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Journal of Chromatography A, 1011 (2003) 227–231
JOURNAL OF CHROMATOGRAPHY A
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Short communication

Utilization of an evaporative light scattering detector for high-performance size-exclusion chromatography of galacturonic acid oligomers

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Received 26 March 2003; received in revised form 2 June 2003; accepted 30 June 2003

Abstract

A high-performance size-exclusion chromatography–evaporative light scattering detector method was used to separate, detect and quantify galacturonic acid (GA) oligomers. In 40 m*M* acetic acid GA monomer, dimer and trimer could be separated with baseline resolution but polygalacturonic acid (PGA) precipitated and could not be eluted from the column. An NH_4OAc, pH 3.7, buffer was developed as the eluent which separated GA oligomers as well as PGA and pectin without precipitation. Linear calibration curves for mono-, di- and tri-GA were produced with this buffer which could be used to estimate masses of tetra-, penta- and hexa-GA, as well as 19mer and 20mer.
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Keywords: Galacturonic acid; Polygalacturonic acid; Pectin; Carbohydrates

1. Introduction

Characterization of pectin fine structure following enzymatic modification has been used to probe bacterial, fungal and plant pectin methylesterase mode of action [1–4]. Common analytical techniques used in these studies are high-performance ion-exchange and high-performance size-exclusion chromatography (HPSEC). Detection methods that have been coupled to these chromatographic methods are refractive index [5], pulsed amperometric detection (PAD) [3,6–9] postcolumn derivatization with UV–Vis detection [4,7,10] and matrix-assisted laser desorption ionisation time-of flight mass spectrometry (MALDI-TOF-MS) [7]. Refractive index detectors are commonly used only with HPSEC because of problems associated with gradient elution [11]. PAD is satisfactory for small galacturonic acid oligomers but loses sensitivity as the oligomer size increases [12,13] which limits quantification to compounds of low molecular mass for which standards can be obtained. Postcolumn derivatization is inherently complex and requires use of strong acids [10,14]. MALDI-TOF-MS is highly specialized and not readily available. One detection method that has not been applied to chromatographic analysis of pectin

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doi:10.1016/S0021-9673(03)01171-3

and oligogalacturonides is evaporative light scattering detection (ELSD); this is a mass detector and is not dependent on the presence of a chromophore in the analyte and the baseline is not affected by gradient elution when volatile components are used [12,13,15]. In this study we investigated the usefulness of an ELSD to detect and quantify galacturonic acid oligomers, polygalacturonic acid and pectin during HPSEC using solutions of acetic acid and ammonium acetate as eluents.

2. Experimental

2.1. Apparatus

The HPSEC system was composed of a Perkin-Elmer Binary LC 250 Pump (Shelton, CT, USA) and either a Rheodyne (Rohnert Park, CA, USA) manual injector with a 20 μl injection loop or Perkin-Elmer Series 200 autosampler (20 μl injections). Detection of analytes was accomplished with an ESA, Model 301 ELSD (Chelmsford, MA, USA). Data collection was accomplished with an A/D converter connected to a Hewlett-Packard (Palo Alto, CA, USA) personal computer using EZ Chrome Elite software (Scientific Software, Pleasanton, CA, USA).

2.2. Chemicals and samples

The tetra-, penta- and hexamers of galacturonic acid (GA) were isolated according to Hotchkiss et al. [5]. Oligogalacturonides with a degree of polymerization (DP) of 19 and 20 were prepared according to Hotchkiss et al. [16]. Working stocks of all chromatography samples were made to 1.0% (w/v) in deionized water and contained 0.02% (w/v) sodium azide. Dilutions of these stocks were made with deionized water. All other samples and chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA) except sodium hydroxide (Fisher, Pittsburgh, PA, USA).

