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Carbohydrate Research 340 (2005) 1193–1199
Carbohydrate RESEARCH

An investigation of pectin methylesterification patterns by two independent methods: capillary electrophoresis and polysaccharide analysis using carbohydrate gel electrophoresis

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Received 24 November 2004; received in revised form 17 January 2005; accepted 29 January 2005

Abstract—The analysis of partially methylesterified oligogalacturonides plays a key role both in the elucidation of the fine structure of the polysaccharide pectin and in the study of pectin-acting enzymes. Experimental methods performing the separation, detection and quantification of oligogalacturonides are, therefore, of crucial importance in the drive to understand structure–function relationships in pectin containing systems, both in vitro and in vivo. In this work standard samples of unesterified and partially methylesterifed galacturonides, and enzymatic digests of several pectin samples possessing distinct intramolecular patterns of methylesterification were studied using capillary electrophoresis (CE) and polysaccharide analysis using carbohydrate gel electrophoresis (PACE). In addition to yielding interesting information regarding the fine structures of the different pectic substrates digested, the study has been used as a vehicle in order to compare the two recently reported methods.

Keywords: Pectin; Oligogalacturonides; Polygalacturonase; Methylesterification; PACE; CE

1. Introduction

The plant cell wall is a complex biological matrix in which hemicelluloses, pectins, cellulose, proteins and lignin all play a role in determining structure and properties.[1] Despite this complexity it is known that the pectin component alone has considerable utility within the cell wall, its fine structure being modified in location and time, by enzymatic processing, in order to tailor its molecular functionality. In particular, understanding the role that the modification of homogalacturonan methylesterification (both level and pattern) plays in determining the properties of pectin networks is a central challenge not only for understanding the mechanical properties of the cell wall but also in order to design polymeric architectures to suit specific industrial applications.[2]

Many methods have been used in order to investigate the composition and structure of pectin, particularly regions of homogalacturonan. The average degree of methylesterification (DM) of pectin samples has been obtained via chemical methodologies,[3,4] gas chromatography,[5,6] and Fourier-transform infra-red[7] and nuclear magnetic resonance (NMR) spectroscopies.[8,9] Attempts to measure the inter-molecular distribution of the degree of methylesterification have revolved around ion exchange chromatography (IEC)[10–12] and capillary electrophoresis (CE),[13] while inferring information regarding the intramolecular distribution has relied on NMR methods[14,15] or fragmentations of the chain followed by product characterisation using high performance anion exchange chromatography (HPAEC) and mass spectrometry (MS).[16,17] Recently, electrophoretic methods have also been reported as additional tools in pectic

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doi:10.1016/j.carres.2005.01.037

oligosaccharide analysis. These include the use of polysaccharide analysis using carbohydrate gel electrophoresis (PACE)[18,19] and CE.[20]

While CE studies of oligogalacturonides have previously been reported using the derivatisation of the reducing function to supply charge and chromophore,[21] the method used here[20,22] uses the unmethylesterified galacturonic acid itself to mobilise the oligomeric species and the UV absorbance of the native carboxylate group for detection. The PACE methodology on the other hand does involve a derivatisation of the sugars by a fluorophore and then a separation by gel electrophoresis.[18] The separation and quantification strategies of the two methods under discussion herein are then entirely different and therefore, comparing enzymatic digest patterns obtained from well characterised pectin substrates by the CE[22] and PACE[18] methodologies forms an ideal cross-validation of the methods and an opportunity to discuss the advantages of each technique. Four pectins, prepared in order to have different total values and distributions of methylesterification have been digested with an *endo*-polygalacturonase (*endo*-PG) and the released oligogalacturonides analysed by the two distinct methodologies in order to obtain structural information regarding the polymeric architectures of the pre-digested substrates.

2. Materials and methods

2.1. Materials

Mono-, di-, tri- and polygalacturonic acid (PGA) and L-methionine ethylester hydrochloride were obtained from Sigma (Poole, Dorset, UK). Samples of single partially methylesterified galacturonides were obtained by fraction collection from an *endo*-PG digest of the C31 pectin sample using HPAEC as described previously.[20]

Pectin substrates derived from lemon peel were obtained from CP Kelco ApS, DK 4623 Lille Skensved (Denmark). These samples were manufactured from the same highly methylesterified mother pectin extraction. Their DM was controlled via treatment with a pectin methyl esterase (PME) of fungal origin (CP Kelco, private communication). This enzyme is believed to generate intrachain distributions of methylesterification that are close to random. Initial characterisation of the DM of the samples used here was carried out by the manufacturer and the values obtained were found to be 31% (C31), 55% (C55) and 65% (±2%). The former two samples were studied without further modification while the later was used as a starting material for the production of samples with controlled intramolecular DM distributions, as described below.

