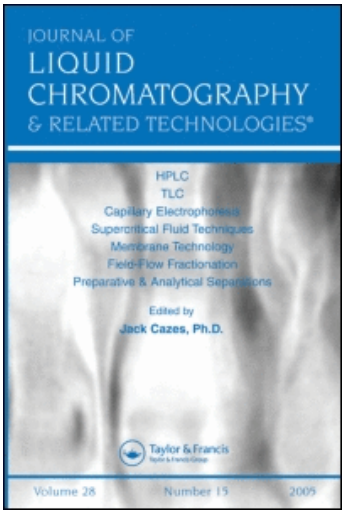

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Separation, Detection, and Quantification of Galacturonic Acid Oligomers with a Degree of Polymerization Greater than 50

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Separation, Detection, and Quantification of Galacturonic Acid Oligomers with a Degree of Polymerization Greater than 50

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Abstract: A high performance anion exchange chromatography (HPAEC)–evaporative light scattering detector (ELSD)-method was developed to detect, separate, and quantify galacturonic acid (GA) oligomers. Following digestion of polygalacturonic acid (PGA) with a monocomponent endo-polygalacturonase (EPG), more than 70 GA oligomer peaks could be resolved using a convex/linear ammonium formate gradient. Linear calibration curves were produced for 0.015–1.0% mono-, di-, and tri-GA. The mass response for mono-GA differed from those for di- and tri-GA, as evidenced from the slope of the calibration curve regression lines (1.611 ± 0.0201 for mono-GA vs. 1.3068 ± 0.0291 and 1.3004 ± 0.0262 for di-, and tri-GA, respectively). The degree of polymerization (DP) appeared to affect mass response as the trend line for log-transformed peak areas of DP 3, 4, 6, and 8 oligomers had a slope of -0.0304 ± 0.0032 ($r^2 = 0.98$). Buffer concentration also affected mass response. ANOVA of peak areas from isocratic elution of trimer and hexamer with 50 mM to 0.8 M ammonium formate indicated mass response was dependent on buffer concentration for each oligomer ($P < 0.005$), although Duncan's Multiple Range Test described concentration ranges within which mass response was not affected ($P < 0.05$).

Keywords: pectin, oligogalacturonic acid, ELSD, evaporative light scattering detection, endo-polygalacturonase, EPG

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INTRODUCTION

Pectin is a complex polysaccharide found in the primary cell walls of angiosperms.[1–3] It is generally accepted that pectin is composed of three distinct domains, i.e., homogalacturonan (HG), rhamnogalacturonan I, and rhamnogalacturonan II,[2] although the macromolecular organization within the cell wall is still poorly understood. The primary source of commercial pectin is citrus fruit peel, most commonly from lemons.[4] Galacturonic acid (GA) comprises 80–90% of citrus pectin on a dry weight basis and is mainly found in the HG regions, which are linear, unbranched polymers of GA[1,2,4,5] of which a variable proportion of GA residues are methylated at their C6 position and acetylated at C2 or C3. Many of the biological and commercial functionalities of pectin are related to the proportion of the GA residues in the HG that contain methyl esters.[4,6] This percentage is commonly known as the pectin's degree of methylesterification (DE). Commercial pectin is divided into two categories: high DE (DE $>$ 50%) and low DE (DE $<$ 50%) pectin. Gelation of high DE pectin requires the addition of sugar and is pH dependent, while low DE pectins typically form gels in the presence of calcium. In addition to the absolute DE, functional properties of pectin also are dependent on the distribution of the methyl esters along the HG stretches.[7,8]

Both random and ordered (blockwise) distribution patterns of methyl esters along the HG regions have been recognized.[9,10] Analyzing these methyl ester distribution patterns is key to understanding their relationship to functional properties. Pectin demethylation can be accomplished by either chemical (i.e., alkaline demethylation) or enzymatic (pectin methylesterase; PME) methods. While chemical demethylation appears to be random, three modes of action have been hypothesized for enzymatic demethylation.[11] These were based on the mode of action of starch degrading enzymes,[12] which are depolymerizing enzymes, while PMEs are methyl ester hydrolases; therefore the comparison may be an over simplification. The three postulated modes of action are: (a) single chain mechanism—processive demethylation of all adjacent methyl esters from an initial binding site; (b) multiple chain mechanism—only a single methyl ester is hydrolyzed for each enzyme binding event; and (c) multiple attack mechanism—a limited number of adjacent methyl esters are hydrolyzed for each binding event.

