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Capillary electrophoresis of homogeneous pectin fractions

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Abstract

Capillary electrophoresis (CE) has been used to characterize pectin fractions obtained from mother samples of varying degrees (and intramolecular patterns) of methylesterification (DM). These samples have been carefully produced by fractionation, (a) from the original mother samples on the grounds of molar mass, and (b) subsequently from the daughter fractions so obtained, on the grounds of charge. The results confirm theoretical predictions regarding the dependence of electrophoretic mobility on molar mass and DM and give further evidence that CE is able to give realistic information regarding the intermolecular DM of pectins.

Keywords: Pectin; Capillary electrophoresis; Degree of methylesterification; Intermolecular DM distributions

1. Introduction

Pectin is a complex carbohydrate polymer that plays an instrumental role in regulating the mechanical properties of the plant cell wall (McCann & Roberts, 1996) and has also found great utility in many diverse areas of science and technology (May, 1990). While the detailed structure of the pectin macromolecular assembly in vivo is still a matter of debate (Vincken et al., 2003) most commercially available pectin samples can be considered as a collection of polymer chains each consisting of extended regions of homogalacturonan interspersed sparsely with regions of rhamnogalacturonan I (Ralet & Thibault, 2002). Even at this level of description complexity and heterogeneity abound. In particular, the distribution of methylesterification both among chains and along individual polymer backbones is a key determinant of molecular functionality. Indeed, cell wall enzymes routinely tailor DM distributions in order to exploit structure–function relationships based on the dependence of molecular association on the pattern of methylesterification (Willats et al., 2001). The measurement of such distributions is then vital in order to understand the role that fine structure modifications play in determining the functionality of pectin both in vivo and in vitro. It is envisaged that ultimately useful pectin structure–function models will not just incorporate information on DM distributions, but will require it in order to correctly predict the properties of pectin samples.

Capillary electrophoresis has recently been shown to be a useful tool for the investigation of pectin methylester distributions (Jiang, Wu, Chang, & Chang, 2001; Ström & Williams, 2004; Williams, Buffet, & Foster, 2002a,b; Williams, Foster, & Schols, 2003; Zhong, Williams, Keenan, Goodall, & Rollin, 1997; Zhong, Williams, Goodall, & Hansen, 1998). Most simply it can be used in order to measure the average DM of a pectin sample, since there is a linear relationship between the electrophoretic mobility and the average charge per residue (Zhong et al., 1997, 1998). While many other methods perform this sample averaged DM measurement equally well (Bédouet, Courtois, & Courtois, 2003; Huisman, Oosterveld, & Schols, 2004; Lévigne, Thomas, Ralet, Quéméner, & Thibault, 2002; Maness, Ryan, & Mort, 1990; Massiot, Perron, Baron, & Drilleau, 1997; Synytsya, Copikova, Matejka, & Machovic, 2003; Rosenbohm, Lundt, Christensen, & Young, 2003), an advantage of the electrophoretic method is its inherent separation quality. For chains with lengths in excess of around 15 residues a symmetrical scaling of charge and

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hydrodynamic friction co-efficient with the degree of polymerisation (DP) is found. This means that larger polymeric chains, regardless of their DP, elute according to their average charge density and, therefore, that each CE migration time marks species with a unique DM. Peak shapes thus reflect the intermolecular methylesterification distribution (the DM distribution among chains) of the sample (Williams et al., 2003).

