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Determination of average degree of polymerisation and distribution of oligosaccharides in a partially acid-hydrolysed homopolysaccharide: A comparison of four experimental methods applied to mannuronan

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Abstract

The average degree of polymerisation (DP) and distribution of oligosaccharides in partially acid hydrolysed mannuronans were quantitatively evaluated by ^{1}H NMR, electrospray ionisation mass spectrometry (ESI-MS), micellar electrokinetic capillary chromatography with UV detection (MEKC-UV), and high-pressure anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Our investigation shows that ^{1}H NMR, MEKC-UV and, in particular, HPAEC-PAD can be used as quantitative tools to aid the investigation of polysaccharide structure, function and synthesis. For the latter two techniques, especially, this represents a significant new development as it enables calculation of the quantity of individual oligomers of nominal DP by direct analysis of a defined oligomer mixture. Appropriate statistical averages of number and weight distributions were also calculated and found to fit very well to predicted Kuhn distributions that assume random depolymerisation.

Keywords: Degree of polymerisation; Alginate; Oligosaccharides; Mannuronan

1. Introduction

Measurement of the average degree of polymerisation (DP) and distribution of oligosaccharides is of fundamental significance in aiding the elucidation of metabolism, structure, synthesis and function of complex carbohydrates. Some analytical techniques, such as osmometry and light scattering, provide number and weight average DPs, respectively. When combined with online size-exclusion chromatography (SEC), light scattering has the potential to give the chain length distribution of complex mixtures, but may suffer from low sensitivity in the low DP range. Generally, SEC has low resolution and usually can not separate individual oligomeric components. A number of other analytical techniques are potentially able to overcome such problems, but their application has so far only been exploited to a limited degree. Examples of such techniques are high-pressure anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) [1–6], capillary electrophoresis (CE) [7,8] and electrospray ionisation mass spectrometry (ESI-MS) [9,10]. As yet, however, none of these techniques have been extended so that the number and weight average DP and distribution of individual oligosaccharides can be fully quantified. Consequently, an evaluation is needed to identify which of the current available techniques are most appropriate for this task.

The present work attempts to do this for four techniques in the case of a partially acid-hydrolysed mannuronan, a bacterial alginate containing exclusively β-1,4-linked mannuronic acid residues. Standards of nominal DP 2–12 were obtained by preparative gel-filtration, and their identity, purity and

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composition evaluated by ^{1}H NMR and ESI-MS. These parameters were further evaluated by micellar electrokinetic capillary chromatography with UV detection (MEKC-UV) and HPAEC-PAD, and then used as a supplement to NMR data as a basis for assessment of the chain length distribution of oligomannuronic acid mixtures obtained after partial acid hydrolysis. We test the hypothesis that quantification is possible for at least three of the evaluated methods.

2. Materials and methods

2.1. Materials

4-Aminobenzonitrile (ABN) and 3-(trimethylsilyl)-propionic-2,2,3,3,-d_4 acid Na salt (TSP) were from Aldrich (St. Louis, MO, USA); H_3BO_3 was from Carlo Erba (Milan, Italy); NH_4Ac, NaAc, and Na_2SO_4 were from SDS (Peypin, France); ^2H_2O and NaO^2H were from Chiron (Trondheim, Norway); HCl was from Merck (Darmstadt, Germany); H_2O Milli-Q (18Ω) was from a Millipore water purifier, (Molshelm, France); all other reagents were from Sigma (St. Louis, MO, USA).

2.2. Preparation of high molecular weight mannuronan

High-molecular weight mannuronan was isolated from the fermentation broth of a mannuronan C-5 epimerase (AlgG) negative strain of *Pseudomonas fluorescens*. Purification and deacetylation were carried out as described earlier [11]. No guluronote signals could be detected by ^{1}H NMR (molar fraction $F_G < 0.001$), indicating that homopolymeric mannuronan had been formed [12].

2.3. Partial acid hydrolysis

Two hundred and fifty milligrams of mannuronan was dissolved in 100 ml H_2O and the solution adjusted to pH 5.6 with 0.1 M HCl, prior to de-oxygenation with nitrogen, followed by incubation at 95 °C for 3.5 h to undergo pre-hydrolysis. The sample was then cooled, pH adjusted to 3.5 with 0.1 M HCl, degassed and hydrolysed as before but for either 3, 5 or 8.5 h (hydrolysis times quoted hereafter refer to these times). After this time samples were again cooled, neutralised by with 0.1 M NaOH, and either freeze-dried directly or, in the case of the sample hydrolysed for 3 h, first dialysed against H_2O to remove the smaller oligomers (DP < 6) and free Na^+ and Cl^-.

2.4. Preparation of purified oligomers

Oligomannuronic acid hydrolysis mixtures (50–200 mg) were chromatographed on two columns of preparative grade Superdex 30 (2.6 cm × 95 cm, serially connected) at a flow rate of 0.8 ml/min with 0.1 M NH_4Ac (pH 4.5) at room temperature. An on-line refractive index (RI) detector (Shimadzu RID-6A) was used to measure the relative concentration of oligomers eluting from the column. Fractions of 4 ml were collected from three successive column runs and pooled on the basis of peaks identified from the RI profile. Fractions from the bottom third of any peak were discarded. These pooled samples were initially stored at 4 °C prior to two cycles of freeze-drying to remove all traces of NH_4Ac and to eventually provide solid purified oligomannuronic acid standards (F2–F12) in the H^+-form.

2.5. ^{1}H NMR

Standards or hydrolysis mixtures (5 mg) were dissolved in a 5 mm diameter sample tube in 600 μl ^2H_2O, and the pH adjusted with NaO^2H to 6.8. One per cent TSP in ^2H_2O (5 μl) was added as an internal reference standard. One-dimensional ^{1}H NMR spectra were recorded on a Bruker DPX 400 spectrometer at 90 °C. Spectra were obtained using a 30° pulse angle, a spectral width of 4789 Hz, a 32 K data block size, and 64 scans after eight dummy scans. No pre-saturation was used. The ratio of total integrated H-1 signals to integrated H-1 signal from the reducing ends was used to determine the number average degree of polymerisation (DP_n), which is defined as [13]:

$$DP_n = \frac{\sum_{i=1}^{n} n_i DP_i}{\sum_{i=1}^{n} n_i}$$

where n_i is the number of moles of an oligomer with i residues, and DP_i is the corresponding DP.