Plant PMEs have been shown to demethylate pectin in an ordered, blockwise fashion (either single chain mechanism or multiple attack mechanism), at least at neutral pH.[9,11,13–16] Indirect statistical methods[11,17,18] and enzymatic methods using exo- and endo-polygalacturonase,[9] which is inhibited by methylated GAs, have been used to estimate lengths of the demethylated blocks in HG regions with varying DEs and probe the mode of action of PME from plants, fungi, and bacteria.[10,11,17,18] All of these methods have been hampered by their inability to directly detect and quantify GA oligomers, larger than a trimer. While pulsed amperometric

detection has been used to detect oligomers up to a degree of polymerization (DP) of 50,[19]it is not suitable for quantification of individual oligomers, for which there are no standards available because detector response strongly decreases with increasing oligomer length. More recently, gel[3] and capillary[20] electrophoretic separations of GA oligomers have been reported. The capillary electrophoresis method does allow for estimates of molar amounts but is limited to oligomers less than DP of 15–20.[20] Cameron et al.[21] have utilized an ELSD coupled to a high performance size exclusion chromatography system to estimate the mass of oligomers up to a DP of 20; however, baseline resolution was lost after a DP of 3, so complex mixtures could not be analyzed. As a mass detector, the ELSD is not dependent on the presence of a chromophore in the analyte and the baseline is not affected by gradient elution when volatile buffer components are used.[22–24]

In this study, we report on results obtained on the separation, detection, and quantification of GA oligomers obtained by coupling an ELSD to a high performance anion exchange chromatography system (HPAEC).

EXPERIMENTAL

Chemicals

All chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise noted. Tetra, penta-, hexa-, 19-, and 20-mer GA oligomers were kindly provided by Dr. Arland Hotchkiss (US Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, Wyndmor, PA, USA). The octamer GA was obtained by digestion of polygalacturonic acid (PGA) with endo-polygalacturonase (EPG; EC 3.2.1.15, Megazyme International Ireland Limited, Bray, Ireland, Lot # 00901) as described subsequently.

Endo Polygalacturonase Digestion

A 200 mL volume of a 2% solution of the potassium salt of PGA (KPGA) in 20 mM potassium acetate, pH 4.7, 0.02% sodium azide and 2.5 mg mL^{-1} bovine serum albumen (Fisher Biotech, Fair Lawn, NJ, USA) was digested with 0.12 Unit mL^{-1} EPG at 37°C for 2 hr. An aliquot (100 mL) was removed after 1 hr and the EPG was inactivated by preheating in a microwave oven for 2–3 min followed by heating in a boiling water bath for 10–15 min. After a total of 2 hr, the remainder of the digest was heat treated as described earlier to inactivate the EPG. Additional KPGA (200 mL) was digested with EPG as described earlier, except that the enzyme was added at 0.05 Units mL^{-1} and the digest was carried out at

room temperature for 3 hr with a 100 mL aliquot being removed after 2 hr. The EPG was inactivated as described earlier.

Apparatus

The analytical HPAEC system was composed of a Perkin–Elmer Binary LC 250 Pump (Shelton, CT, USA), a Perkin–Elmer Series 200 Autosampler, and a CarboPac PA1 (4 × 250 mm, Dionex Corporation, Sunnyvale, CA, USA) anion exchange column. Detection of analytes was accomplished with an ELSD (ESA, Inc. Model 301, Chelmsford, MA, USA). Data collection was accomplished with an A/D converter connected to a Hewlett Packard (Palo Alto, CA, USA) personal computer using EZChrom Elite software (Scientific Software Inc., Pleasanton, CA, USA).

The preparative HPAEC system was composed of a DEAE-Sephacel column (26 × 370 mm), connected to an FPLC chromatography system (Amersham Biosciences, Piscataway, NJ, USA), coupled to a Dionex ED-50 Electrochemical Detector operated in the integrated amperometric mode with a wave form of +0.1 V for 0.48 sec, +0.95 V for 0.12 sec, and −0.80 V for 0.07 sec. A postcolumn addition of 100 mM K_2CO_3 at 0.3 mL min^{-1} was used to enhance detector response.

