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Capillary electrophoresis studies of pectins

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Abstract

Capillary electrophoresis (CE) has been used to study a variety of pectins that originate from different sources (citrus, apple and beet) and have different anhydrogalacturonic acid (AGU) contents (86.2–63.2%). This CE method can be used for the quantitative detection of aqueous solutions of pectins in the concentration range 0.5–5 mg mL^{-1} using a 50 mM pH 7.0 phosphate background electrolyte and UV detection at 192 nm. Using lemon pectin calibration standards in the degree of esterification (DE) range 31.1–75.8%, the technique was used to determine the DE of a further 11 pectin samples. Results are shown to be in excellent agreement with those obtained by titration, irrespective of the pectin type and neutral sugar content. The method also allows determination of the intermolecular DE distribution (variation in DE between molecules), from the CE peak shape. Results for the DE distribution are compared with those obtained by ion-exchange chromatography in combination with size-exclusion chromatography (IEC-SEC). Both DE and DE distribution are obtained from CE in approximately 2 h, inclusive of sample preparation and calibration, in contrast to 2 days for the IEC-SEC method.

Keywords: Pectin; Degree of esterification; Capillary electrophoresis; DE distribution

1. Introduction

Pectin is a biopolymer found in the cell walls of many living plant tissues and is composed mainly of GalA residues. Rha is an additional minor constituent of the pectin backbone and other neutral sugars such as Ara, Gal and Xyl are commonly found in side-chains. Chains typically consist of 200–1000 GalA units linked together by α-(1→4)-glycosidic bonds and occur in varying degrees of esterification (DE). It is well known that pectin can be used to form aqueous gels, and this property is commonly exploited by the food industry [1]. In terms of gelling properties pectin can be divided broadly into two categories, high DE and low DE. The characterisation of the DE distributions of pectin samples is thus of primary importance in understanding and controlling the nature of their gels and is of great commercial relevance.

At present the classical titration method [2,3] is still the official method used for the determination of DE [4–6]. Alternatives involve the liberation of methanol by de-esterifying enzymes or by acid or alkali treatments, and subsequent quantification of the methanol by chromatography [7,8]. Recently methods have been described using size-exclusion

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chromatography (SEC) and a dual detection system monitoring refractive index and conductivity [9,10]. The carboxylate density, defined as mmole of carboxyl groups per gram of pectin, is calculated by comparison with calibration standards of known composition. The conversion into DE, however, requires that the AGU of the sample is known.

Chromatographic methods for obtaining the intermolecular DE distribution (variation in DE between molecules) involve binding pectin onto an ion exchange column under conditions of low ionic strength and subsequent elution of the pectin with a buffer, the concentration and ionic strength of which is gradually increased. Fractions are collected and classically the pectin mass in the column eluates has been determined by a colorimetric assay with meta-hydroxy diphenyl (MHDP) while the DE has been inferred from the elution time [11].

However, it should be noted that the ion-exchange chromatography (IEC) method is not without its problems. Pectins with blockwise intramolecular DE distributions are found to elute from the column in an irregular manner, which is potentially a serious problem, as the intramolecular charge distribution will be polydisperse to some degree even in a sample in which all chains possess the same average charge per residue. The binding of pectin to the column will be a function of its degree of polymerization since the equilibrium constant for the binding of a polyanionic molecule to a polycationic IEC stationary phase depends on electrostatic interactions summed over all groups. In summary, it is an oversimplification to assume that the IEC method discriminates purely on the grounds of average molecular charge density.

In an attempt to correct for such problems approximately 30 eluates from the IEC were analysed by SEC with a dual detection system monitoring refractive index and conductivity [10]. In this method the DE of the eluates is actually measured, using a calibration linking refractive index/conductivity ratio to DE, developed using pectin standards, rather than inferred from the IEC elution time. However, the eluates from IEC may still be polydisperse with respect to DE and therefore the DE distribution derived from the average DE values is still an approximation.

Electrophoretic methods can provide information complementary to SEC. An early application to pectic molecules used analytical scale paper electrophoresis and preparative column electrophoresis [12]. The focus of this paper was on cell-wall polysaccharide structure and information on the DE distribution was not obtained since all samples were initially de-esterified. Rapid and routine methods for the determination of the DE distribution of pectin samples, that provide reliable results regardless of the polydispersity in intramolecular charge distribution and molecular weight, are of great potential importance. Unlike many other natural polysaccharides, pectins possesses both charge and UV chromophore, which are prerequisites for the direct use of free solution capillary electrophoresis (CE) [13–15]. We have recently presented a preliminary study using CE as a rapid method for the quantitative analysis of aqueous solutions of pectins in the concentration range 0.5–5 mg mL^{-1}. The technique also permits the determination of DE since there is a linear relationship between the electrophoretic mobility and the average charge per residue, calculated as $z = -(100 - \mathrm{DE})/100$. For a fixed charge the mobility was found not to depend significantly on the intramolecular pectin charge distribution so that CE has the advantage, in contrast to other methods, that no problems are encountered with pectins possessing a blockwise charge distribution.

