au working electrode), a GPM-2 gradient pump and a Gilson 202 Fraction Collector. A 5 mL/min nonlinear potassium acetate buffer (pH 7.5) mobile phase gradient was used. This gradient was produced by mixing 2 M potassium acetate (A) with water according to the following schedule: 20% A at 0–1 min, 30% A at 30 min, 36% A at 45 min, 36% A at 60 min, 37% A at 72 min, 38% A at 84 min, 39% A at 95 min, 46% A at 100 min, 100% A at 110 min, and 20% A at 111 min. The next injection was made 10–15 min following the end of this gradient. No hydroxide was added post-column. The PED integrated amperometry potential sequence used was: 0.1 V at 0–0.48 s, 0.95 V at 0.49–0.61 s, and −0.8 V at 0.62–0.69 s.

Chromatograms were collected with a Chrom Perfect Direct (Justice Innovations) chromatography data system that included a DT2804 A–D board. Fractions (1 min, maximum) were collected using the peak-sensing mode of the fraction collector. Like fractions were pooled, dialyzed (1000 MWCO, Spectrum) and lyophilized. Peak purity was established by analytical-scale HPAEC–PAD using a nonlinear 0.025–0.5 M potassium oxalate buffer (pH 6) gradient.[17]

## 3. Results and discussion

Using a nonlinear potassium acetate buffer (pH 7.5) gradient and a 21-mm CarboPac PA1 column, we separated 50 mg of a mixture of oligogalacturonic acids that was enriched in DP 10–20 (Fig. 1). The PAD detector was relatively insensitive to the mobile phase gradient requiring only minimal baseline adjustment under these conditions (auto offset was pressed at 42 and 90 min in Fig. 1). It was possible to separate higher (up to 70 mg) sample loads of this mixture, but with decreased peak resolution. At 100 mg/mL this sample was not completely soluble in water. The loading capacity of the preparative CarboPac PA1 column was 8–10-fold greater than the 6-mg loading capacity reported for the semipreparative CarboPac PA1 column.[6] However, it is far less than the 375 mg sample (mixture of DP 2–7 oligogalacturonic acids) load that was used with a preparative aminopropylsilica gel column.[5] The separation of oligogalacturonic acids with the preparative CarboPac PA1 column and a nonlinear potassium acetate gradient agreed with the previously reported anion-exchange parameters in that capacity factors were inversely proportional to buffer concentration and pH.[18] At pH 7.5, a very shallow potassium acetate buffer gradient was required for resolution of individual oligogalacturonic acids (Fig. 1). We previously demonstrated that oligogalacturonic acids were more soluble in a potassium oxalate (pH 6) mobile phase buffer (separation up to approximately DP 50) compared to a sodium acetate (pH 5) mobile phase buffer (separation only up to DP 19).[15] These observations were confirmed by a report that oligogalacturonic acids are more soluble in the potassium salts of mobile phase buffers and represent the best choice for HPAEC–PAD purification of these oligosaccharides.[6] Unfortunately, the PAD background signal of the oxalate buffer is too high at pH 6 to allow for detection of oligogalacturonic acids (rotating disk electrode (RDE) analysis performed by Dr William LaCourse, University of Maryland, Baltimore County). Therefore, we used a pH 7.5 potassium acetate mobile phase buffer and observed good PAD response for the DP