It is worth noting that the validity of such a methodology for obtaining intermolecular DM information from CE hinges crucially on two major assumptions. Firstly, that contributions to peak widths arising from chromatographic factors are small compared to the breadth of the intermolecular mobility (charge) distribution, and secondly that the mobility is not significantly dependent on the intramolecular distribution of charge (methylesterification), so that the migration behaviour is determined simply according to the chain-averaged charge density. The first point has been largely addressed by observing the invariance of peak widths to changes in injection time, monitoring the co-injection and subsequent resolution of discrete samples, and performing calculations of the relative contributions expected from the relevant band-broadening mechanisms (Zhong et al., 1997, 1998). The second point has been addressed by studying commercial pectin samples fractionated according to their calcium sensitivity (Zhong et al., 1998; Williams et al., 2002a) and, more recently, home-made samples molecularly engineered to have a random or block wise DM distribution by exploiting demethylesterification using chemical saponification in contrast to processive enzymes (Williams et al., 2003). It was concluded that there was no statistically significant difference between the electrophoretic mobilities of pectins of equivalent DM with differing intramolecular arrangements of methylesterification. It is interesting to note that recent work using Ion Exchange Chromotography (IEC) has also suggested that the elution can be largely governed by charge density independently of charge distribution patterns (Ralet & Thibault, 2002).

While the success of the CE work to date implies that indeed the electrophoretic mobility is independent of the molecular weight (above DP ~ 15); that is, polymeric chains of the same DM will elute together regardless of their length, here we explicitly investigate this experimentally by studying samples fractionated solely on the grounds of molar mass. Furthermore, while the co-injection and subsequent separation of pectin mixtures has provided strong evidence that the CE peak width is a reasonable reflection of the intermolecular DM distribution, collecting and re-injecting fractions from the leading and following edges of a single peak has not been possible owing to the small sample volume that can be collected from a single CE run. In this work, however, in the same spirit, we have taken pectin fractions homogenous in molar mass, and subsequently fractionated these samples again, now on the grounds of charge (using IEC). These samples, homogenous with respect to both mass and charge, have then been further studied by CE.

2. Experimental

2.1. Materials

A commercial pectin (L72) from Mexican lime peel (*Citrus aurantifolia*), with a DM of 72 was esterified in acid-methanol medium to give a pectin (E81) of a DM of 81. A series of pectins with defined DM were prepared by enzymatic treatment of E81 as fully described previously (Limberg et al., 2000). F-series samples were prepared using a fungal pectin methylesterase (f-PME) from *Aspergillus niger* purified from Pektolase™ (Danisco, Brabrand, Denmark) and P-series samples were prepared using a plant pectin methylesterase (p-PME) purified from orange peels as described by Christensen, Nielsen, Kreiberg, Rasmussen and Mikkelsen (1998). Those pectin samples (mother samples) were fully characterised in a previous study (Ralet, Dronnet, Buchholt, & Thibault, 2001). Mother samples (F69, F58, F43 and P60) were fractionated by Size Exclusion Chromatography on a column (92 × 5 cm) of Sephacryl S-500 to give daughter samples. A detailed description of chromatography conditions and physico-chemical characterisation of recovered daughter samples has been published elsewhere (Ralet & Thibault, 2002). The intermediate size fractions (pectin-S500(3)), representing the bulk of the samples, were further purified by Ion Exchange Chromatography on DEAE-Sepharose CL-6B to give granddaughter fractions as fully described elsewhere (Hellin, Ralet, Bonnin, & Thibault, in press). The isolation procedure of daughter and granddaughter samples is summarised in Fig. 1.

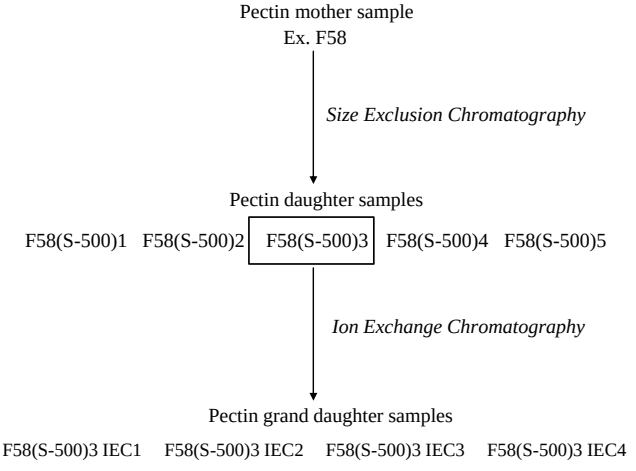

Fig. 1. Schematic diagram of the fractionation procedures employed for the production of homogeneous samples.