The aims of this paper are twofold. Firstly, to test the CE method on a range of pectin samples that have varied neutral sugar contents and secondly, to investigate the possibility of obtaining intermolecular DE distributions directly from CE peak shapes.

2. Experimental

Samples.—All pectin samples were supplied and initially characterised by Copenhagen Pectin A/S. Fractions of varying DE were obtained via de-esterification using the enzyme pectin esterase of *Aspergillus*. The details of all samples used in this study are given in Table 1 (lemon peel pectins used as calibration standards) and 2 (all other samples).

Characterisation.—Preliminary characterisation was carried out using the titration method referred to in the introduction [2,3]. In addition, selected samples were also subjected to analysis by an IEC-SEC method. The IEC used a Protein-Pac column (14.5 cm long and 13 mm internal diameter) and a Waters 600 gradient pump. The concentration of the buffer (CH_3COONa, containing 0.1%

Table 1
Characteristics of the pectins available for calibrations

Source	AGU / % w/w	DE / %
Lemon	84.5	75.8
Lemon	85.1	70.3
Lemon	89.0	55.8
Lemon	86.4	47.2
Lemon	85.7	42.6
Lemon	85.2	31.1

C_6H_5COONa, adjusted to pH 4.40) was varied from 0.025 to 3.0 M. The sample loading was 70 mg in 20 mL of 0.025 M buffer. The flow and linear gradient were set to 2 mL min^{-1} and 0.01 M mL^{-1}, respectively, and 7 mL fractions were collected. After application of the sample the column was pre-flushed with 80 mL of 0.025 M buffer before starting the gradient and between runs cleaning was carried out with 50 mL of 0.10 M NaOH. The subsequent SEC of the eluates used a Chrompack P300 column with a 0.075 M CH_3COONa buffer at pH 4.40. 200 μL were injected and a flow rate of 1.3 mL min^{-1} was used. Detection was via conductivity and refractive index measurements. Data collection and analysis were carried out using Waters *Millenium* software.

Capillary electrophoresis.—All samples were supplied as powders and solutions were prepared by heating in de-ionised water at 60 °C for 30 min. All sample solutions were 2.5 mg mL^{-1} unless otherwise stated. A phosphate buffer at pH 7.0, used as the CE background electrolyte (BGE), was prepared by titrating aqueous 50 mM NaH_2PO_4 with 1 M NaOH. At pH 7.0 pectin ($pK_a = 3 - 4$) is fully charged, and although pectin is susceptible to base-catalysed β-elimination above pH 4.5, no problems were encountered during run times of between 5 and 20 min in the CE capillary.

Experiments were carried out on an automated CE system (Beckman P/ACE 5000), equipped with a diode array detector (DAD), thermostatted at 30 °C. A fused silica capillary (Composite Metals, Hallow, UK) of internal diameter 100 μm and a total length of 57 cm (50 cm from inlet to detector) was used. All new capillaries were conditioned by rinsing for 30 min with a 0.1 M NaOH solution. Between runs the capillary was washed for 2 min with 0.1 M NaOH, 1 min with water and finally 2 min with BGE solution. Detection was carried out using UV absorbance at 192 nm with a bandwidth of 5 nm. The DAD spectra confirmed that all CE peaks analysed corresponded to pectin. Samples were loaded hydrodynamically (10 s at 3450 Pa, giving an injection volume of 190 nL), and electrophoresed across a potential difference of 15 kV. All experiments were carried out at normal polarity (inlet anodic) unless otherwise stated. Electrophoretic mobilities, μ, were determined from migration times of the pectins and a neutral marker, t and t_o, respectively, using the equation:

$$\mu = \mu_{obs} - \mu_{eo} = \left(\frac{lL}{V}\right)\left(\frac{1}{t} - \frac{1}{t_o}\right) \qquad (1)$$

where L is the total length of the capillary, l the distance from the inlet to detector, V the applied voltage, μ_{obs} the observed mobility and μ_{eo} the mobility of the electroosmotic flow (EOF) [13]. The neutral marker position was taken as that of an observed absorbance dip resulting from the passage of water (from the injected sample plug) past the detection window. This was confirmed by studies with mesityl oxide as a UV absorbing neutral marker. In CE experiments carried out under normal polarity, as in this work, anionic polysaccharides, such as pectin, migrate after the neutral marker. The observed mobility, μ_{obs}, is the vector sum of μ_{eo} and μ (eq (1)) and although μ is negative it is smaller in magnitude than μ_{eo}.

