Contactless Conductivity: An HPLC Method to Analyze Degree of Methylation of Pectin

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Pectin is a complex polysaccharide composed of at least five different sugar moieties where 80% to 90% of its dry weight is anhydrogalacturonic acid (AGA). The majority of the AGA is found in homogalacturonan (HG) regions of pectin as unbranched polymers of AGA in which a variable proportion of the AGA residues contain a methyl ester at their C6 position (Ridley et al., 2001; Vincken et al., 2003). Pectin’s functional properties and reactivity toward calcium and other cations are largely dependent on the amount of unmethylated galacturonic acid subunits and their distribution pattern within the HG stretches (Powell et al., 1982; Willats et al., 2001).

Thus, degree of methylation (DM) is an important parameter for characterization of pectin. Methods of analysis require time-consuming steps of purification and sample preparation and relatively large sample sizes such as the titrimetric method (Codex, 1981). In some methods a combination of two methods of analysis is required to determine DM; often these are quantitation of galacturonic acid (Blumenkrantz et al., 1973; Luzio, 2004) and measurement of liberated methanol (Klavons et al., 1986). Several methods of detection have been used in combination with liquid chromatography for characterization of pectin DM. Conductivity detection has been reported in combination with gel permeation chromatography (GPC) or HPLC of pectin DM, but this system was based on conventional electrodes in contact with the mobile phase and the use of a reference cell was not described (Ploger, 1992).

More recently capacitively coupled contactless conductivity detection (C4D) with the axial electrode configuration was introduced in 1998 as a quantification method for capillary electrophoresis (Zemann et al., 1998). Its universality allows the detection of small inorganic ions as well as organic and biochemical species. First reports on C4D in electrophoretic separations were published in the early 1980s using a radial C4D cell for the isotachophoretic determination of small anions (Gas et al., 1980). The main advantage of C4D is that the electrodes are not in contact with the mobile phase and do not degrade with time as compared to conventional conductivity probes. Due to its robustness, minimal maintenance demands, and low cost, the use of this detector has been steadily growing. Applications have recently been extended to other analytical methods such as ion chromatography, high-performance liquid chromatography (HPLC), and flow-injection analysis. A commercial unit for C4D has been introduced (Kubán et al., 2009). Contactless conductivity detection, based on an AC voltage capacitively coupled into the detection cell, is more easily implemented and a more robust alternative to conventional conductivity detection. In addition, differential units are commercially available with a reference electrode that should improve sensitivity.

The objective of this study was to develop a new method for determination of pectin DM using rapid sample analysis by means of SEC and detection by C4D and differential refractometry in series.

**Material and Methods**

**Materials.** Unless otherwise noted all chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

**Chemicals and Reagents.** Buffer for the mobile phase was 1 mM ammonium formate, pH 6.5 (no. 09735, ≥99% purity; Fluka BioChemika, Steinheim, Switzerland), in high purity deionized-
distilled water. Pectins tested were ≥85% DM (P-4516), 55% to 70% DM (P-9436), 20% to 34% DM (P-9311), and polygalacturonic acid (P-7151). Pectins were dissolved at room temperature at a concentration of 0.5% in mobile phase.

C4D and RI Analysis by SEC

Conductivity is affected by mobility, molecular size and shape, molecular charge, temperature, and is proportional to concentration. High molecular weight ions such as pectin have lower mobility that lessens the signal response relative to low molecular weight salt ions. In addition, higher DM pectins have lower charge density that also lowers response relative to lower DM pectins. The influence of pectin DM on conductivity parameters has been reported (Recape et al., 1989). Elsewhere it was found that the equivalent conductivity of the monomeric unit inserted in the polyion increased with decreasing degree of esterification (Thibault et al., 1985). Since the RI provides accurate concentration data during isocratic runs and is typically used with SEC systems, the RI was tested to determine if it could be used to calibrate the C4D against concentration effects under isocratic conditions in SEC and to observe relative responses. C4D measurement under SEC conditions provides several advantages. SEC serves to separate out low molecular weight salt ions, which introduces error into the calibration of high molecular weight pectins. In addition SEC provides a broad range of concentrations during elution for calibration. Finally SEC is done under temperature control which aids in calibration since conductivity is affected by temperature.

The SEC system consisted of a pump (Model 1000D syringe pump; ISCO, Lincoln, NE) and an inline filter (0.02-μm pore size, Anodisc 25; Whatman, Maidstone, UK). Mobile phase was purged with helium prior to loading into the syringe pump. Mobile phase flow rate was 0.60 mL/min. Samples (50 μL) were injected using an autosampler (Series 200; Perkin Elmer, Waltham, MA) onto a set of three SEC columns (PL-aquagel-OH 50 and 60, 8-μm pore size, 300 × 5.7 mm Polymer Laboratories Inc., Amherst, MA; and TSK-GEL, 10-μm pore size, 600 × 7.5 mm TOSOHaas, Montgomeryville, PA) with an operating range of 100–10,000,000 Da. The columns were connected in series (largest pore size first), enclosed in a column incubator at 27.0 ± 0.1 °C. Three detectors were present in line in the following order: Contactless Conductivity detector (C4D), multangle laser light scattering (MALLS), and differential refractive index (RI). The MALLS (DAWN® EOS, Wyatt Technologies, Santa Barbara, CA) was equipped with a K5 flow cell and a He–Ne laser light source (L = 633 nm). Prior to the measurements, the MALLS was calibrated using 0.2-mm filtered HPLC quality toluene and was used to determine number average molecular weight (Mn). The RI (Optilab® DSP, Wyatt Technologies, Santa Barbara, CA) had a P100 cell (10-mm path length) operating at 633 nm and a constant temperature of 42 °C. The Contactless Conductivity Detector (Model C4D, eDAQ Inc., Colorado Springs, CO) was equipped with a planar headstage (ET123) with contactless probes for ion chromatography/HPLC was set at a gain setting of zero, amplitude of 100 V, and frequency of 500 kHz. The C4D unit was controlled with eDAQ communication software version 1.4. Liquid flow from the pump was routed through the reference side of the planar headstage before entering the autosampler. Liquid flow exiting the last SEC column was sent through the sample side of the planar headstage which was installed in the column incubator to maintain temperature control.