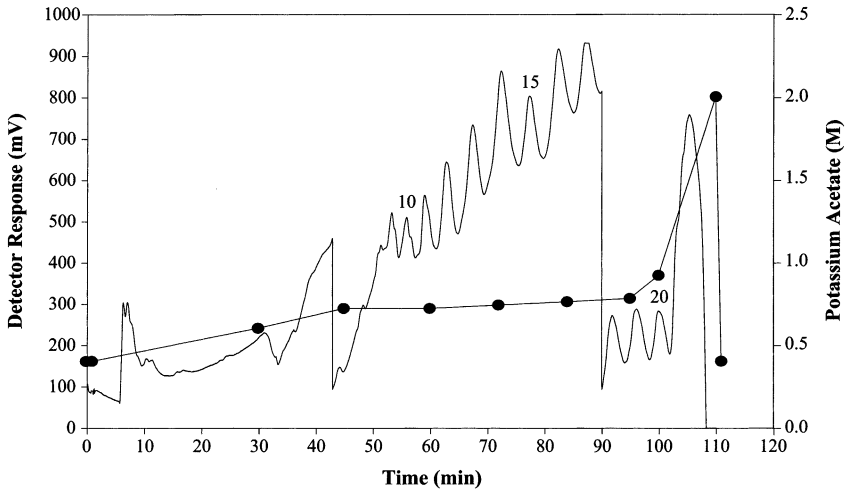


Fig. 1. Preparative HPAEC–PAD of a DP 10–20-enriched oligogalacturonic acid mixture. Chromatographic conditions included a 21-mm i.d. CarboPac PA1 column, a nonlinear potassium acetate (pH 7.5) mobile phase gradient (●; 5 mL/min), no post-column addition of hydroxide and a 50-mg sample load. The numbers above the peaks refer to their degree of polymerization.

Table 1
Amounts of oligogalacturonic acids isolated by preparative HPAEC–PAD

| DP | Total weight of fractions [a] (mg) | 85+% pure [b] (mg) | 90+% pure [c] (mg) |
|---|---|---|---|
| 7 | 2.2 | 0.5 | 0.5 |
| 8 | 6.5 | 6.1 | 3.9 |
| 9 | 14.0 | 5.5 | 1.1 |
| 10 | 26.0 | 9.8 | 2.0 |
| 11 | 37.0 | 16.8 | 6.1 |
| 12 | 47.3 | 6.7 | 5.9 |
| 13 | 41.5 | 4.3 | 1.6 |
| 14 | 32.2 | 5.0 | |
| 15 | 27.9 | 2.7 | |
| 16 | 26.7 | | |
| 17 | 20.7 | 6.1 | 4.1 |
| 18 | 15.1 | | |
| 19 | 15.8 | 2.3 | |
| 20 | 10.5 | 2.2 | 2.2 |
| 21 | 3.5 | | |

[a] Cumulative weight of oligogalacturonic acids fractions collected from up to 90 HPLC runs using a 21-mm i.d. CarboPac PA1 column.
[b] Amount of total weight recovered that was greater or equal to 85% purity (analytical HPAEC–PAD peak area basis).
[c] Amount of total weight recovered that was greater or equal to 90% purity.

10–20-enriched oligogalacturonic acid mixture without post-column addition of hydroxide. Detection of oligogalacturonic acids at lower pH values was possible; however, their detector response decreased to near-background levels at pH 6 (confirmed by RDE analysis). PAD detection of DP 10–15 oligogalacturonic acids in a pH 8 potassium acetate mobile phase buffer has also been reported.[6] Therefore, while the sensitivity of PAD carbohydrate detection is greater at high pH, this condition is not required for preparative separations, where sensitive detection is not necessary. PAD detection of carbohydrates at near-neutral pH is an excellent choice for preparative separations since it avoids base-catalyzed degradation reactions that can occur at high pH.

Preparative CarboPac PA1 fractions were pooled at various intervals and analyzed for purity following dialysis and lyophilization. Although conditions for chromatography and fraction collection were kept constant, like DP fractions showed differences in purity due to run-to-run variation in column performance and mobile phase composition. Multi-milligram amounts of DP 8–20 oligogalacturonic acids were isolated (Table 1) with an isolation efficiency range of 0.1–0.6 mg/peak/run. Those preparative CarboPac PA1 fractions isolated during optimal preparative HPAEC–PAD system performance had peak purity values that exceeded 85% (Table 1). The peak purity of representative fractions (Fig. 2) isolated from 10 preparative CarboPac PA1 runs