3. Results and discussion

Fig. 1 shows pectin mobility, calculated as described in the preceding section, plotted versus the average charge per residue, z, (calculated as $z = -(100 - DE)/100$), for a set of well characterised lemon peel pectins of varying DE. These particular three samples were selected based on our previous observation that the resultant CE peaks can be clearly resolved [16]. Hence, their mobilities could be obtained from a single electropherogram recorded using a mixture of the three pectins, each of concentration 1.7 mg mL^{-1}. This simultaneous injection methodology ensured a time-efficient calibration. The electrophoretic mobility is seen to scale linearly with the average charge per residue over the range studied. In our preliminary communication no statistically-significant difference was found between the electrophoretic mobility of samples of the same DE, irrespective of the intramolecular distribution of esterified groups or GalA content.

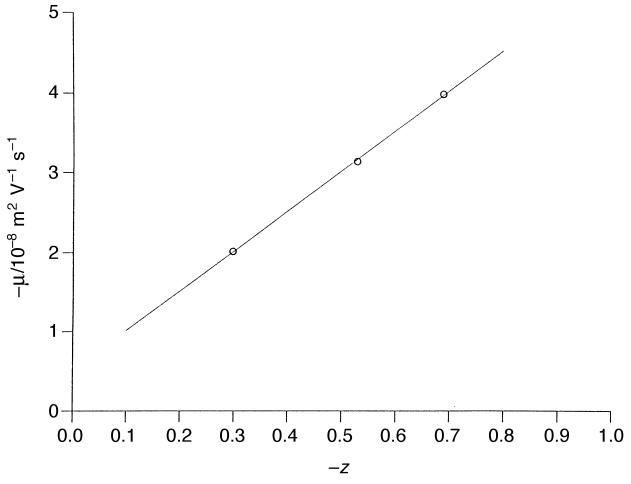

Fig. 1. Pectin mobility versus the average charge per residue, z, (calculated as $z = -(100 - \mathrm{DE})/100$), measured for a set of well-characterised lemon peel pectins of varying DE (○). The solid line is the result of a linear regression analysis

To study the effect of variation of neutral sugar content, which varies according to pectin source, Table 2 shows results from citrus, apple and beet pectins. The quoted uncertainty values are 95% confidence limits based on single runs, calculated from the uncertainties in the calibration (that is, uncertainties in values of the quantities that parameterise the relationship between μ and z). This analysis assumes that the uncertainty in the measurement of the mobility of an unknown pectin sample is negligible compared with the uncertainties arising from the subsequent calculation of DE. Triplicate runs of the same sample gave mobilities reproducible to within 1%. The simple, rapid calibration and analysis described here already yields uncertainties comparable to those obtainable by other methods (the titration results are typically found to have 95% confidence limits in the region of $\pm 1.7\%$, based on five operators each performing four repeat experiments). The precision of the CE method could be improved further, though at the expense of time, by using more points in the calibration.

All DE values obtained by the CE method were found to be in good agreement with those determined by the suppliers using the titration method. This is particularly interesting as these pectins have a wide range of neutral sugar contents. Electrophoretic mobility is determined by the charge to size ratio in the free solution mode of CE used in these experiments, and the results imply that the inclusion of further neutral sugars, indicated by a reduction in AGU, does not significantly alter the hydrodynamic properties of the molecule.

From a pragmatic point of view this invariance of electrophoretic mobility to neutral sugar content is extremely useful. It should be noted that this study encompasses a broad range of pectin types and it is not expected that other commercially-relevant pectins would have an AGU outside the region of study. Well-defined standards can be used for calibration and subsequently the DE of pectins from any source with any neutral sugar content and intramolecular charge distribution can be determined. Indeed, the determination can be successfully carried out without recourse to these properties of the sample.