Preparative Anion Exchange Chromatography

After loading 20 mL of EPG digested KPGA, the column was washed with 160 mL H_2O. Bound oligomers were eluted with a step/linear gradient of potassium acetate (50 mM over 200 mL, 50–100 mM over 2 mL, 100–200 mM over 200 mL, 200–300 mM over 500 mL, followed by an isocratic step at 300 mM for 320 mL before re-equilibrating at 50 mM potassium acetate) at a flow rate of 2 mL min^{-1}. Fractions of ~20 mL were collected, to which 80 μL of glacial acetic acid had been added to reduce the pH to ~5.0 in the final fraction volume. Fractions within individual peaks were pooled, volumes were decreased to ~10 mL by evaporation, and two volumes of ethanol were added to precipitate GA oligomers. After centrifugation at 15,000 × g for 30 min at 4°C, the pellets were washed with 10 mL 80% ethanol and centrifuged as described earlier. Resulting pellets were re-solubilized in a minimal volume of 0.02% sodium azide and stored at 4°C.

High Performance Anion Exchange Chromatography

An ammonium formate (Fluka Biochemika, Buchs, Switzerland) gradient (50 mM for 5 min, 50–600 mM convex gradient over 55 min, 600–800 mM

linear gradient over 120 min, 800–850 mM linear gradient over 15 min, followed by 50 mM for 15 min, total run duration of 210 min at 1 mL min^{-1}) at pH 4.4 was used to separate the GA oligomers. Isocratic elution was also performed for tri- and hexa-GA from 50 mM to 0.8 M ammonium formate. The nebulizer and the evaporation chamber temperatures of the ELSD were set at 60°C and 152°C, respectively. The photomultiplier sensitivity was set at 700 V. Either air or nitrogen was used as detector carrier gas at a pressure of 138 kPa.

RESULTS AND DISCUSSION

Over 70 peaks (Figure 1) could be resolved by the HPAEC–ELSD after injecting 70 μL of a 2% EPG digest of KPGA (containing a 1 : 2 ratio of 1 hr digest : 2 hr digest) onto the CarboPac PA1 column. Figure 2 demonstrates which chromatographic peaks correspond to mono-GA through hexa-GA and the 19- and 20-mers. These standards were injected both individually and as a mixture and consistently eluted with retention times indicated in Figure 2. The 19- and 20-mers had slightly longer retention times when injected individually or as a mixture of the two oligomers. Detection of 70 oligomers is 20 more than previously reported by HPAEC,[19] 50 greater than by capillary electrophoresis,[20] and 30 greater than that resolved by carbohydrate gel electrophoresis.[3] The two peaks eluting at ~9 and 13 min (Figures 1 and 2) were identified as K^+ and Na^+ salts based on retention times of peaks obtained from the injection of potassium and sodium acetate standards. When used in association with PME demethylation and controlled EPG digestion of pectin, this HPAEC–ELSD methodology will be extremely useful for directly identifying demethylated and methyl-protected fragment lengths, which will aid in elucidating the PME mode of action under a variety of physical and chemical conditions.

Linear calibration curves of log-transformed data could be constructed with mono-GA (intercept = 7.7602 ± 0.0201, slope = 1.6111 ± 0.0185, $r = 0.997$), di-GA (intercept = 8.1542 ± 0.0268, slope = 1.3068 ± 0.0291, $r = 0.992$), and tri-GA (intercept = 8.1484 ± 0.0241, slope = 1.3004 ± 0.0262, $r = 0.993$) between 0.015% and 1.0% (Figure 3). The slope and Y-intercept of the mono-GA regression line differed from the di- and tri-GA regression lines. Similar results were obtained for calibration curves obtained with two other ELSDs from different manufacturers (data not shown). These data suggest that the DP of the GA oligomer or the concentration of the elution buffer might affect detector response.[22] In order to investigate the possible causes for the difference in detector response for mono-vs. di- or tri-GA, we compared detector response for replicate injections of 0.2% (w/v) mono-GA through hexa-GA and octa-GA (Figure 4) and for