The 65% DM citrus pectin was extensively esterified with methanolic sulfuric acid at 4 °C for 7 days, using a method based on that of Heri et al.[23] In order to generate a random distribution of methylesterification along the polymeric chains this highly esterified starting material was de-esterified by base saponification until an average DM value of 30% was obtained (designated R30), as described previously.[13] In contrast, in order to generate an extreme blockwise distribution of methylesterification along the polymeric chains de-esterification of the highly methylesterified pectin was carried out using a PME derived from orange peel, until an average DM of 30% was reached (designated E30), as previously described.[13] The absence of *endo*-PG activity in the PME sample was confirmed by carrying out control experiments with PGA. Solutions of the final pectic materials were prepared by heating in buffer solutions at 60 °C for 20 min.

2.2. Enzymatic fragmentation

endo-PG (EC 3.2.1.15) from *Aspergillus japonicus* was obtained from Sigma (Poole, Dorset, UK). Digests with this enzyme were carried out by incubating 1 mg of substrate (PGA, C31, C55, E30 and R30) with 0.1 U, in 2 mL of 100 mM ammonium acetate buffer pH 5.0, for 5 h at 20 °C.

2.3. Demethylesterification of oligosaccharides/polysaccharides

In order to deesterify partially methylesterified poly- and oligosaccharides, samples were treated by 1 M NaOH (200 mM final concentration). NaOH (20 or 50 μL) solution was used before or after *endo*-PG hydrolysis, respectively. The treatment was performed at room temperature for 2 h before adjusting the pH to 5 with HCl (0.5 M) using a pH paper. Subsequently the solutions were dried for storage before redissolution followed by *endo*-PG treatment or derivatisation of samples.

2.4. Capillary electrophoresis analysis

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). Detection was carried out using UV absorbance at 191 nm with a bandwidth of 2 nm. Samples were loaded hydrodynamically and typically electrophoresed across a potential difference of 20 kV. The CE data presented has been obtained from six runs, three each at two ionic strengths of BGE (90 and 30 mM), and two of these being spiked with the internal L-methionine ethylether hydrochloride

standard. The details of the developed quantification scheme are elaborated elsewhere.[22]

2.5. Polysaccharide analysis by carbohydrate gel electrophoresis

Derivatisation was carried out in the tubes initially containing the dried polysaccharides, oligosaccharides or monosaccharides. The chemical procedure and the analysis were performed as previously described.[18] To separate $(GalU)_5Me_2$ and $(GalU)_2$, a different percent of acrylamide (21% acrylamide containing 0.7% *N*,*N*′-methylenebisacrylamide) was used for the resolving gel with a stacking gel previously described.[18] The samples were electrophoresed initially at 200 V for 30 min and then at 1000 V for 2 h using the same running buffers. The PACE data are generated from 10 to 12 loadings and in each gel standards of GalU and $(GalU)_3$ were run concurrently in different lanes. The figures given in this publication are a representative example of gels obtained. Gels were scanned and quantified as previously described.[19]

3. Results

All uncertainties presented are standard deviations (SD). For samples containing multiple species the relative concentrations of each oligomer to the total has also been calculated. In this case the mean values and associated standard deviations have been obtained by averaging the relative concentrations for each individual dataset.

3.1. Quantification of unmethylesterified standards by CE and PACE

Commercially available samples of mono-, di- and trigalacturonic acid were run and quantified, either as separate solutions or in mixtures of known (by weight) concentrations. The results are shown in Table 1 and help to establish the level of uncertainty associated with the methodologies themselves (i.e., not related to the preparation of the digests). In addition to the comparison of absolute concentration the relative concentrations of the different oligogalacturonides were also considered. Such relative concentrations potentially represent important information in themselves, for example, regarding the specificity and mode of action of degrading enzymes, and can be seen to be well recovered by both techniques.

3.2. Quantification of individual partially methylesterified OGAs by CE and PACE

The analysis of individual methylesterified oligogalacturonides was carried out and the results are shown in Table 2. Several concentrations were prepared by dilution of the collected samples. It can be seen again that within the experimental uncertainties there is reasonable agreement between the techniques.