Intermolecular DE distributions can be determined from detailed analysis of the CE peaks. The distributions are derived from the CE data directly from the electropherogram as follows: (i) the time variable was converted via mobility, and subsequently z, into DE and (ii) the absorbance values were normalised by dividing the amplitude of each point by the corresponding migration time, to account for the differing lengths of time spent in the detector [17]. The effect on the peak shape of a

Table 2
Degree of esterification (DE) of pectin samples measured by titration, capillary electrophoresis and ion-exchange/size-exclusion chromatography (IEC-SEC). Samples labelled (a) and (b) are pre and post treatment with the enzyme polygalacturonase

Source	AGU / % w/w	Titration DE / %	CE DE / %	IEC-SEC DE / %
Lemon	86.2	66.1	65.2 ± 2.9	67.9
Lemon	85.0	70.0	70.0 ± 2.0	—
Lemon(a)	83.7	72.7	72.0 ± 2.0	73.0
Lemon(b)	83.6	72.5	72.0 ± 2.0	73.0
Lemon	82.4	75.6	75.4 ± 1.8	75.8
Lemon	80.8	71.0	72.6 ± 1.9	72.8
Lemon:orange 1:1	74.2	57.8	60.1 ± 2.1	—
Lemon:orange 1:1	73.6	59.8	61.5 ± 2.1	—
Lemon:orange 1:1	72.5	63.1	63.1 ± 3.0	—
Beet	64.6	55.4	55.8 ± 2.0	—
Apple	63.2	68.4	68.8 ± 2.0	—

possible variation in UV absorption coefficient as a function of DE was investigated, since in our preliminary CE study a slightly lower normalised peak area was found, for a given pectin concentration, for a 70.3% DE pectin compared to a 31.1% DE sample [16]. However, the application of such a correction to a representative peak was found to make no significant difference to the peak shape and it was therefore deemed to be unnecessary.

Fig. 2 shows DE distributions as measured by CE and by IEC-SEC, obtained from lemon peel pectin samples (Table 2) of average DE (a) 71.0, (b) 66.1 and (c) 75.6. The distributions obtained by CE and IEC-SEC were independently normalised to give the area under the curves as 100%. In order to compare the distributions obtained by the different methods the magnitude of the IEC/SEC maximum was taken as unity and the CE peak was scaled appropriately in order to obtain the same area under the graph in both cases.

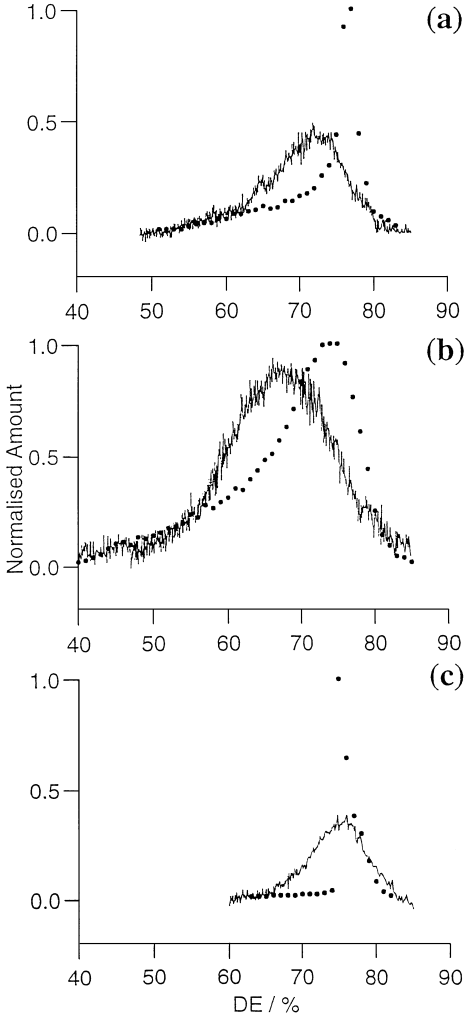

Fig. 2. Peak shapes as measured by CE (—) and by IEC-SEC (•) obtained from lemon peel pectin samples of DE (a) 71.0 (b) 66.1 and (c) 75.6. The IEC/SEC maximum has been set to unity and the CE peak scaled appropriately in order to obtain the same area under the graph in both cases