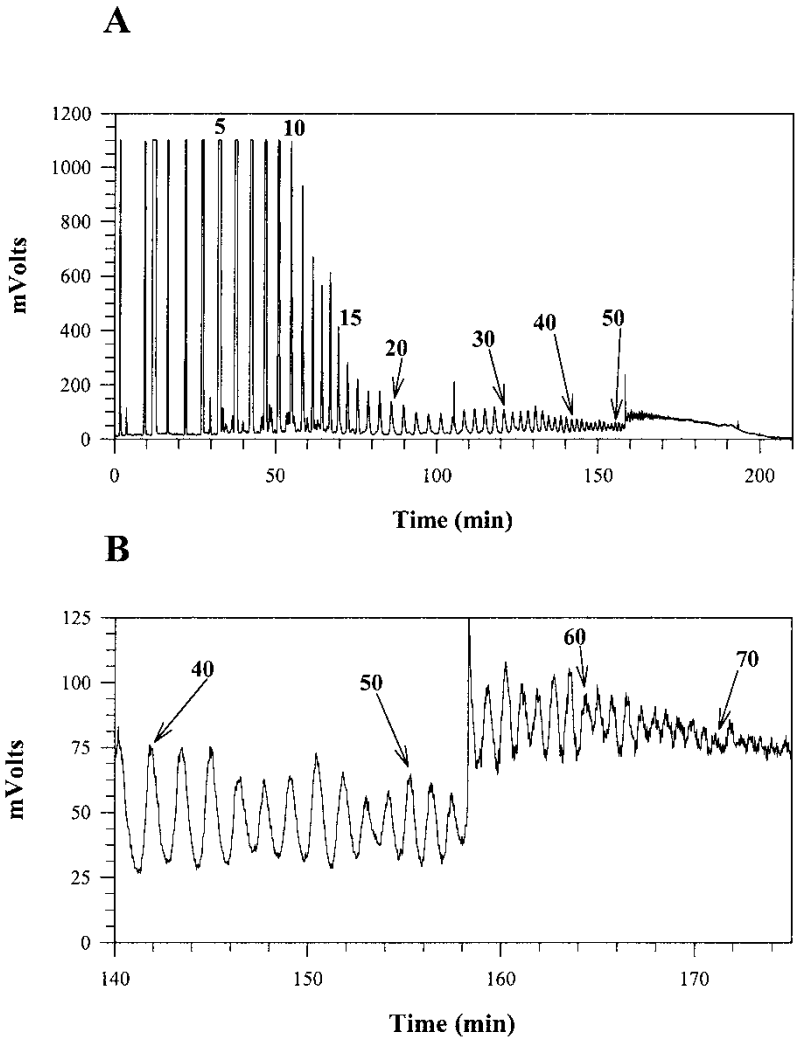

Figure 1. HPAEC/ELSD chromatogram of 70 μL of a 2 : 1 mixture of a 1–2 hr EPG digest of 2% KPGA. Numbers indicate DP of designated peaks. A. Full scale chromatogram, peaks at ~9 and 13 min are K^+ and Na^+, respectively. B. Zoom of chromatogram in A showing DP 40–70.

replicate injections of trimer and hexamer at varying concentrations of the ammonium formate buffer (Figure 5).

Absolute and log-transformed peak areas for equal concentrations of mono-GA through hexa-GA and octa-GA reinforced the observation from calibration curves that detector response for mono-GA was dramatically

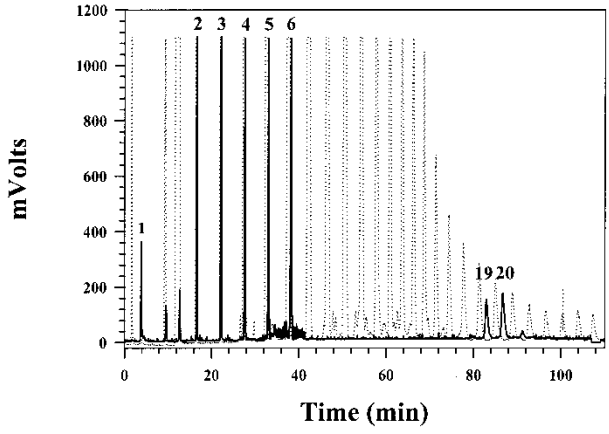

Figure 2. HPAEC/ELSD chromatogram from 15 μL of 0.1% 1–6 and 19 and 20 DP GA oligomers (——) overlaid on the chromatogram from Figure 1 (– – –). Numbers indicate DP of the oligomer.

lower than the larger DP oligomers (Figure 4). Peak areas for di- and tri-GA were very similar, but began an apparent trend toward lower values as the oligomer DP increased. Although material was not available to perform sufficient replication for ANOVA, regression analysis of the peak area means for DP 2, 3, 4, 5, 6, and 8 oligomers gave a slope of -0.0295 ± 0.0127 and an