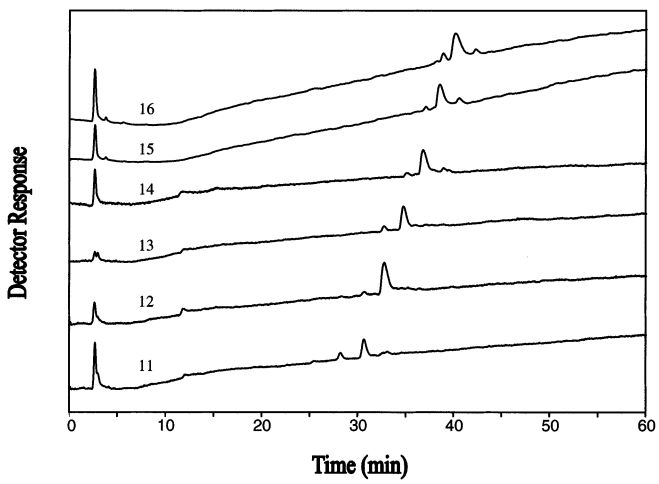


Fig. 2. Analytical HPAEC–PAD of representative HPAEC–PAD-purified oligogalacturonic acids that were isolated from a series of 10 preparative CarboPac PA1 runs. Chromatographic conditions included a 4-mm i.d. CarboPac PA1 column, a nonlinear potassium oxalate (pH 6) mobile phase gradient (0.8 mL/min), post-column addition of hydroxide and a 50-μg sample load. The numbers above the chromatograms refer to the degree of polymerization of the major peak.

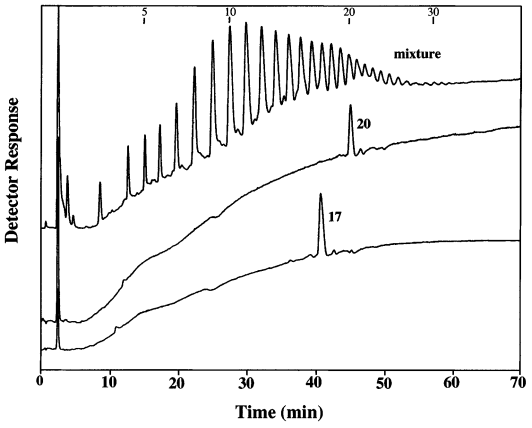


Fig. 3. Peak purity of the preparative HPAEC–PAD-purified DP 17 and 20 oligogalacturonic acids. A mixture of oligogalacturonic acids is included (top chromatogram) for reference. Conditions were the same as in Fig. 2.

was as follows: DP 11, 73%; DP 12, 92%; DP 13, 86%; DP 14, 86%; DP 15, 80%; DP 16, 81%. The peak purity of DP 17 and 20 preparative CarboPac PA1 fractions (91 and 92%, respectively) is illustrated in Fig. 3. While smaller amounts of the isolated oligogalacturonic acids had peak purity that exceeded 90% (Table 1), it should be emphasized that the HPAEC–PAD system is a more selective and sensitive separation and detection method than others used to determine peak purity of oligosaccharides isolated by preparative HPLC.[5] Therefore, the amounts in Table 1 with 85% or better purity are equivalent to the most pure oligosaccharides that were previously isolated.[5] Fractions with less than 85% peak purity were reinjected on the preparative CarboPac PA1 column to further purify the oligogalacturonic acids present.