In order to confidently interpret the CE peak width it is important to calculate the contributions that are characteristic of the CE separation [13]. The total variance of the observed CE peak width can be decomposed in the following manner:

$$\sigma^2_{obs} = \sigma^2_D + \sigma^2_{inj} + \sigma^2_T + \sigma^2_{DE} \quad (2)$$

where σ^2_{obs} is the observed variance, σ^2_{DE} is the variance due to the DE distribution, and σ^2_D, σ^2_{inj} and σ^2_T are variances resulting from diffusion, injection length and sample heating respectively [13]. In order to be confident that the peak shape reflects the real DE distribution, σ^2_D, σ^2_{inj} and σ^2_T must be shown to be small in comparison to σ^2_{obs}. As shown in the Appendix, the sum of these contributions, $\sigma^2_D \sim 10^{-7}\,m^2$, $\sigma^2_{inj} \sim 5 \times 10^{-4}\,m^2$, and $\sigma^2_T \sim 10^{-9}\,m^2$, is of the order of 3% of the observed variance, $\sigma^2_{obs} \sim 5 \times 10^{-2}\,m^2$, so that it is fair to assume that the width is predominantly determined by the polydispersity of the charge to size ratio within a particular pectin sample.

Table 2 gives the average DE values of the pectin samples, calculated from the measured IEC-SEC DE distribution. This calculation involves averaging the IEC-SEC distribution in the following manner:

$$\overline{DE} = \sum_{i=1}^{n}(w_i \times DE_i)/\sum_{i=1}^{n} w_i \quad (3)$$

where w_i is the mass of a fraction with degree of esterification DE_i and n is the number of fractions. Strictly, this type of analysis should also be applied in the determination of average DE from the distributions determined by CE. However, owing to the near symmetrical nature of the CE peaks, values calculated in this manner were expected to be close to those obtained simply from the position of the peak maxima. Preliminary investigations showed that indeed the difference between two such values was negligible and so each value determined by CE presented in Table 2 was derived from the position of the peak maximum.

It can be seen from Table 2 that the results of the average DE obtained by titration, IEC-SEC and CE methods are all in good agreement, for the three samples whose distributions are shown in Fig. 2. It has been demonstrated that the results

obtained by the CE method are unaffected by differences in intramolecular charge distribution or neutral sugar composition and furthermore that it is not necessary to know these properties of the sample to obtain the DE. The spans of the DE distributions predicted by CE and IEC-SEC are also in good agreement (IEC-SEC points not shown were zero readings). For measuring the span the CE method has the advantage of being significantly easier and considerably faster.

There is, however, a considerable difference in the shapes of the distributions as determined by CE and IEC-SEC. Two further pectin samples were examined, one of which had been treated with the enzyme polygalacturonase (PG). The enzyme attacks the pectin chain, hydrolysing glycosidic linkages next to free carboxyl groups, and producing small fragments (DP 1–3) of low DE which are subsequently lost during precipitation of the treated sample. These samples are detailed in Table 2 as pre (a) and post (b) enzyme treatment. The DE distributions obtained are shown in Fig. 3. It is interesting to note that the IEC-SEC method shows a significant change in the distribution with PG treatment, in contrast to the CE result.

An explanation of this disagreement could be that the somewhat broader CE peak widths are not determined predominantly by the intermolecular DE distribution, but by some other factor. Other major peak broadening factors, however, have been discussed in the Appendix and found not to make a significant contribution. All evidence suggests that the CE peak shapes should indeed be a good reflection of the intermolecular DE distribution.

Alternatively, the IEC-SEC distributions may be significantly affected by other properties of the pectin sample. It should be noted that the average DE measured by all three methods is unchanged by the enzyme treatment, as shown in Table 2. This must indicate that the lost low DE sections were not large enough fractions of the pectin chains from which they were cleaved to significantly alter the average charge per residue of the remnants. (Otherwise the average DE would have been observed to be substantially raised). The fact that, despite this finding, the IEC-SEC method shows a distinct change in the DE distribution casts doubt on interpreting changes solely in terms of average molecular charge density.

At pH 4.4 (used throughout the IEC-SEC work) the pectin samples may not be completely ionised.