The mobile phase was 1 mM ammonium formate pH 6.5 at 0.6 mL/min. Pectin solutions were micro-centrifuged at high speed before injection. Data was collected at 1.25-s intervals. Results were processed using the software provided by the manufacturer (DNDC for Windows ver. 5.90.03, and Astra for Windows ver. 4.90.07, Wyatt Technologies). Processed data were exported to a spreadsheet and adjusted for inter-detector delay volume and baseline. Pectin DM values were determined by a modified chromatography method that had been previously described (Levigne et al., 2002).

Results and Discussion

As previously noted, RI provides accurate concentration data during isocratic runs and is typically used with SEC systems, the RI was tested to determine if it could be used to calibrate the C4D against concentration effects under isocratic conditions in SEC and to observe relative responses. To test C4D response with pectin under SEC conditions 100 μL of 0.5% pectin (55% to 70% DM) was injected into the chromatography system. The C4D voltage response was compared against the RI voltage response. Figure 1 shows a typical SEC chromatogram observed for this high DM pectin. The voltage response of the C4D detector was less than the voltage response of the RI in the elution volume region (20 to 30 mL) where high molecular weight pectin elutes. In comparison, the low molecular weight salts, which elute between 30 and 35 mL showed a higher volt-

![Fig. 1. Representative curve of interferometric refractive index detector response (——) (V) without detector delay and C4D response (○○○○○○○○) (V) without detector delay relative to elution volume (mL) from SEC column of a pectin with a DM value of 54.1 (sd = 1.2). Pectin elution from 20 to 30 mL elution volume.](image-url)
age response for the C4D than the RI and this is probably a result of the higher mobility of the low molecular weight salts which results in a stronger signal response on C4D at a given concentration. Similar results were observed for other pectins tested which had lower or higher DM values (data not shown). Since these pectins were randomly deesterified, the average ester content of each molecule at a specific DM would be similar regardless of molecular weight (Mw). This has been observed with this pectin by graphing DM vs. Mw from the SEC run and observing a horizontal response (data not shown).

Relative response was measured for C4D response versus RI response during SEC using chemically deesterified pectins. Data for selected SEC runs are shown in Figure 2 for pectins tested at 94.0, 54.1, 22.1 and 0.0 DM. The 0.0 DM pectin tested was polygalacturonic acid. Data were plotted as C4D voltage vs. RI voltage without delay. As observed all plots indicate a linear response for a specific DM value. These data have been corrected for baseline and inter-detector delay volumes to the nearest 12.5 μL elution volume. Nonlinearity was observed for C4D and resulting plot indicated least squares fit to linear equation of y = –5.96 × 10⁻³x + 0.592 with r² = 0.998. High r² is consistent with selection of linear fit. Useful signals were observed for all runs and standard deviations were small when compared to the mean values. Linear fit was independent of the observation that these pectins had a wide range of Mn values from 1.48 × 10⁴ to 114 × 10⁴ as determined by MALS. In addition there was a high level of polydispersity for each polysaccharide. For example the 94.0 DM sample had a weight average molecular weight range of approximately 1 x 10⁴ to 1 x 10⁶ daltons (data not shown). These data indicate that mobility in the oscillating electrical field for the conductivity measurements (which uses alternating current) is not largely affected by the molecular sizes being studied. Finally, these data indicate that this method is useful for pectin DM determinations regardless of DM values, even high DM values such as the 94.1 % DM sample where C4D response was very small (see Fig. 2).

**Conclusion**

A method has been developed for determination of pectin DM using SEC to separate high molecular weight pectins from low molecular weight salts with detection by differential contactless conductivity and differential refractometer in series. DM can now be determined by conductivity simultaneously to measurement of molecular weight by MALLS during SEC for pectins which do not contain high molecular weight contaminants. This will be important when studying bimodal populations where DM may not be evenly distributed across populations of varying molecular weight. In addition this provides and independent facile method for determination of DM and will enable efficient analysis when confronted with large numbers of samples for DM determinations.

<table>
<thead>
<tr>
<th>Degree of methylation (%)</th>
<th>Response conductivity /RI</th>
<th>Mn (Daltons)</th>
<th>Percent error</th>
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<tbody>
<tr>
<td>0</td>
<td>0.561</td>
<td>1.48(4%)</td>
<td></td>
</tr>
<tr>
<td>22.1</td>
<td>0.62</td>
<td>81.2(1%)</td>
<td></td>
</tr>
<tr>
<td>54.1</td>
<td>0.264</td>
<td>114(0.9%)</td>
<td></td>
</tr>
<tr>
<td>94.0</td>
<td>0.031</td>
<td>24.1(2.9%)</td>
<td></td>
</tr>
</tbody>
</table>

*Table 1. Conductivity response (V) divided RI response (V) for pectins at specific degrees of methylation for triplicate runs. Data fitted in Excel to linear equation y = –5.96 × 10⁻³x + 0.592 with r² = 0.998. Degree of methylation was determined by a modified chromatography procedure (Levigne et al., 2002) using triplicate runs. Mn is weight average molecular weight determined by MALS with calculated percent error in parentheses.*
Literature Cited