2.2. DM determination

For the mother and daughter samples, carboxylic functions were quantified at the neutralisation point by conductimetric titrations with three bases (KOH, LiOH, $Ca(OH)_2$) of known molarity and total carboxylic functions by colorimetry (Thibault, 1979) on the same solutions after saponification. DM was calculated as:

$$\mathrm{DM} = 100 \times (\text{total carboxylic functions} - \text{free carboxylic functions})/\text{total carboxylic functions}$$

For the main granddaughter fraction, DM was calculated after HPLC determination of methanol released by alkaline de-esterification of pectins (Levigne et al., 2002). Isopropanol was added as an internal standard and DM was calculated as the molar ratio of methanol to galacturonic acid.

2.3. Capillary electrophoresis

Experiments were carried out using an automated CE system (HP 3D), equipped with a diode array detector. Electrophoresis was carried out in a fused silica capillary of internal diameter 50 μm and a total length of 46.5 cm (40 cm from inlet to detector). The capillary incorporated an extended light-path detection window (150 μm) and was thermostatically controlled at 25 °C. Phosphate buffer at pH 7.0 was used as a CE background electrolyte (BGE) and was prepared by mixing 0.2 M Na_2HPO_4 and 0.2 M NaH_2PO_4 in appropriate ratios and subsequently reducing the ionic strength to 50 or 90 mM. At pH 7.0 the unmethylated galacturonic acid residues are fully charged and while the polymers are susceptible to base-catalysed β-elimination above pH 4.5, no problems for DM determination were encountered during the CE runs of some 20 min at room temperature, as assessed by the good agreement between DM values obtained by CE and other techniques. Some small reduction is average molecular weight cannot be ruled out by the CE experiments alone, and indeed it is the insensitivity of the technique to polymer DP that allows information on the intermolecular DM distribution to be extracted, as is discussed in detail herein. However, it is worth noting that we see no evidence for the generation of any oligomeric species. All new capillaries were conditioned by rinsing for 30 min with 1 M NaOH, 30 min with a 0.1 M NaOH solution, 15 min with water and 30 min with BGE. Between runs the capillary was washed for 2 min with 1 M NaOH, 2 min with 0.1 M NaOH, 1 min with water and 2 min with BGE. Detection was carried out using UV absorbance at 191 nm with a bandwidth of 2 nm. Samples were loaded hydrodynamically (various injection times at 5000 Pa, typically giving injection volumes of the order of 10 nL), and typically electrophoresed across a potential difference of 20 kV. All experiments were carried out at normal polarity (inlet anodic) unless otherwise stated. Electrophoretic mobilities, μ, are related to the migration times of the injected samples relative to a neutral marker, t and t_0, respectively, by the equation:

$$\mu = \mu_{obs} - \mu_{eo} = (lL/V)(1/t - 1/t_0) \quad (1)$$

where L is the total length of the capillary, l is the distance from the inlet to detector, V is the applied voltage, μ_{obs} is the observed mobility and μ_{eo} is the mobility of the electroosmotic flow (EOF) (Grossman & Colburn, 1992; Weinberger, 2000). All these anionic polysaccharides migrated after the neutral marker. The observed mobility μ_{obs} is the vector sum of μ_{eo} and μ and since μ is negative and smaller in magnitude than μ_{eo} the anions having the most negative mobility have the smallest μ_{obs} and thus the longest migration times.

3. Results and discussion

Figs. 2–4 show the DM distributions measured by CE for the fractions obtained from samples F43, F58 and F69,