2.3. LC conditions

Two Tosoh Biosep TSK-Gel SE G3000PW (60× 0.75 cm, 10 μm bead size) columns (Tosoh Biosep, Montgomeryville, PA, USA) connected in series, and a Tosoh Biosep TSK-Gel Guard PWH column (7.5× 0.75 cm, 12.5 μm bead size) were used. A flow-rate of 1 ml min^{-1} was used for all chromatographic runs which were conducted at ambient temperature. The nebulizer temperature and the evaporation chamber temperature of the ELSD were set at 40 and 55 °C, respectively. The photomultiplier sensitivity was set at 600. Nitrogen was used as the carrier gas at a pressure of 138 kPa. The mobile phases used for isocratic elution were acetic acid (0–100 m*M*) or NH_4OAc (50 or 500 m*M* at pH 4.0 and pH 5.5; 25, 40, 50, 100 or 250 m*M* at pH 3.7). Dextran (M_r 580 000, Sigma–Aldrich) was used for V_0 determination. All peak area or retention time data are based on the mean of two to twelve replicates.

2.4. Colorimetric assay

The concentrations of tetra-, penta- and hexameric acid were verified by the colorimetric method of Scott [17] which uses 3,5-dimethylphenol.

3. Results and discussion

3.1. Acetic acid as the mobile phase

A flat baseline could be obtained with water as the mobile phase, however k (Fig. 1) and R_s values for mono-, di- and tri-GA were low due to repulsion of negatively charged samples by anionic groups in the column packing. Acidifying the mobile phase with 10 m*M* acetic acid resulted in a substantial decrease in sample ionization and an increase in both k (Fig. 1) and R_s values. Further increasing the acetic acid concentration resulted in increased k values although the curves plateaued above 40 m*M* acetic acid (Fig. 1) and R_s values decreased. The k values for the neutral sugar glucose were only slightly affected by the acetic acid concentration (Fig. 1), due to the lack of interaction with charged groups on the stationary phase. As a compromise between decreasing R_s and increasing k, a concentration of 40 m*M* acetic acid was chosen for further evaluation.

Although nearly identical linear calibration curves could be constructed for mono-, di- and tri-GA with 40 m*M* acetic acid (data not shown) attempts to detect high methoxy pectin and PGA were unsuc-

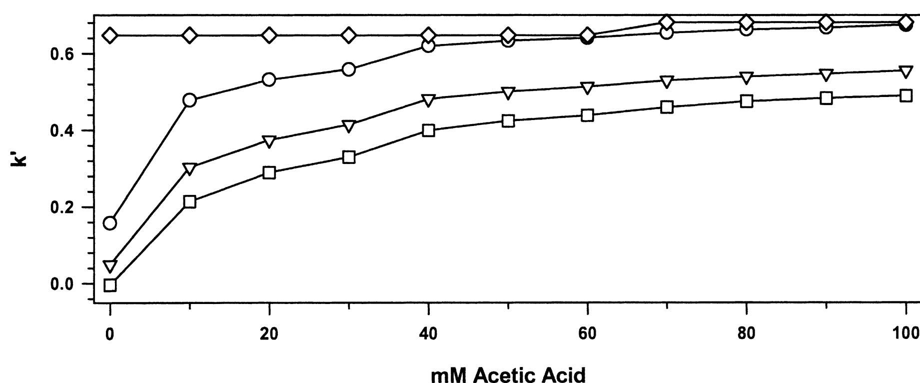

Fig. 1. Values of k for 0.1% mono-GA (○), di-GA (∇), tri-GA(□) and glucose (◊) in a 0–100m*M* acetic acid mobile phase. The guard column was changed at 70 m*M* acetic acid.

cessful. Repeated attempts for elution resulted in an increased column backpressure and plugged guard column. The addition of the 0.1% PGA to acetic acid greater than 30 m*M* in a microfuge tube produced a visible precipitate, suggesting that the PGA and high methoxy pectin precipitated in the guard column, resulting in the increased backpressure. Since precipitation of PGA and possibly larger oligomers of GA limit the use of dilute acetic acid as an eluent we extended our studies to ammonium acetate solutions as a mobile phase.