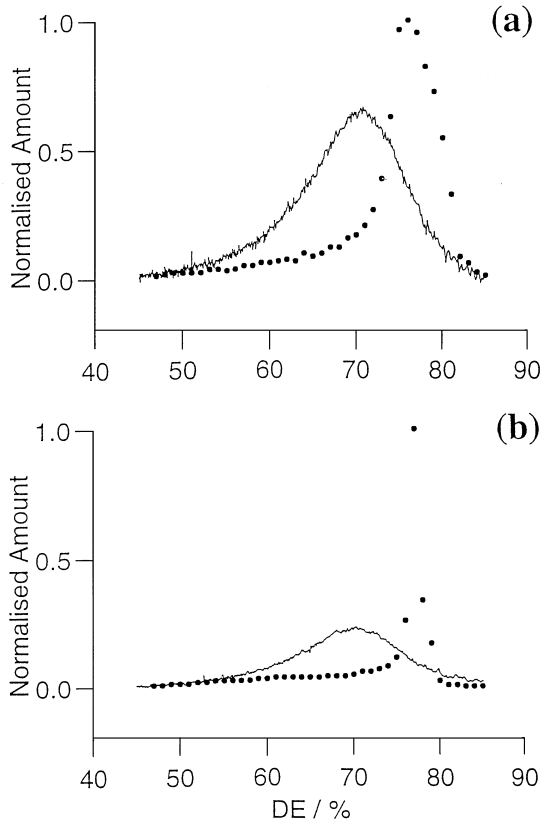

Fig. 3. Peak shapes as measured by CE (—) and by IEC-SEC (•) obtained from a lemon peel pectin (a) untreated and (b) treated with polygalacturonase. The IEC/SEC maximum has been set to unity and the CE peak scaled appropriately in order to obtain the same area under the graph in both cases

The proton dissociation of these polyacids is complex [18] and the apparent pK_a values are themselves a function of DE and depend to some extent on the intramolecular charge distribution. However, identical results have been obtained at pH 5.5 so that the interpretation of IEC results is not thought to be complicated by the pH conditions.

Further conclusive evidence that the CE peak width is a measure of the real DE distribution could be obtained by collecting fractions from the CE peak and reanalysing selected eluates. This would, however, require a signal to noise ratio far in excess of that routinely obtained with UV absorbance. Appropriate levels could be achieved in future work by derivatising the sample with a fluorescent label and using laser induced fluorescence (LIF) detection [19,20]. Alternatively, the use of other detection methods could be investigated, for example monitoring refractive index [21,22].

4. Conclusions

It has been demonstrated that capillary electrophoresis provides a simple, rapid method for the quantitative detection and separation of pectins in aqueous solution in the concentration range 0.5–5 mg mL^{-1} according to degree of esterification. This is true irrespective of the intramolecular charge distribution and neutral sugar content. Furthermore, this technique also allows the direct measurement of the intermolecular DE distribution. The method has a number of advantages over the more conventionally used IEC-SEC method, including a vast improvement in speed (2 h; c.f. 2 days), ease of use, and economy of materials.

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APPENDIX

The contribution to the peak width resulting from diffusion of the analyte out of the sample zone during migration can be readily calculated from the well known one dimensional diffusion equation $\sigma_D^2 = 2Dt$, where D is the diffusion coefficient and σ_D^2, which is the mean square distance travelled, is the variance of the resultant diffusion profile. Once the variance has been calculated then the peak width at half height can be obtained simply by assuming a Gaussian peak shape. Even if the diffusion coefficient is as large as 10^{-10} m^2 s^{-1}, which is unlikely for these large biomolecules, then the predicted peak width at half height is of the order of 1 s (after conversion from distance to time using the velocity of the analyte). This is negligible compared to the observed pectin peaks, which are typically in the region of 120 s.

The variance due to the finite injection volume is $\sigma_{inj}^2 = l^2/12$ where l is the injection plug length [13]. Thus, an injection volume of 190 nl in a 100 μm i.d. capillary gives a variance of the order of 1% of that observed experimentally. Indeed, peak widths were not found to be significantly altered when the injection time was reduced from 10 to 7 or 5 s.

The band broadening contribution resulting from the presence of a temperature profile within

the capillary was also calculated using the equation [13]:

$$\sigma_T^2 = \frac{r^6 E^6 \kappa_e^2 \Omega_T^2 \mu_{obs}^2 t}{1536\,D\,k_b^2} \quad \text{(A1)}$$

where r is the internal radius of the capillary, E the electric field strength, κ_e and k_b are the electrical and thermal conductivities of the buffer respectively, Ω_T the temperature coefficient of the electrophoretic mobility, μ_{obs} the observed electrophoretic mobility, t the migration time, and D the diffusion coefficient of the analyte. For a 100 μm diameter capillary, with typical experimental conditions: $E = 2.6 \times 10^4\,V\,m^{-1}$, $\kappa_e = 0.87\,\Omega^{-1}\,m^{-1}$ (calculated from the measured current), $\Omega_T = 0.02\,K^{-1}$, $k_b = 0.6\ W\,m^{-1}\,K^{-1}$ and $D = 10^{-10} m^2\,s^{-1}$ the resultant calculated peak width at half height is of the order of 10 ms, which again is negligible.