2.6. ESI-MS

Standards (1 mg) were dissolved in 1 ml 50% MeOH/H_2O containing 1% NH_3 and 120 μM of *p*-nitrophenyl-β-D-glucuronide (internal standard). Mass spectra of these oligomers were recorded in negative ion mode on an API-I PE SCIEX quadrupole mass spectrometer equipped with an articulated ion spray connected to a syringe pump for sample injection. The injection flow rate was 0.1 ml/h and the electrospray voltage was −5000 V. The mass spectra of the standards were recorded on successive days in order to exclude contamination in the flow-injector from the previous sample. The analysed molecular weight (m/z) range was 150–2400. Considering the standards contained traces of other oligomers ±1 DP, evaluation of the amount of oligosaccharide in each fraction was carried out by MEKC-UV before calculating the molar response factors (MRFs). These were calculated by: $MRF_x = (I_x/I_{i.s.})/([x]/[i.s.])$ where I is the absolute peak intensity and i.s. the internal standard. Molar response factors obtained for F2–F7 were fitted to a linear function ($MRF = 0.184 - 0.022x$, $R^2 = 0.986$). These values were then employed to calculate DP_n.

2.7. MEKC-UV

Standards (1 mg/ml) or hydrolysis mixtures (4 mg/ml) were derivatised with 0.5 M ABN in the presence of 0.16 M $NaCNBH_3$ in 1 ml MeOH/CH_3COOH (95/5) for 15 min at 90 °C. These preparations were then diluted five times with H_2O and filtered through a 0.2 μm pore size membrane prior to injection into the capillary electrophoresis system. The system consisted of an Applied Biosystems HPCE Model 270A-HT which comprised an on-column UV detector and a hollow silica column 72 cm in length, i.d. 50 μm and o.d. 375 μm, cut from a 10 m long fused, polyimide coated, silica tube (Supelco, St. Louis, MO, USA). The window for UV detection was prepared by burning 0.5 cm of the polyimide coating off the silica column 22 cm from the column outlet. Before sample injection, a 4-min conditioning of the capillary with the running buffer (660 mM H_3BO_3, pH 8 containing 75–100 mM SDS) was necessary, preceded by a 2-min wash with 0.1 M NaOH at vacuum pressure 67.6 kPa. Samples were loaded under vacuum at a pressure of 16.9 kPa for 1.5 s and all runs performed at 30 °C at 18 kV. Detection of derivatised sample was made by continuously monitoring UV absorbance at 285 nm and data collected and processed by Turbochrom Navigator 4.0 software. Quantification of the amount of each standard was calculated by dividing the integrated areas of identified peaks by their migration time (A/t). Response factors were independent of chain length in the range of 2–12. Calibration was linear between a sample concentration of 0.2 and 6 mM. Limits of detection were in the μM range and evaluated using the method suggested by Miller & Miller [14].

Once the relative content of each oligomer had been determined, we calculated DP_n as before, and in addition the weight average DP (DP_w), which is defined as [13]:

$$DP_w = \frac{\sum_{i=1}^{n} w_i DP_i}{\sum_{i=1}^{n} w_i}$$

where w_i is the mass of an oligomer with i residues, and DP_i is the corresponding DP.

Given that the relative content of each oligomer had been determined, the number and weight distributions where directly obtained. The number distribution is the relative number of oligomers (mole fraction) corresponding to each DP:

$$X(i) = \frac{n_i}{\sum_{i=1}^{n} n_i}$$

Accordingly, the weight distribution is the relative weight of oligomers (weight fraction) corresponding to each DP:

$$W(i) = \frac{w_i}{\sum_{i=1}^{n} w_i}$$

The experimentally obtained distributions were compared to the theoretical distributions (Kuhn distributions) corresponding to a random depolymerisation of linear polymers, for which the following equations apply [13]:

$$W(i) = i\alpha^2(1-\alpha)^{i-1}$$

$$X(i) = \alpha(1-\alpha)^{i-1}$$

Here, α is the number of broken linkages divided by the total number of linkages originally present. Moreover, $\alpha = DP_n^{-1}$.

2.8. HPAEC-PAD

A stock solution of 1 mg/ml GalAα(1 → 4)GalA was prepared in H_2O for use as an internal standard. Oligomannuronic acid standards (1 mg) were dissolved in 1 ml of 0.15 M NaOH and rapidly diluted 100-fold with H_2O/10 μl internal standard stock. Hydrolysis mixtures (1 mg) were dissolved in 1 ml H_2O, containing 10 μl of internal standard stock if the results were to be used for quantitation. All samples were injected via a 25 μl loading loop. An additional experiment confirmed exposure to such alkaline conditions did not cause the oligomers to undergo β-elimination before injection. The chromatography unit consisted of a Dionex BioLC system (Sunnyvale, CA, USA) coupled to a Dionex AS50 autosampler. The HPLC system was equipped with an IonPac AS4A (4 mm × 250 mm) anion-exchange column connected to an IonPac AG4A (4 mm × 50 mm) guard-column. Chromatography was performed at room temperature and at a flow rate of 1 ml/min. Buffer A was 0.15 M NaOH, prepared from a carbonate-free 50% (w/w) NaOH solution, and buffer B was 1 M NaAc in 0.15 M NaOH (buffer A). The buffers were continuously degassed and maintained under helium pressure by a Dionex degassing module. Linear gradients of acetate were produced to elute the samples by increasing the concentration of buffer B from 0 to 100% over 80 min. Column effluent was monitored with a pulsed amperometric detector on an Au working electrode and Ag/AgCl reference electrode. The sequence of potentials applied to the electrode were: $E1 = 0.05$ V (480 ms, integrating from 280 to 480 ms), $E2 = 0.6$ V (120 ms), and $E3 = -0.8$ V (300 ms) at a sensitivity of 100 nC. Data acquisition and integration was performed using Dionex PeakNet software.