The 5 mL/min preparative flowrate used was considerably lower than the 'optimal' preparative flowrate predicted from the following formula:[19]

$$F_p = (ID_p/ID_a)^2 \cdot (L_p/L_a)$$

where $F$, $ID$, $L$, p and a correspond to flowrate, internal diameter, column length, preparative and analytical columns, respectively. According to this formula, a 1 mL/min flowrate on a 4 mm i.d. analytical column corresponds to 'optimal' flowrates of 28.1 mL/min on a 21 mm i.d. preparative column and 5 mL/min on a 9 mm i.d. semipreparative column. Both 'optimal'[6,11] and 'suboptimal'[7,10] flowrates were reported for the isolation of oligogalacturonic acids and rhamnogalacturonan oligosaccharides with preparative and semipreparative CarboPac PA columns. Therefore, our use of a 'suboptimal' flowrate for the preparative isolation of larger oligogalacturonic acids with the preparative CarboPac PA1 column is in line with previous literature reports. The above relationship appears to overestimate the actual optimal flowrate for preparative versions of CarboPac PA columns. This may be due to the pellicular design of the CarboPac PA stationary phases that requires less mass transfer for the analyte to reach ion-exchange sites than fully functionalized stationary phases.

For comparison, milligram amounts of oligogalacturonic acids were also isolated from the 21-mm i.d. CarboPac PA1 column using a high-pH (12.7) mobile phase (nonlinear potassium acetate gradient in 100 mM KOH) at 10 mL/min and a nonlinear potassium acetate (pH 8) mobile phase gradient (without post-column addition of hydroxide) at 15 mL/min with a Gilson preparative HPLC as previously published,[5] except that the PED detector and Chrom Perfect data acquisition system (see Section 2) were included. Under these conditions the highest isolated peak purity was 90% (DP 11). Therefore, changing to a high-pH mobile phase or increasing the flowrate did not improve the peak purity of the oligogalacturonic acids isolated from the preparative CarboPac PA1 column. We also tried to use a 21 mm i.d. aminopropylsilica gel (Dynamax 60A) column to isolate DP 10–20 oligogalacturonic acids using a nonlinear potassium acetate (pH 5) mobile phase gradient at 15 mL/min and pulsed amperometric detection without post-column addition of hydroxide. However, the peak purity of the milligram amounts of oligogalacturonic acids isolated did not exceed (highest peak purity 83% for DP 12) that obtained using the preparative CarboPac PA1 column.

Therefore, the method reported here (preparative HPAEC–PAD, 21 mm i.d. CarboPac PA1 column, pH 7.5 nonlinear potassium acetate gradient, 5 mL/min) represents several improvements compared to previously reported methods. It is the first method for preparative-scale purification of DP 10–20 oligogalacturonic acids by HPAEC–PAD.

While preparative CarboPac PA1 and PA100 columns were used to purify RG oligosaccharides,[10,11] both of these methods utilized a high-pH mobile phase, and no details were reported for loading capacity, amounts of oligosaccharides isolated, or their purity. While Spiro et al.[6] reported isolation of approximately 70 mg each of DP 10–15 oligogalacturonic acids (purity demonstrated only for DP 13) using a semipreparative CarboPac PA1 column, they could not purify $DP > 15$ oligogalacturonic acids because of irreversible binding of these oligosaccharides to the Q-Sepharose gel used as their first chromatographic step. During the course of preparative injections, we observed a decrease in peak symmetry over time. This decrease in column performance may have been due to retention of small amounts of oligogalacturonic acids during each run, a phenomenon that has been reported.[13] Column performance was restored by washing the column with 0.1 M NaOH. We also observed that $DP \geq 17$ oligogalacturonic acid fractions precipitated when refrigerated overnight in the potassium acetate buffer mobile phase. The precipitation of these larger oligogalacturonic acids facilitated their recovery since the precipitate was easily collected by centrifugation. The oligogalacturonic acids readily went into solution following the addition of 1–2 mL of water, and subsequent desalting by dialysis did not take as long because of the low combined fraction volume. Finally, we collected up to 14 preparative peaks/run in our preparative CarboPac PA1 method in contrast to the one semipreparative peak/run collected by Spiro et al.[6] Therefore, our method is simpler, faster, has higher sample loading capacity and allows for the isolation of higher DP oligogalacturonic acids compared to previous methods. However, while we have isolated multi-milligram quantities of up to DP 20 oligogalacturonic acids by our method, more research will be required to obtain gram quantities of these oligosaccharides.

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