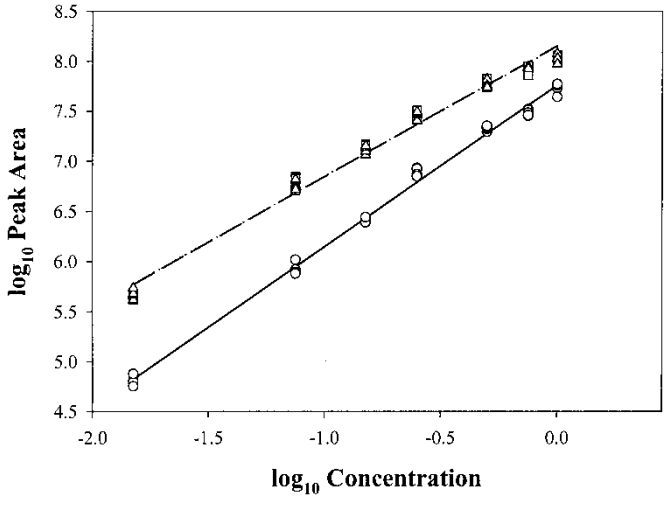

Figure 3. Scatter graph and regression lines for calibration curves ($n = 4–5$ for each concentration level) obtained with 0.015–1.0% mono- (○, —), di- (□, – –), and tri-GA (Δ, . . .).

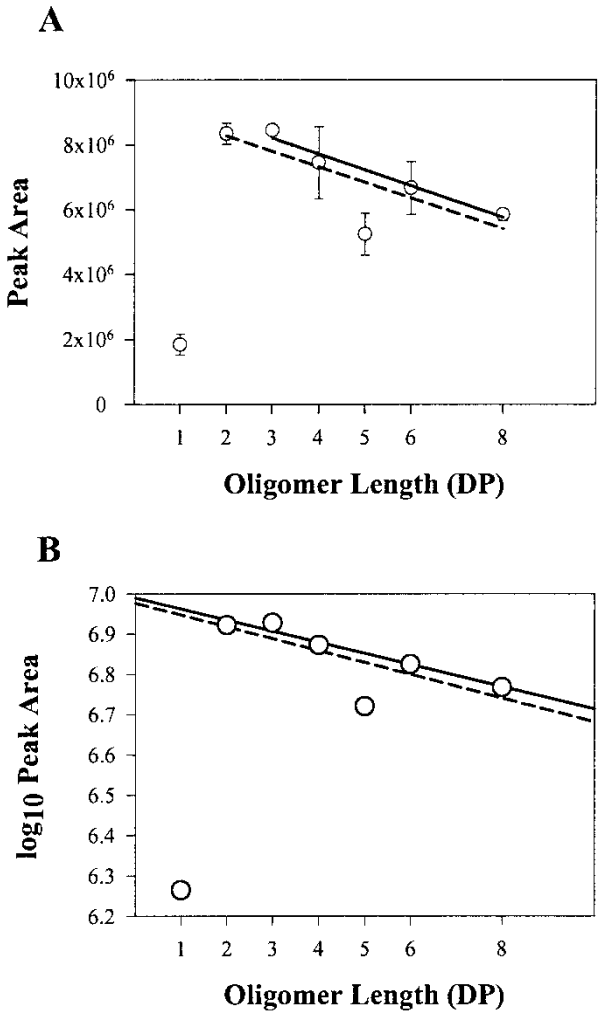

Figure 4. Effect of oligomer DP on peak areas obtained with 0.2% samples. A. Average peak areas ± S.E. for GA oligomers with DP of 1–6 and 8 ($n = 2$–4), and regression lines obtained for DP 2–6, 8 (———) and DP 3, 4, 6, 8 (– – – –). B. Regression lines (as indicated in Figure 4A) for log-transformed peak areas.

r-value of 0.7581. Removing the mono-, di-, and penta-GA values resulted in a slope of -0.0304 ± 0.0032 with an *r*-value of 0.9889. While these results do support a hypothesis that DP may affect detector response, they do not address the possible effect of buffer concentration. Such large discrepancies in peak area for mono- vs. di- or tri- GA were not observed with HPSEC–ELSD using 50 mM ammonium acetate, pH 3.7, buffer.[21]