In order to take into account each oligomannuronic acid standard contained significant amounts of oligomers with ±1 DP, MRFs were determined for each standard in the DP range of 2–9 by using eight linear equations with eight unknowns. First, a unique RF (f_x) was assigned to each DP by $f_x = (A_x/A_{\text{i.s.}})(m_{\text{i.s.}}/m_x)f_{\text{i.s.}}$, where m (total moles injected) $= m_{\text{DP}-1} + m_{\text{DP}} + m_{\text{DP}+1}$ (sum of moles for each DP found in the sample). It then goes that $m_x = (A_x/A_{\text{i.s.}})m_{\text{i.s.}}f_{\text{i.s.}}(1/f_x)$. By setting $f_{\text{i.s.}} = 1$ (arbitrary) an equation is derived for each DP:

Monomer : $A = a_1x_1$

Dimer : $B = b_1x_1 + b_2x_2 + b_3x_3$

Trimer : $C = c_2x_2 + c_3x_3 + c_4x_4$

etc. . . . where $A = (m_{\text{total}}/m_{\text{i.s.}})(1/f_{\text{i.s.}})$ (for monomer), B is same for dimer, given $a_1 = (A_1/A_{\text{i.s.}})$ and $x_1 = (1/f_1)$.

Solving these equations yields individual response factors. Another and simpler approach was to define $A = (A_{DP-1} + A_{DP} + A_{DP+1})$, which in practice turned out to yield essentially the same result as the rigorous method. The reason being the errors introduced by including the DP − 1 and DP + 1 peaks are of the same magnitude and have opposite signs, and therefore effectively cancel out.

After determining MRFs for F1–F9 their dependence on DP were described by fitting the data to an exponential function ($MRF = 0.9793DP^{0.8399}$, $R^2 = 0.9939$). This relationship was used to calculate MRF for oligomers of higher DP values, as well as for the monomer (DP = 1). These MRFs were then used to calculate the chain length distributions of oligomers from the hydrolysis mixtures. In accordance with observations made by other workers [1,15–18] we also fitted our data to an asymptotic function ($MRF = A \cdot DP^{-1} + B$).

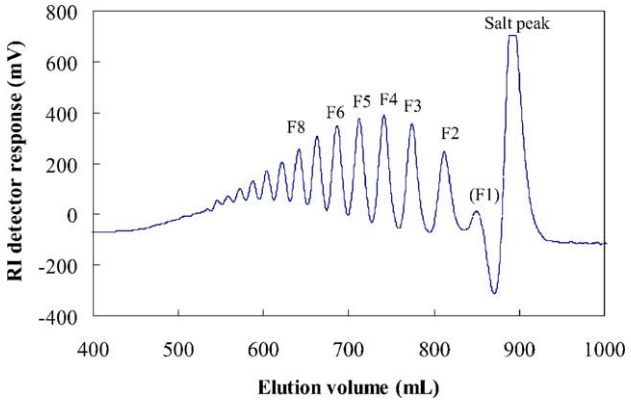

Fig. 1. Preparative Superdex 30 chromatography of a 3 h partially hydrolysed mannuronan eluted with 0.1 M NH_4Ac at 0.8 ml min^{-1}. The eluent was monitored on-line with a refractive index detector.

3. Results and discussion

3.1. Preparation of oligomannuronic acid standards

Preparative gel-filtration chromatography of partially acid-hydrolysed mannuronan on Superdex 30 yielded a series of distinct and well-resolved peaks (Fig. 1) similar to previous work on other homopolysaccharide oligomer mixtures [19]. Excluding the salt peak, 14 distinct peaks were resolved with areas roughly representative of the amounts of oligomer. Each peak (F2–F12) was presumed to represent pure oligomer from dimer to dodecamer consecutively with only negligible contributions from homologues one monomer unit apart. The monomer fraction (F1), however, was not studied further because it partially co-eluted with the salt peak. Some separations were also performed using 0.05 M Na_2SO_4 as the mobile phase in accordance with previous work [20] resulting in slightly better resolution (data not shown). On the other hand, sulphate salts are considerably more difficult to remove than the volatile NH_4Ac, especially at low DP were dialysis leads to substantial loss

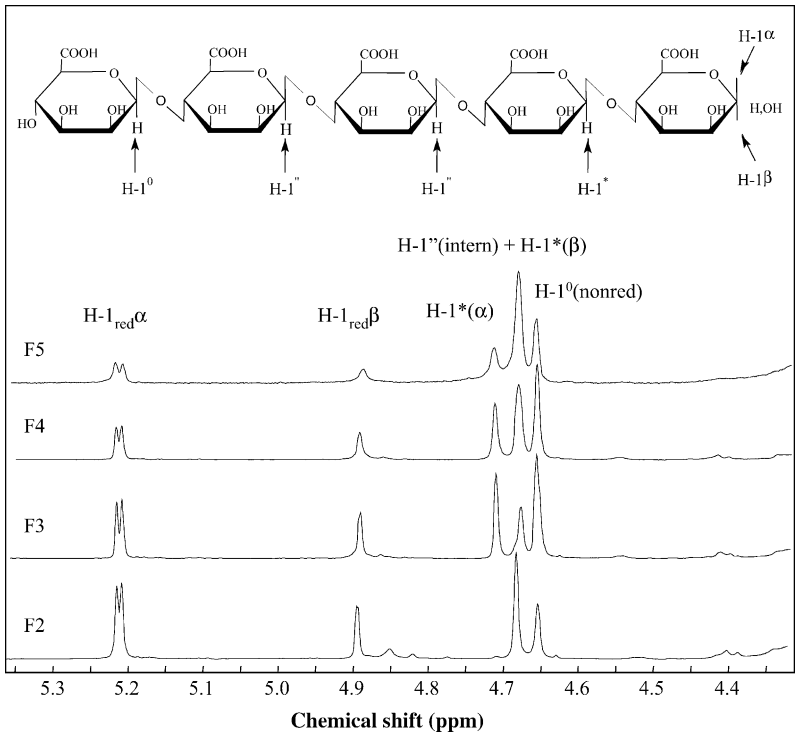

Fig. 2. H-1 region of a 400 MHz ^1H NMR spectra of oligomannuronic acid standards F2–F5 obtained from a 3 h partial hydrolysis. The prefixes 'red, non-red and intern', respectively denote the reducing end, non-reducing end and internal residues. The numbers correspond to the proton assigned to the signal.

of oligomers, and therefore the use of Na_2SO_4 was precluded in favour of NH_4Ac. In this respect, gel-filtration offers some advantages over the preparative purification of homopolysaccharide oligomer mixtures by anion-exchange chromatography, utilising gradients of non-volatile salts as counter ions [2,21]. Yet in principle, optimised preparative anion-exchange chromatography may offer a complementary [22] or alternative method to gel-filtration [23].