3.2. Ammonium acetate as the mobile phase

A mobile phase of ammonium acetate also produced a flat baseline at all concentrations (25–500 m*M*) and pH values (3.7, 4.0 and 5.5) tested (data not shown). It also did not cause precipitation of PGA or pectin. Baseline separation (R_s>1.3) of mono-, di- and tri-GA was achieved at all pH values and ammonium acid concentrations tested. Of the pH values tested 3.7 produced the highest R_s values and this pH was chosen for tests of a wider range of ammonium acetate concentrations. Increasing the ammonium acetate concentration from 25 to 250 m*M* resulted in increased k values and a concomitant decrease in R_s values as had been observed with dilute acetic acid (data not shown) but baseline separation on mono-, di- and tri-GA was observed in the concentration range of 25–100 m*M* ammonium acetate. A concentration of 40 m*M* was chosen for subsequent chromatography because it was an acceptable compromise between increasing k values and decreasing R_s values. Calibration curves (0.05–0.75% w/v) for mono-GA (intercept=6.5965±0.0115, slope=1.3760±0.0293, r=0.993), di-GA (intercept=6.6220±0.0124, slope=1.2861±0.0351, r=0.996) and tri-GA (intercept=6.6123±0.0418, slope=1.4592±0.1066, r=0.994) with 40 m*M* ammonium acetate as the mobile phase produced regression lines with very similar slopes. At a photomultiplier setting of 600, a 20-μl injection and the 40 m*M* ammonium acetate (pH 3.7) buffer, concentrations below 0.01% (w/v) could not be integrated. The HPSEC–ELSD calibration curves lost linearity above 0.75% (w/v). Using these calibration curves to estimate the mass of tetra-, penta- and hexamer GA, as well as the 19 and 20 DP oligomers, from chromatographic peak areas indicated they were within 12% of the expected concentration for tetra-, penta- and hexameric GA but deviated by as much as 26% for the DP 20 oligomer (Table 1). Since discrepancies were found between colorimetric and HPSEC–ELSD concentration estimates we began a search for a suitable compound to be used as an internal standard in conjunction with a mobile phase of 40 m*M* ammonium acetate, pH 3.7. Glucose could be used if an unknown sample contained no neutral sugars but would not be of use if they were present (intercept=6.4795±0.0355, slope=1.3872±0.0591, r=0.999). Compounds tested included acidic, neutral and cationic substances of low molecular mass. We were looking for a compound with an elution time between glucose (37.18

Table 1
Comparison of concentration estimates (mean±SE) for GA oligomers from a colorimetric assay and HPSEC–ELSD calibration curves obtained with 40 m*M* ammonium acetate (pH 3.7) as the mobile phase. Concentration values are in mg ml^{-1}; all samples were prepared at a concentration of 1 mg ml^{-1}

DP	Colorimetric assay	HPSEC–ELSD method		
		Mono-GA curve	Di-GA curve	Tri-GA curve
4	0.95±0.07[a]	1.12±0.04[b]	1.08±0.08[b]	1.09±0.04[b]
5	0.87±0.08[a]	1.04±0.07[b]	1.00±0.07[b]	1.02±0.06[b]
6	0.90±0.08[a]	0.94±0.15[a]	0.88±0.15[a]	0.91±0.13[a]
19	1.04±0.05[a]	0.89±0.03[c]	0.84±0.03[c]	0.87±0.03[c]
20	1.14±0.04[a]	0.78±0.01[d]	0.74±0.01[d]	0.78±0.01[d]

[a] $n=3$.
[b] $n=2$.
[c] $n=12$.
[d] $n=11$.

min) and sodium, potassium or lithium acetate (46.33–46.64 min). All neutral sugars (erythrose, fructose, arabinose and mannose) and polyols (mannitol, xylitol and erythritol) tested had k values (0.66–0.71) very close to glucose ($k=0.68$) and two polyols (ethylene glycol and 1,3-propanediol) were too volatile for the ELSD. Organic (glucuronic, aspartic, glutaric and malonic) acids had k values (0.50–0.70) close to galacturonic acid or glucose, which left only lysine ($k=0.79$) or urea ($k=0.99$) as possible candidates as an internal standard. However, lysine does not produce a symmetrical peak in this system leaving only urea (Fig. 2) as the only suitable standard so far identified. A urea calibration curve was constructed (intercept=4.8534±0.0275, slope=1.6479±0.0700, $r=0.994$) but it illustrated a more than two-fold lower detector response compared to GA oligomers, glucose and PGA.