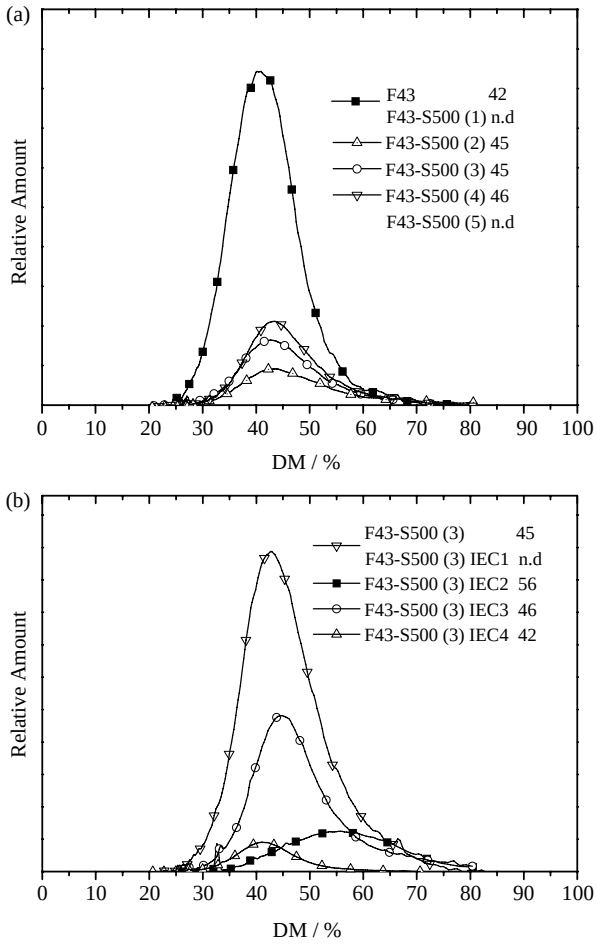

Fig. 2. Intermolecular distributions of the degree of methylesterification measured by CE from fractions obtained (a) from the sample F43 on the grounds of molar mass and (b) from the fraction F43-S500(3) using IEC. The number average degrees of methylesterification are shown.

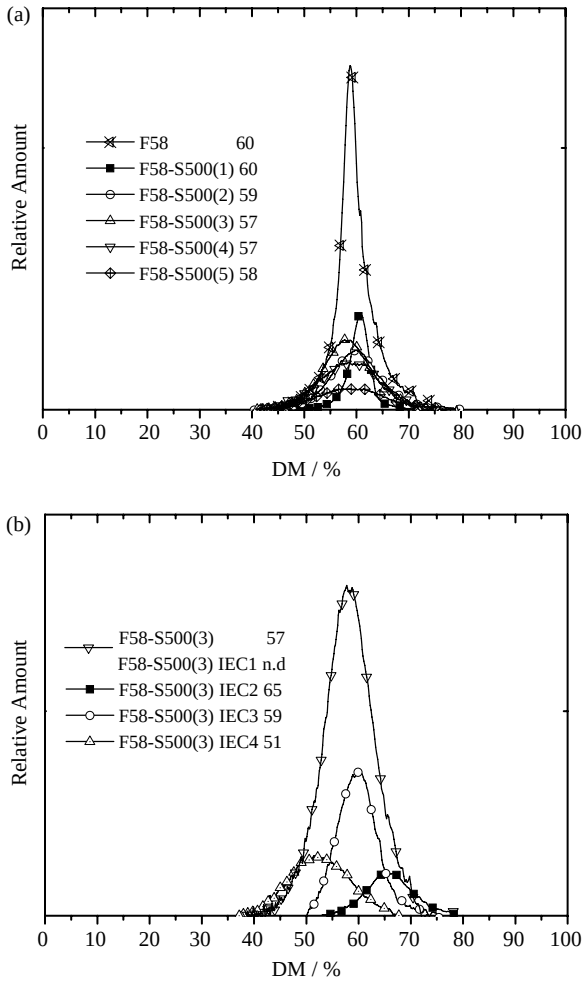

Fig. 3. Intermolecular distributions of the degree of methylesterification measured by CE from fractions obtained (a) from the sample F58 on the grounds of molar mass and (b) from the fraction F58-S500(3) using IEC. The number average degrees of methylesterification are shown.