3.2. ^{1}H NMR analysis

The H-1 region of the ^{1}H NMR spectrum obtained for standards F2–F5 is shown in Fig. 2. Fraction F2 gives rise to four signals only. The two anomeric proton signals at 5.21 ppm (H-$1_{red}\alpha$) and 4.89 ppm (H-$1_{red}\beta$) have previously been assigned for mannuronic acid oligomers [12], and are also found in the spectra of F3–F5. The resonance at 4.68 ppm has the same intensity as the H-1α signal of the reducing end, and the resonance at 4.65 ppm has the same intensity as the H-1β signal of the reducing end. Taken together these results unequivocally show that F2 primarily comprised the ManAβ(1 → 4)ManA dimer where the two additional resonances originate from H-1 of the non-reducing end, each corresponding to the α- and β-anomer, respectively. It then follows by induction that the Superdex fractions F3–F5 principally correspond to the trimer, tetramer and pentamer, respectively, whereas F1 corresponds to the monomer. The identities of F3–F5 were confirmed by further analysis of the NMR spectra, where two novel peaks in the anomeric region occurred for DP > 2. The resonance at 4.71 ppm has for all DPs, the same intensity as the H-1α resonance, and is therefore assigned to the H-1 of the residue adjacent to the reducing end of the α-anomer. H-1 of the non-reducing end now appears as an apparently single resonance at 4.65 ppm because the influence of the reducing end on its chemical shift is negligible. The remaining internal H-1 resonances, including that of the residue adjacent to the reducing end of the β-anomer, overlap to form an apparently single resonance at 4.68 ppm.

For each oligomeric preparation, DP_n was obtained as the ratio between the total H-1 intensities to the total intensities of the H-1 signals from the reducing ends (Table 1). The estimated DP_n values were not integers, but in most cases slightly below the theoretical value. Consequently, these fractions did not comprise pure oligomers, but were slightly contaminated with predominantly smaller oligomers. The proportions of minor oligomers, however, could not be determined by this method. Furthermore, if DP_n exceeds 20, estimation is less accurate due to the low proportion of reducing ends.

Table 1
Estimated DP_n of oligomannuronic acid standards F2–F5 obtained after 3 h of hydrolysis

Method	Standard			
	F2	F3	F4	F5
^{1}H NMR	1.85	2.81	3.86	5.15
MEKC-UV	1.97	2.96	3.93	4.70
HPAEC-PAD	1.92	2.97	3.97	4.98
ESI-MS	1.86	2.71	3.72	4.35

3.3. ESI-MS analysis

The negative ion mode mass spectra of standards F2–F5 display signals attributable to the main deprotonated oligomers $[M–H]^-$ (where M is the main solute molecule) and adducts with $Na[M–2H + Na]^-$ or $K[M–2H + K]^-$ (Fig. 3). The most abundant oligomer in the standards corresponds to the most abundant deprotonated ion, such as that at m/z 720.9 in standard F4. Also, in the same standard lower and higher homologues are present at 369.0 (DP 2), 545.1 (DP 3) and 897.3 (DP 5) with the latter present in only trace amounts (Fig. 3). Similar results were

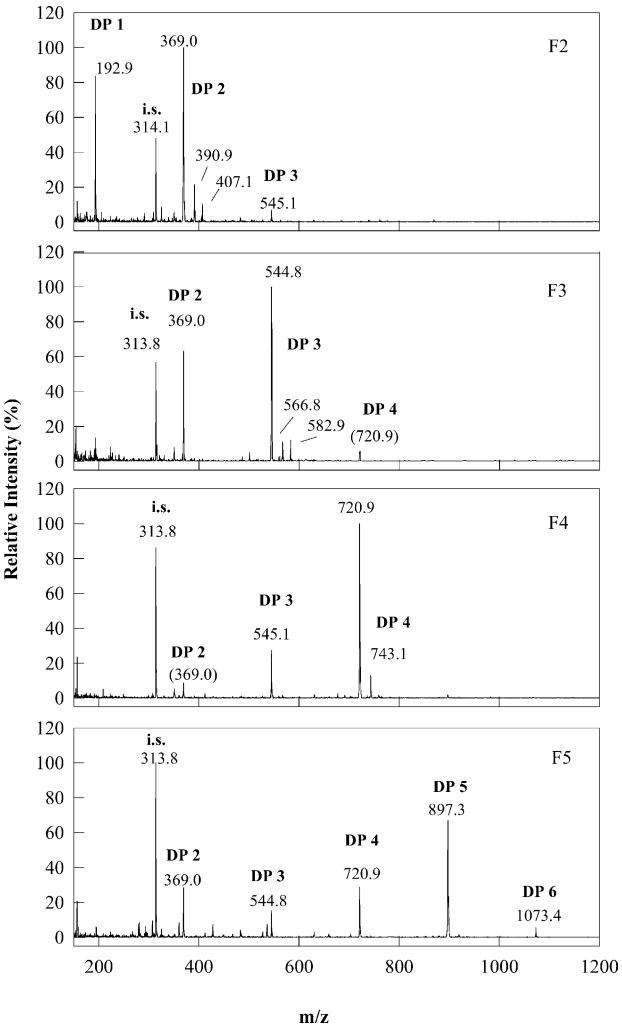

Fig. 3. Negative-ion ESI mass spectra of oligomannuronic acid standards F2–F5 obtained from a 3 h partial hydrolysis. Initials i.s. denote the internal standard, while DP and the respective integer (1–6) refers to the degree of oligomer polymerisation.

obtained for fractions F2, F3, F5 and F6, whereas mass spectra of standards F7–F12 were more complex due to an increase in double-charging phenomena that hindered a reliable purity assessment. Analysis of the mass spectra of the same standards (F2–F12) in the positive-ion mode was more sensitive, but even more complex (result not shown), because of a greater amount of ion adduction. This reinforces the contention that negative-ion mode ESI-MS is better suited for the analysis of acidic carbohydrates such as polyuronans [10]. Determination of DP_n was therefore only attempted for negative-ion mode spectra. It was not possible to accurately evaluate DP_n of standards >F6 or hydrolysis mixtures, which is why we now evaluate other quantitative techniques as complementary alternatives.