3.3. Pectin fine structure by CE and PACE

Finally, the PACE and CE methods have been used in order to investigate the intramolecular methylester

Table 2. Concentration values obtained using PACE and CE for partially methylesterified oligogalacturonides, purified from HPAEC

OGA	Concentration/mM	
	CE	PACE
$(GalU)_3Me$	0.22 ± 0.03	0.17 ± 0.03
$(GalU)_3Me$	0.78 ± 0.08	0.85 ± 0.09
$(GalU)_4Me$	0.63 ± 0.05	0.70 ± 0.05
$(GalU)_4Me_2$	0.17 ± 0.01	0.17 ± 0.01
$(GalU)_4Me_2$	0.05 ± 0.01	0.05 ± 0.01
$(GalU)_5Me$	0.43 ± 0.08	0.59 ± 0.04
$(GalU)_5Me_2$	0.11 ± 0.01	0.15 ± 0.02
$(GalU)_6Me_2$	0.06 ± 0.01	0.05 ± 0.01
$(GalU)_6Me_2$	0.27 ± 0.04	0.35 ± 0.04

These fractions ran as a single peak in both techniques and were diluted to give a variety of concentrations. All uncertainties presented are standard deviations (SD).

Table 1. Concentration values and relative amounts obtained using PACE and CE compared to the known values (by weight) of commercial GalU with DP 1–3

Standard	DP	Concentration/mM			Relative concentrations/%		
		Weight	CE	PACE	Weight	CE	PACE
Individual	1	9.8 ± 0.2	9.4 ± 0.5	9.0 ± 1.0	—	—	—
	2	8.5 ± 0.2	8.2 ± 0.4	8.2 ± 1.2	—	—	—
	3	5.7 ± 0.1	5.5 ± 0.3	5.9 ± 0.6	—	—	—
Mix I	1	3.92 ± 0.08	3.7 ± 0.3	3.4 ± 0.3	63 ± 2	63 ± 6	66 ± 8
	2	1.70 ± 0.03	1.66 ± 0.05	1.3 ± 0.1	27.1 ± 0.7	28 ± 1	25 ± 3
	3	0.65 ± 0.01	0.59 ± 0.07	0.40 ± 0.05	10.4 ± 0.3	10 ± 1	9 ± 3
Mix II	1	1.96 ± 0.04	1.97 ± 0.06	1.5 ± 0.1	40.7 ± 1.2	43 ± 1	39 ± 1
	2	1.70 ± 0.03	1.62 ± 0.05	1.6 ± 0.2	35.3 ± 0.9	35 ± 1	39 ± 2
	3	1.16 ± 0.02	1.05 ± 0.04	0.9 ± 0.1	24.1 ± 0.6	22 ± 1	22 ± 1

The single compounds were run individually and then as mixtures (Mix I and Mix II) of all three oligosaccharides. All uncertainties presented are standard deviations (SD). For samples containing multiple species the mean relative concentrations and associated standard deviations have been obtained by considering the relative concentrations for each individual dataset.

distribution of the pectin samples E30, R30, C31 and C55 by quantifying the oligogalacturonides present in the *A. japonicus endo*-PG digests of these substrates. As detailed in the experimental section C31, C55 and R30 are believed to have largely random distributions of methyl groups along the homogalacturonan backbone (although the former two were generated by a fungal enzyme and the latter by a simple base saponification), and in contrast, E30 that was prepared using a processive PME and contains long stretches of unmethylesterified galacturonic acid.[13]

PGA was included in the study in order to compare the *endo*-PG digest of a completely unmethylesterified sample with those obtained from other methylesterified substrates. A simple fingerprint of the oligogalacturonic acids (OGAs) of DP 1–3 was obtained after the enzymatic hydrolysis of PGA, as can be seen in Figures 1 and 2, detected by PACE and CE, respectively. Interestingly, the E30 digest has a very similar pattern of oligosaccharides produced, again visualised by both techniques, confirming its blocky nature, whereas the R30, C31 and C55 digests show not only these unmethylesterified OGAs, but many other species, corresponding to partially methylesterified digest fragments. In the case of C55 particularly, a smear is evident in the PACE data, corresponding to many OGAs that were not separated into individual bands. Similarly in CE, a series of small peaks can be observed in the electropherogram of the C55 digest, between 5 and 6.5 min, overlying a broader feature, (more clearly seen in Fig. 4), corresponding to this population of larger unresolved material.