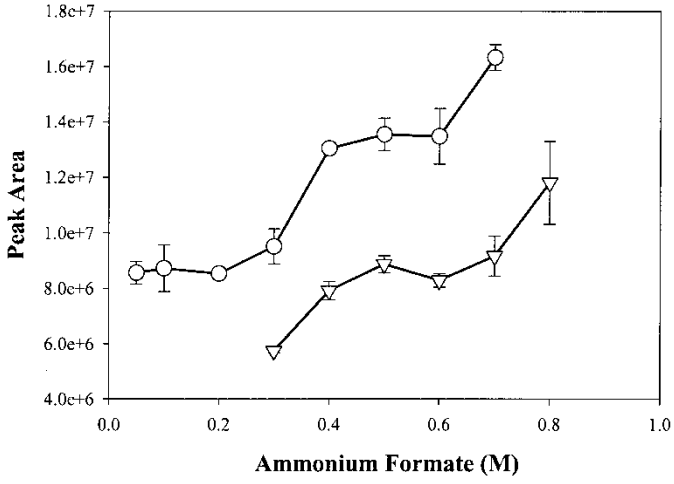

Figure 5. Effect of ammonium formate concentration on peak areas (mean ± S.E.) for 0.2% DP 3 (○; $n = 5$) and DP 6 (▽; $n = 3$) oligomers.

To study the effect of buffer concentration on detector response, peak areas from the isocratic elution of DP 3 and 6 oligomers, at ammonium formate concentrations of 50 mM to 0.8 M, were compared (Figure 5). ANOVA indicated that peak area means for either tri-GA or hexa-GA differed significantly (F value = 26.42, $P > 0.001$ for tri-GA; F value = 7.75, $P > 0.002$ for hexa-GA) through the buffer concentration range tested (Table 1). Duncan's multiple

Table 1. Peak areas (mean ± SE) for tri- and hexa-GA at different concentrations of ammonium formate, pH 4.4

Buffer concentration (% 1 M ammonium formate)	Peak area	
	Tri-GA ($n = 5$)	Hexa-GA ($n = 3$)
5	8,562,227 ± 402,402^{A}	NA
10	8,721,620 ± 844,717^{A}	NA
20	8,532,293 ± 75,917^{A}	NA
30	9,509,871 ± 632,586^{A}	5,739,369 ± 70,108^{D}
40	13,035,980 ± 204,232^{B}	7,918,168 ± 335,286^{E}
50	13,534,611 ± 583,228^{B}	8,870,199 ± 304,885^{E}
60	13,470,197 ± 998,734^{B}	8,284,172 ± 238,128^{E}
70	16,323,697 ± 471,606^{C}	9,160,589 ± 716,594^{E}
80	NA	11,803,363 ± 1,489,686^{F}

Note: Means with different superscripts within each oligomer are significantly different. NA, not applicable.

range test ($\alpha = 0.05$) identified three buffer concentration ranges within which means were similar. For tri-GA, there was no difference between 5–300 mM and 0.4–0.6 M ammonium formate. The mean for 0.7 M differed from the other two groups. Hexa-GA would not elute from the column satisfactorily below 0.3 M and the peak area mean for 0.3 M ammonium formate differed from the group at 0.4–0.7 M. The mean at 0.8 M differed from the 0.4–0.7 M group. These data suggest that calibration curves from commercially available standards could be used to estimate masses associated with peak areas for oligomers that elute between 50 mM and 0.3–0.4 M ammonium formate (tetramer elutes at ~0.4 M). Similarly, a calibration curve for a single, larger DP oligomer that elutes above 0.4 M ammonium formate could be used to estimate oligomer masses eluting in the higher DP range (29-mer elutes at ~0.7 M).

The ability to estimate masses and number of molecules of these larger DP oligomers would be of tremendous benefit for studies on the mode of action of PMEs, structural mapping of pectin, and for tailoring pectin functional properties for a variety of industrial and food purposes.

CONCLUSIONS

Coupling an ELSD to an HPAEC system has enabled us to extend the detection limits of GA oligomers up to a DP of 70, an increase of 40% over the previous limit.[19] In addition, this HPAEC–ELSD approach provides for the potential to estimate masses associated with each oligomer by constructing as few as two calibration curves, one for oligomers eluting <0.3–0.4 M ammonium formate and one for oligomers eluting >0.4 M. We were able to construct linear calibration curves for mono-, di-, and tri-GA, but the mass response for mono-GA was lower than the larger DP oligomers. Detector response also was affected by elution buffer concentration, as evidenced by isocratic elution of either tri- or hexa-GA with buffer concentrations ranging from 50 mM to 0.8 M ammonium formate. ANOVA and Duncan's multiple range tests identified two regions of buffer concentration where peak areas were statistically equivalent.

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