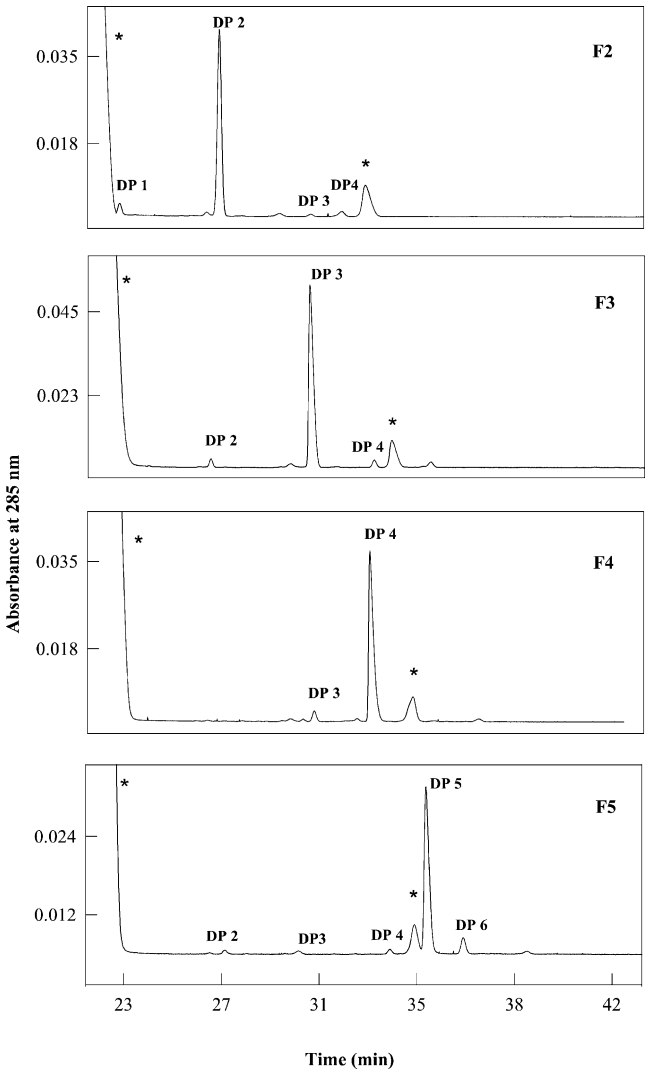

Fig. 4. MEKC-UV electropherograms of ABN-derivitised oligomannuronic acid standards F2–F5 obtained from a 3 h partial hydrolysis and detected by their UV absorbance at 285 nm (75 mM SDS in buffer). The symbol* denotes absorbance from free ABN. The acronym DP and the respective integer (1–6) refer to the assigned degree of oligomer polymerisation for each peak.

3.4. MEKC-UV analysis

Fig. 4 shows the analysis by MEKC-UV of the same standards (F2–F5). To achieve optimal resolution of oligomers, the ideal buffer was found to be concentrated H_3BO_3 in the presence of SDS. This was due to the different extent of complexation of saccharidic compounds [24,25] with alkaline borate, and to the selective interaction with SDS micelles [26,27]. This resolution compared well with the one obtained by capillary electrophoresis of an oligosaccharide mixture of galacturonic acid [8]. The values of DP_n for standards (F2–F5) obtained by MEKC-UV were consistent with those achieved by ^{1}H NMR (Table 1). The purity of the fractions decreased upon increasing DP_n, following a linear trend ($y = 101.48x - 1.64$; $R^2 = 0.94$, where y is the percentage in weight of the main component in a fraction with x nominal DP) from 95% in F2 to 74% in F12. The MRFs in MEKC-UV of different standards were independent of chain length because there was only one ABN chromophore attached to each oligomer. This represents an advantage over chromatographic techniques such as HPAEC-PAD where MRFs must be determined for each oligomer [15]. Therefore, an immediate visual impression of the molar number distribution of oligomers in a hydrolysis mixture is conveyed (Fig. 5).

However, analysis of oligomannuronic acid hydrolysis mixtures by MEKC-UV was not sensitive enough to detect small amounts of oligomers with DP above 12 and 19 for the 8.5 and 5 h hydrolysis times, respectively, and above DP

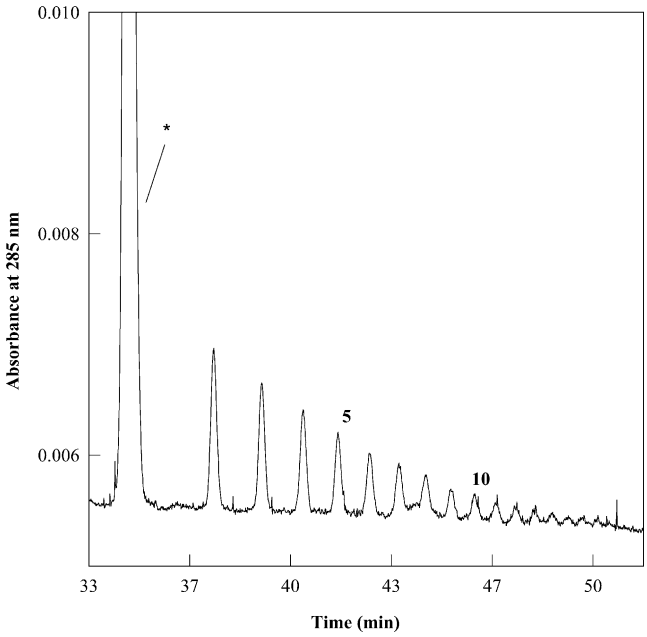

Fig. 5. MEKC-UV electropherogram of a oligomannuronic acid 3 h hydrolysis mixture derivitised with ABN (100 mM SDS in buffer). Oligomers were detected by their UV absorbance at 285 nm. The symbol (∗) denotes absorbance from free ABN. Numbers labelling the peaks refer to the assigned degree of polymerisation.