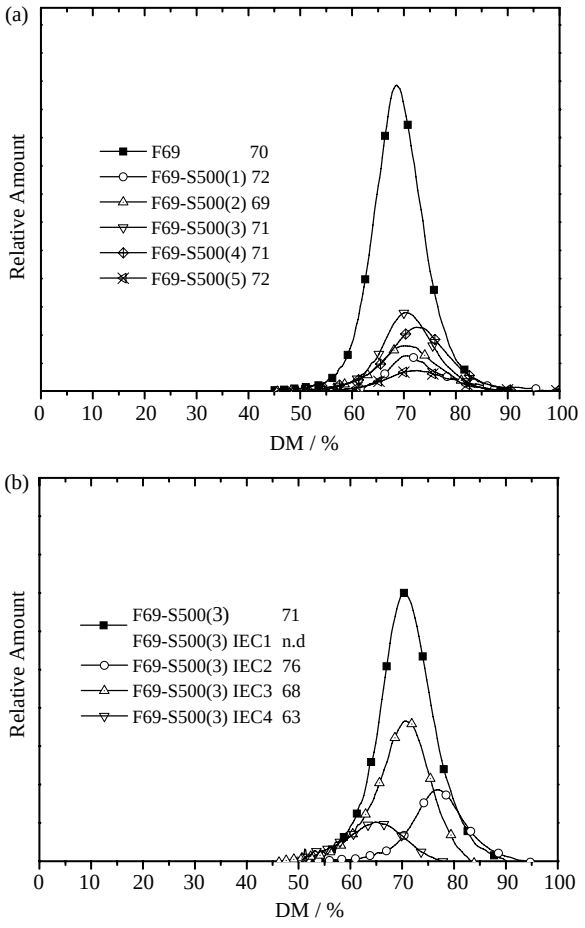

Fig. 4. Intermolecular distributions of the degree of methylesterification measured by CE from fractions obtained (a) from the sample F69 on the grounds of molar mass and (b) from the fraction F69-S500(3) using IEC. The number average degrees of methylesterification are shown.

respectively: (a) results obtained from the mother sample and daughter samples obtained by fractionation on the grounds of molar mass, and (b) results obtained from a single molar mass fraction and granddaughter fractions obtained by further fractionation on the grounds of charge, as described in Section 2. Briefly, the distributions are obtained by normalizing the electropherogram absorbance according to the migration time (Goodall, Williams, & Lloyd, 1991), and transforming migration time via electrophoretic mobility (using Eq. (1)) to degree of methylesterification. This is discussed in detail elsewhere (Williams et al., 2003). Following this data processing, the relative amount of each fraction has been scaled by the yield of that component, in order to facilitate the comparison of the sum of the fraction distributions with that of the mother sample. From the distributions obtained a number average DM was calculated for each sample and is shown in Table 1, compared with the values obtained by titrimetry or HPLC, as described in Section 2. In a small number of cases (indicated by n.d) a fraction was not successfully run in CE owing to lack of material or problems with dissolution. These fractions all had a minimal yield ($<10\%$).

It can be seen from the table that the number average degrees of methylesterifiaction for all samples are in excellent agreement between techniques. Furthermore, it is clear from the figures that, in all cases, the yield weighted sum of the CE determined distributions of the component fractions give reasonable approximations to the distributions of the original samples from which they were fractionated. These facts alone give strong supporting evidence that the distributions are good representations of the real DM distributions of the samples. However, the work undertaken here allows us to explicitly investigate the underlying assumptions of the methodology. Specifically, it can be seen from Figs. 2–4(a) that separating the mother, fungal demethylesterified, pectin samples on the grounds of molecular weight gave fractions that had similar intermolecular distributions of the degree of methylesterification. This provides explicit evidence supporting the conjecture regarding the negligible dependence of

Table 1
The number average degree of methylesterification (DM) of the pectin samples F43, F58, F69 and P60; and fractions obtained (i) from these mother samples on the grounds of molar mass (S-500(1-5)) and (ii) from the homogeneous molar mass fraction 3 on the grounds of charge (S-500(3) IEC1-4). Uncertainties are estimated at around 2%