Using 40 m*M* ammonium acetate, pH 3.7, as the mobile phase we were able to detect peaks for citrus pectin, PGA. and DP 19 and 20 oligomers (Fig. 2). Using Dextran 580 (M_r 580 000) to determine the void volume (22.07 ml) and the linear column calibration curve (log molecular mass vs. elution volume) obtained from GA oligomers 1–6 plus the GA 19mer and 20mer (intercept=8.0566±0.0490, slope=−0.1744±0.0017, $r=0.9996$), the pectin eluted as a M_r 13 700 peak and the PGA eluted at V_0 as a peak of at least M_r 15 700 peak (DP≅88). Comparison of chromatograms for a commercial citrus pectin to sodium, potassium and lithium PGA indicated the potassium and lithium PGA had undergone degradation (data not shown). Chromatography of tetra-, penta- and hexa-GA resulted in poorer resolution than observed with acetic acid as the mobile phase (Figs. 1 and 2). The k values for these oligomers in ammonium acetate were 0.34, 0.32 and 0.30, respectively. Oligomers of DP 19 and 20 both had a k value of 0.17, indicating they were retained by the column matrix.

4. Conclusion

Oligomers of GA were detected and quantified

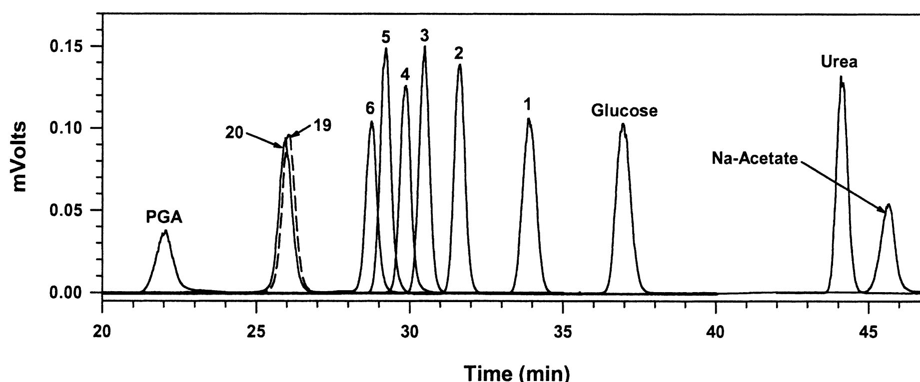

Fig. 2. Chromatographic overlay of 0.1% Na-acetate, glucose, mono-GA (1), di-GA (2), tri-GA (3), tetra-GA (4), penta-GA (5), hexa-GA (6), 19-GA (19), 20-GA (20) and PGA plus 1.0% urea in 40 m*M* ammonium acetate, pH 3.7.

using an evaporative light scattering detector coupled to HPSEC. Both PGA, medium and shorter chain oligomers could be visualized with the same buffer system. Both medium and shorter chain oligomers were retained by the column. Baseline resolution was obtained for mono-, di- and tri-galacturonic acid (M_r 194.2, 370.3, 546.4, respectively) with an ammonium acetate mobile phase. Linear calibration curves could be constructed with log transformed data from mono-, di- and tri-galacturonic acid, neutral sugars and urea. Estimates, based on peak area, of tetra-, penta- and hexa-GA (M_r 722.5, 898.6 and 1074.7, respectively) concentration calculated from mono-, di- and tri-GA calibration curves were in close agreement to weight-based and colorimetric data when ammonium acetate was the mobile phase but detector response was reduced for oligomers of DP 19 (M_r 3364) and 20 (M_r 3540; as well as PGA and pectin), which had concentration estimates up to 27% lower that weighed concentrations. Urea was found to be a suitable compound for use as an internal standard although the slope of its calibration curve was greater than that for GA oligomers or neutral sugars. Results from these studies indicate that an evaporative light scattering detector has great potential utility when detection and quantification of oligogalacturonides, for which no standard is available, is desirable.

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