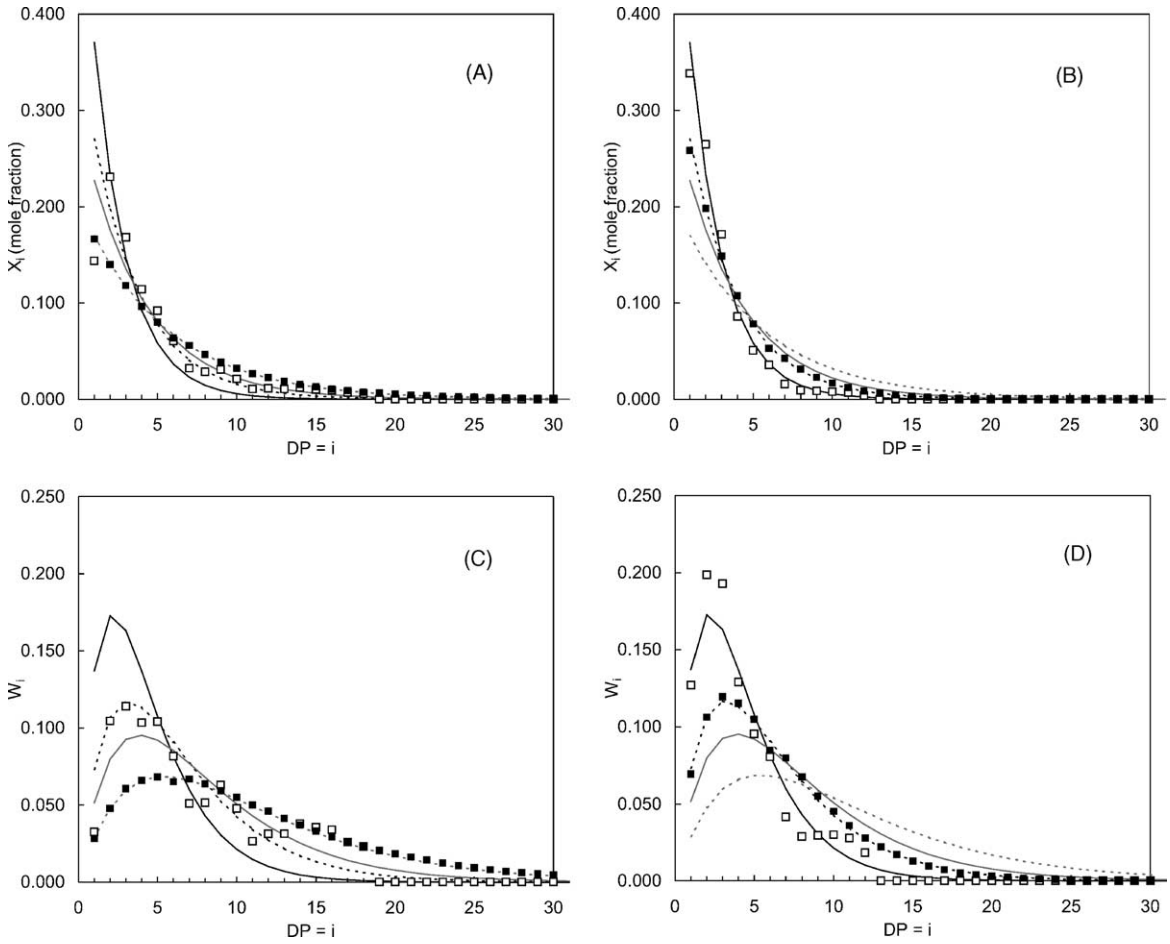

Fig. 6. Number (A and B) and weight (C and D) distribution of oligomers in mannuronic acid hydrolysis mixtures that were hydrolysed for 5 (A and C) and 8.5 h (B and D) and analysed directly. Filled squares (■) are data from HPAEC-PAD while open squares (□) are from MEKC-UV. Theoretical Kuhn distributions for $DP_n = 2.7$, 3.7, 4.4, and 5.9 are plotted as black solid, black dashed, grey solid and grey dashed lines respectively.

17 for 3 h of hydrolysis (Fig. 5). Omitting these oligomers in the calculation of DP_n averages and relative distributions introduces systematic errors. The presence of such errors is suggested by the lower DP_n values obtained by MEKC-UV as compared to ^{1}H NMR (Table 2). It is also evident from Fig. 6 that calculations of DP_n and the weight average degree of polymerisation, DP_w, for hydrolysis mixtures may be further influenced by uncertainties in the peak areas and the relatively high S/N ratio in peaks for DPs above 7. This is shown by lack of fit to a monotonous curve (Fig. 6).

Table 2
Estimated DP_n and DP_w for oligomannuronic acid hydrolysis mixtures

Method	DP_n			DP_w		
	3[a]	5[a]	8.5[a]	3[a]	5[a]	8.5[a]
^{1}H NMR	10.8	7.2	4.4	–	–	–
MEKC-UV	–	4.4	2.7	–	7.4	4.2
HPAEC-PAD	10.1	5.9	3.7	–	10.4	6.3
ESI-MS	–	–	–	–	–	–

[a] Hydrolysis time (h).

3.5. HPAEC-PAD analysis

Analysis of the standards F2–F5 by HPAEC-PAD shows a high resolution separation of oligosaccharides was achieved on an IonPac anion-exchange column eluted with an acetate gradient in 150 mM NaOH (Fig. 7). Analysis of oligomannuronic acid hydrolysis mixtures (Fig. 8) showed that oligomers up to around DP 50 could be separated in 70 min under the present conditions. This result matches earlier attempts at separating oligogalacturonic acids [1] and glucose oligomers in hydrolysis mixtures [4] with HPAEC-PAD. In contrast to previous studies using HPAEC-PAD to separate galacturonic acid oligomers [1], this alkaline mobile phase had no adverse influence on retention time. Moreover, all samples were fully soluble at alkaline pH, and we found no evidence for oligomer degradation due to β-elimination at room temperature during the 80-min period of chromatography (Fig. 8). In an earlier attempt, using the same IonPac column, we used $K_2C_2O_4$ (pH 7.8), instead of alkaline acetate, to make various counter-ion gradients between 0 and 0.5 M, as previously recommended for optimal separation of oligogalacturonic acids up to DP 50 [1,6]. The use of