Samples	DM Titrimetry or HPLC/%	DM CE/%	M_w (1000 g/mol)
F43	42[a]	42	109[a]
F43 S-500(1)	46[b]	n.d.	279[b]
F43 S-500(2)	45[b]	45	158[b]
F43 S-500(3)	45[b]	45	109[b]
F43 S-500(4)	45[b]	46	82[b]
F43 S-500(5)	41[b]	n.d.	59[b]
F43 S-500(3) IEC1	n.d.	n.d.	n.d.
F43 S-500(3) IEC2	n.d.	56	n.d.
F43 S-500(3) IEC3	45[c]	46	47[c]
F43 S-500(3) IEC4	n.d.	42	n.d.
F58	57[a]	60	117[a]
F58 S-500(1)	61[b]	60	300[b]
F58 S-500(2)	59[b]	59	152[b]
F58 S-500(3)	57[b]	57	104[b]
F58 S-500(4)	58[b]	57	69[b]
F58 S-500(5)	57[b]	58	54[b]
F58 S-500(3) IEC1	n.d.	n.d.	n.d.
F58 S-500(3) IEC2	n.d.	65	n.d.
F58 S-500(3) IEC3	59 [c]	59	44 [c]
F58 S-500(3) IEC4	n.d.	51	n.d.
F69	67[a]	70	117[a]
F69 S-500(1)	70[b]	72	268[b]
F69 S-500(2)	67[b]	69	133[b]
F69 S-500(3)	68[b]	71	108[b]
F69 S-500(4)	68[b]	71	89[b]
F69 S-500(5)	69[b]	72	81[b]
F69 S-500(3) IEC1	n.d.	n.d.	n.d.
F69 S-500(3) IEC2	n.d.	76	n.d.
F69 S-500(3) IEC3	70[c]	68	72[c]
F69 S-500(3) IEC4	n.d.	83	n.d.
P60	59[a]	62	94[a]
P60 S-500(1)	68[b]	68	280[b]
P60 S-500(2)	60[b]	65	117[b]
P60 S-500(3)	58[b]	62	67[b]
P60 S-500(4)	61[b]	64	50[b]
P60 S-500(5)	60[b]	63	36[b]
P60 S-500(3) IEC1	n.d.	74	n.d.
P60 S-500(3) IEC2	n.d.	66	n.d.
P60 S-500(3) IEC3	58 [c]	59	47 [c]
P60 S-500(3) IEC4	n.d.	53	n.d.

All missing samples had minor yields (<10%). n.d., not determined.
[a] Values from (Ralet, Dronnet, Buchholt, & Thibault, 2001).
[b] Values from (Ralet & Thibault, 2002).
[c] Values from (Hellìn et al., in press).

electrophoretic mobility on degree of polymerization under the conditions utilized in these experiments. This can be seen in contrast to Figs. 2–4(b), that clearly show that samples obtained by fractionation on the grounds of charge do migrate differently as expected, and indicate that indeed the leading edge of peaks contains material less anionic in nature (higher DM), while trailing parts of the peaks contain more highly anionic polymers.