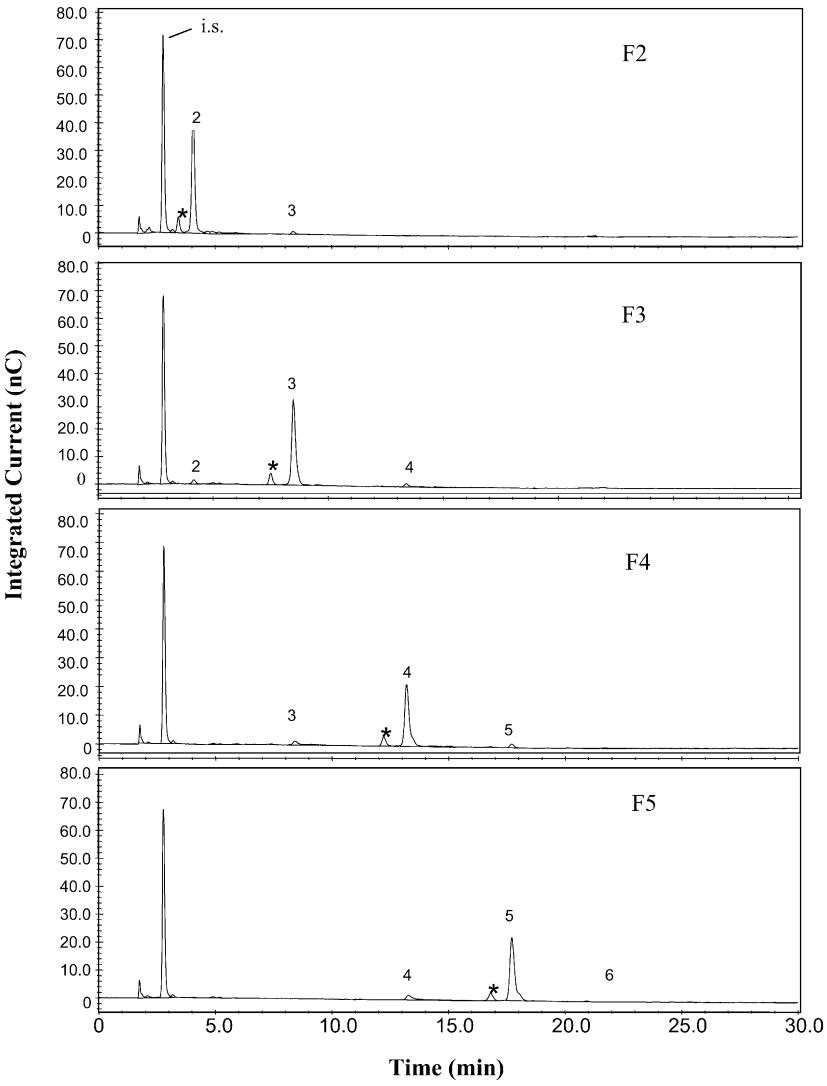

Fig. 7. HPAEC-PAD of oligomannuronic acid standards F2–F5 separated on an IonPac AS4A column using an acetate gradient in NaOH. The symbol (∗) represents an unidentified peak. Initials i.s. denote the internal standard. Peak labels (2–6) refer to the respective assigned degree of polymerisation of oligomers responsible for the peak.

this buffer scheme, including post-column addition of base, was abandoned because resulting chromatograms displayed apparently higher ratios of homologue to major oligomers, in excess of those observed by other techniques (result not shown). At present, we have no definitive explanation for this phenomenon, but it underlines the fact that assessments of purity and chain length distribution should be confirmed by alternative analytical methods.

As with MEKC-UV, it was found that each oligomannuronic acid standard (F2–F12) was contaminated with roughly equal amounts of their adjacent homologues 1 DP apart. Standard F2 had, for example, a purity of 95%, and subsequent oligomer purity decreased at a linear rate ($y = -0.037x + 1.027$; $R^2 = 0.99$) with every unit of nominal increase in DP as with MEKC-UV. As a consequence of the approximate equal contribution of adjacent oligomers, the calculated average DP for each fraction is slightly misleading as is also the case with MEKC-UV and ^{1}H NMR (Table 1, Figs. 4 and 7).

In contrast to MEKC-UV, it is known for PAD detection that MRFs depend on chain length [15]. Indeed, a strong increase in the molar response factor with DP was observed in the range investigated (Fig. 9). It is thought that the decrease in the weight-based response factor with DP reflects that the molar flux of solute to the surface of the PAD detector cell is a function of the diffusion coefficient, which decreases with increased degree of polymerisation [15]. However, the literature does not provide exact relationships between DP and MRF, so we made a standard curve by fitting the experimental data to an exponential function (see methods and Fig. 9). MRFs obtained for various amylopectin oligomers in the DP range of 2–17 [4,28] also fitted this function, whereas MRFs obtained in other similar studies did not (Fig. 9). These other workers found that MRFs for glu-

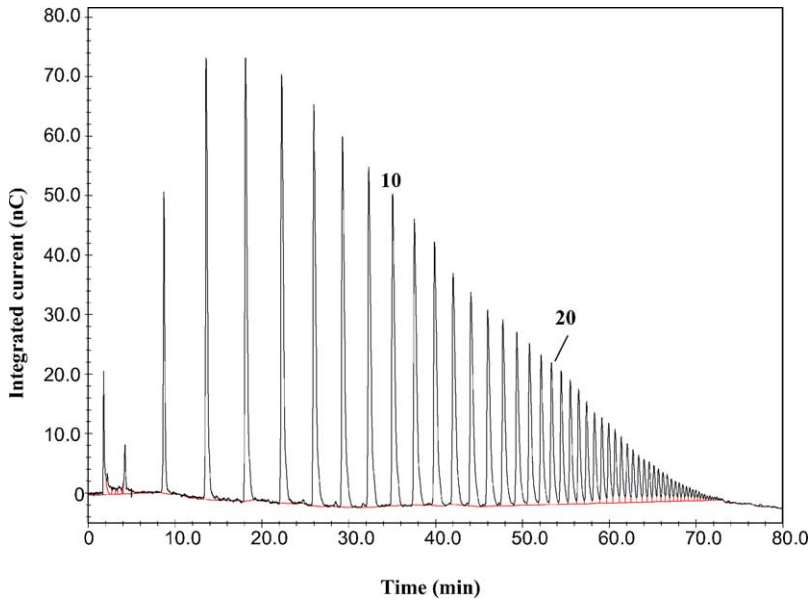

Fig. 8. HPAEC-PAD of an oligomannuronic acid 3 h hydrolysis mixtures without an internal standard and separated on an IonPac AS4A column using an acetate gradient in NaOH. Peak labels refer to the respective assigned degree of polymerisation of oligomers responsible for the peak.

cose oligomers [16,17] and inulin oligomers [18] were best described by asymptotic functions [16–18] (Fig. 9). Similar observations were also made in the smaller 1–7 DP range for oligomers of glucose [15,29], and also for oligomers of galacturonic acid [1]. In yet another study, MRFs calculated for a series of starch oligomers (DP 1–50) were found to fit a linear function [3]. With these observations in mind, we also fitted our data to an asymptotic function (see methods, Fig. 9). When applying these two extrapolations to the HPAEC-PAD data for two of three mannuronic acid hydrolysis mixtures (5 and 8 h), we obtained the results shown in Fig. 6. Assessment of DP_n and DP_w in these samples, where only a small number of large chains (DP < 20) were found, proved that whichever MRF extrapolation function was applied (exponential or asymptotic), it had little effect on the results calculated. Indeed, we found that calculated number and weight distribution of oligomers in various hydrolysis mixtures agreed very well with the theoretical Kuhn distribution assuming random depolymerisation (Fig. 6). Minor deviations were noted for the weight distribution and DP_w of the least degraded sample. In this case it is evident that contributions from oligomers with DP > 30 should have been included for correct estimates of DP_w. The DP_n values, calculated on the basis of exponentially fitted MRFs, were

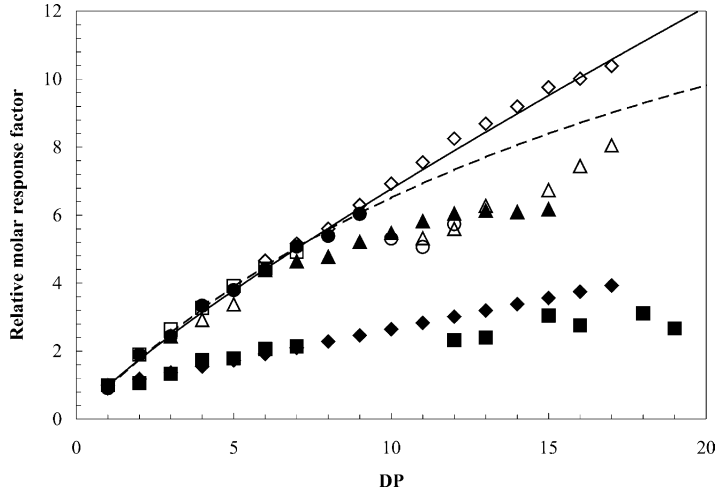

Fig. 9. Molar response factors (filled circle, ●) calculated for oligomannuronic acid standards F2–F9 and open circle (○) F10–12 obtained by 3 h of hydrolysis, separated by HPAEC and detected by PAD, as a function of their degree of polymerisation. Exponential (—) and symptotic (- - -) fits were applied for extrapolation for use in calculating DP_n and DP_w in oligo(ManA) hydrolysis mixtures. The symbols (filled diamond (◆), open square (□), filled square (■), filled triangle (▲), open triangle (△), open diamond (◇) are the MRFs normalised against our data obtained from other studies of carbohydrate oligomers from references [3,4,16–18,28] respectively.

Table 3
Comparative summary of the capability of each technique to determine the average degree of polymerisation and distribution of oligosaccharides in a partially acid hydrolysed homopolysaccharide

DP	Preparative Superdex 30	HPAEC-PAD	MEKC-UV	ESI-MS	^{1}H NMR
1	Co-elutes with salt peak—pure monomer cannot be quantitatively isolated	Standard not available. *Identification*: made by elution order. *Quantification*: assumes same molar response factor as internal standard	Standard not available. *Identification*: made by elution order. *Quantification*: assumes same molar response factor as other oligomers	*Identification*: direct from *m*/*z*. *Quantification*: assumes same molar response factor as internal standard	Standard not available
2–9	Resolution and peak separation acceptable, but decreases with increasing DP. Contamination with homologue oligomers identified by ESI-MS and quantified by HPAEC-PAD and MEKC-UV	*Identification*: as above. Excellent peak separation. *Quantification*: standard curve based on essentially pure standards and internal std. Need to calculate molar response factors for each DP	*Identification*: as above. Good peak separation. *Quantification*: Standard curve based on essentially pure standards. No need to calculate molar response factors as it is independent of DP	*Identification*: as above. *Quantification*: only possible up to DP 5 via an internal standard	*Identification*: spectral chemical shifts and coupling constants. *Quantification*: by determination of the ratio of total integrated H-1 signals to integrated H-1 signals from the reducing ends
10–20	Oligomers obtained with a decreasing resolution of peak separation with increasing DP	*Identification:* as above. *Quantification*: as above, but unless purified standards are available, molar response factors can only be found by extrapolation from oligomers DP 2–9	*Identification*: as above. *Quantification*: as above, but increasingly poorer peak separation with increasing DP	*Identification*: as above. *Quantification*: not achieved	*Identification*: as above. *Quantification*: as above
20–50	Cannot be obtained in a pure form	*Identification*: as above. *Quantification*: as above, but extrapolation of molar response factors from DP 2–9 becomes very uncertain in the absence of an exact theory for the RF-DP relationship	*Identification*: not possible in the low concentration range of the studied hydrolysis mixtures. *Quantification*: as for identification	*Identification*: not practically possible. *Quantification*: not practically possible	*Identification*: as above. *Quantification*: as above, apart from decreasing reducing end group signal with increasing DP increases uncertainty
Other comments	Might be useful to also incorporate a round of preparative anion-exchange chromatography		Sensitivity can be improved by derivatising oligosaccharides with a fluorophore and using laser induced fluorescence detection		Only possible to assess number average molecular weight. However, the distribution could be extrapolated from the Kuhn distribution

somewhat below those obtained by NMR, but showed better agreement with NMR than did the values obtained by MEKC-UV (Table 2).

4. Conclusion

Table 3 provides a summary of the capabilities of each technique used in this study. Each technique offers a number of advantages and disadvantages. With NMR it was possible to determine the number average degree of polymerisation, DP_n, of oligosaccharide mixtures up to approximately 20, but not to directly evaluate the molecular weight distribution. The converse is true for ESI-MS, but reliability in quantification decreased upon increasing size of oligosaccharides. On the other hand, HPAEC-PAD and MEKC-UV permitted determination of oligomer distributions and appropriate statistical averages. In contrast to HPAEC-PAD, MEKC-UV molar response factors were independent of oligosaccharide chain length. MEKC-UV was less effective than HPAEC-PAD in detecting high-molecular weight components present in low amount, biasing the weight average degree of polymerisation, DP_w, towards lower values. For HPAEC-PAD response factors were calculated directly for oligomers with degrees of polymerisation 2–9, and extrapolated by an exponential function for oligomers of higher DP. Experimentally determined molecular weight distributions were in good agreement with those predicted from theoretical Kuhn distributions of random depolymerisation.

Careful consideration should therefore be made to select the technique most appropriate to satisfy the aims of the study. Wherever possible, we would strongly recommend to use more than one technique to arrive at firm conclusions, especially with purity assessments. In the future we intend to extend our approach to study the chain length distribution of oligosaccharides in partially hydrolysed heteropolysaccharides such as alginate and other naturally occurring complex carbohydrates.

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