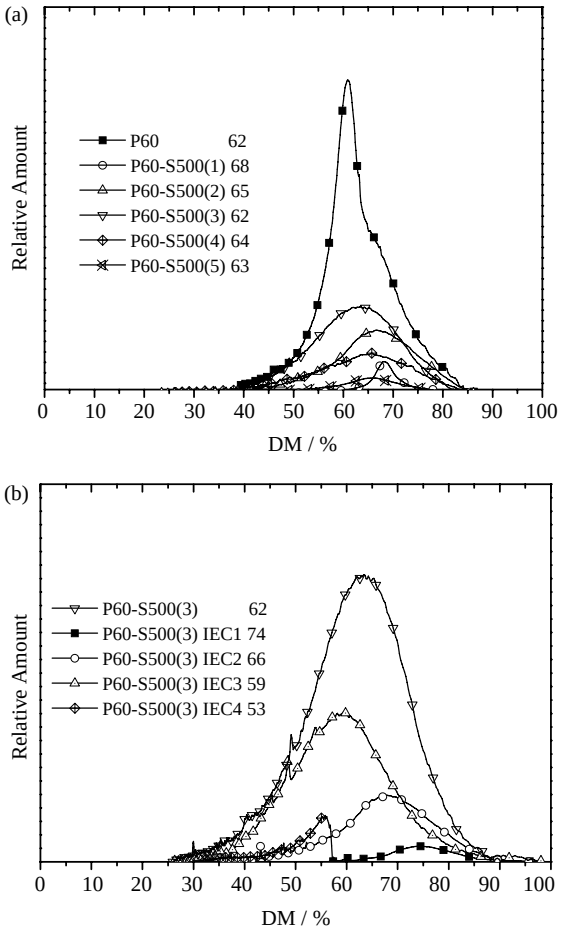

Fig. 5. Intermolecular distributions of the degree of methylesterification measured by CE from fractions obtained (a) from the sample P60 on the grounds of molar mass and (b) from the fraction P60-S500(3) using IEC. The number average degrees of methylesterification are shown.

Fig. 5 shows distributions obtained from CE ((a) and (b) as for Figs. 2–4) for fractions derived from a mother pectin, P60, that has been produced by demethylesterification using a p-PME, as described in Section 2. It is observed that, in general, these samples gave significantly broader DM distributions than those produced with fungal PME. This effect of broader peak widths in the intermolecular DM distribution being generated by more processive enzymes has been observed previously by both CE and by IEC (Williams et al., 2003; Ralet & Thibault, 2002) and is believed to be a consequence of the enzymatic mode of action. Once again the number average degrees of methylesterification for all samples are in good agreement between techniques (Table 1) and the yield weighted sum of the CE determined distributions of the component fractions give reasonable approximations to the distributions of the original samples from which they were fractionated.

However, in contrast to the minimal effect of molar mass based fractionation observed for all the components generated by fungal PME, for the sample demethylesterified

with plant PME the fraction with the largest molar mass clearly has a significantly higher DM than the other factions (Fig. 5(a) and Table 1). It should be noted that this was also found to be the case as measured by titrimetry. The results from the fungal pectin samples clearly suggest that it is not simply that the larger molar mass fractions appear somehow to be more methylesterified owing to their molar mass, but rather support the hypothesis that the highest molar mass fraction obtained from the P60 sample actually does have a greater DM than the other fractions. Further supporting evidence comes from the high DM shoulder visible in the distribution of the mother sample in Fig. 5(a), that is clearly consistent with the presence of a component with higher DM.

Perhaps the simplest hypothesis to explain this is that the higher molar mass material in the original sample has a subtly different fine structure (possibly involving branching or side-chains) so that the pectin–plant PME interaction is affected in a detrimental fashion leaving this fraction of the sample less demethylesterified than the rest when the desired sample average DM is reached and the reaction is curtailed. Such an explanation relies on the fungal PME not exhibiting similar behaviour. We postulate that this might reflect differences in the subsite architecture of the enzymes, in particular the tolerances of the binding and/or catalytic action for certain fine structural features. How such differences might be manifest would also depend on the rates of reaction under which the samples were demethylesterified. If the plant PME reaction was comparatively fast and processive then it is more likely that the effect of any fine structural features unfavourable for the enzyme–pectin interaction will be magnified compared with a comparatively slower non-processive demethylesterification in the case of the fungal enzyme.

The sample characterisation carried out in this work supports that carried out previously and gives further evidence of the usefulness of CE in the characterization of pectin samples. Using careful fractionation procedures to obtain relatively homogeneous sample properties has enabled theoretical predictions with regard to the dependence of electrophoretic mobility on molar mass and degree of methylesterification to be investigated experimentally. Furthermore, by studying sample sets originating from mother pectins with different patterns of methylesterification it has been confirmed that information on the intermolecular DM distribution of pectin samples can be obtained independently of their intramolecular organization.

The fact that the higher molecular weight fraction of P60 has been found to be of a higher DM, in agreement with previous work, is interesting and suggests (i) that the fine structure of the high molecular weight fraction would be an interesting target for further investigation and (ii) that the p-PME sensitivity of pectin fractions could be an interesting fine-structure discriminant.

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