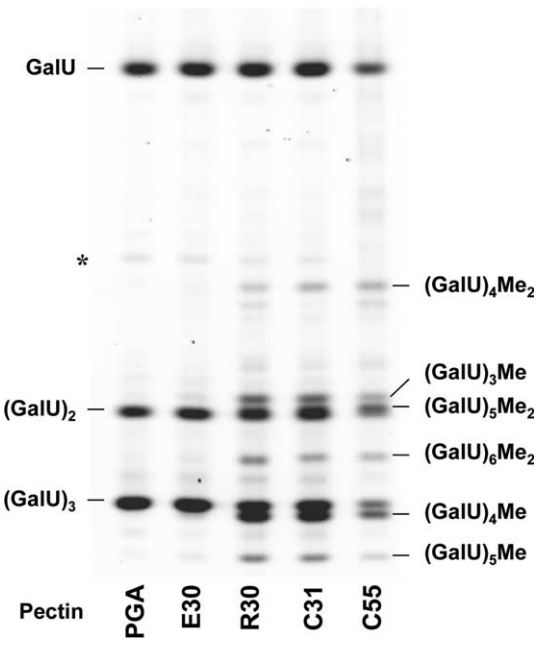

Figure 1. *endo*-PG fingerprint of five pectic substrates (PGA, E30, R30, C31 and C55), digested as described in the text, analysed by PACE. Identification of the bands has been carried out using standards. In these conditions, there is co-migration of $(GalU)_5Me_2$ and $(GalU)_2$. Other conditions as indicated in the material and method section were used to separate them for quantification. ⁎ Unspecific band.

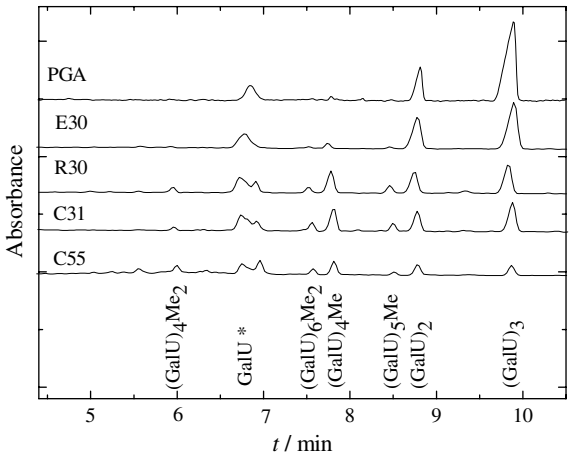

Figure 2. CE electropherograms (in 90 mM BGE) obtained from running the *endo*-PG digests of five pectic substrates (PGA, E30, R30, C31 and C55), as described in the text. * This peak contains GalU, $(GalU)_3Me$ and $(GalU)_5Me_2$, which were resolved in 30 mM BGE for quantification.

In order to investigate the less well-characterised larger fragments in the digests (detected only as a smear) the methyl groups were removed from all oligosaccharides using a base treatment with NaOH (after de-naturation of the *endo*-PG in order to prevent further processing on removal of the protecting methyl moieties). A similar experiment in which the pectin was demethylesterified prior to hydrolysis was also performed in order to verify that the demethylesterification protocol with NaOH does not substantially degrade the pectin, and that the enzyme produced the same final digest pattern in solutions of higher ionic strength. In this case the final fingerprint was identical (data not shown), as expected, to that obtained from PGA hydrolysed by *endo*-PG. For pectin substrates this post-digest experimental demethylesterification procedure has the effect of pooling the large number of potential partially methylesterified oligomers into more concentrated populations based solely on their degree of polymerisation. Interestingly by demethylesterification, many large unmethylesterified OGAs were detected. In PACE, oligomers of at least DP 18 were detectable in the C55 digest (Fig. 3), while in the corresponding CE experiment oligomers of around DP 15 could also be visualised but with limited resolution (Fig. 4). In the PACE conditions previously described[18] the OGA's migrate faster as the DP increases from 1 to 4 and subsequently slower as the DP increases above 5, yielding a turning point. In the PACE conditions used, DP 3 and 7 and also DP 2 and 11 co-migrated although if their separation is indispensable for a future study, different conditions of electrophoresis could be used such as described in Goubet et al.[18]

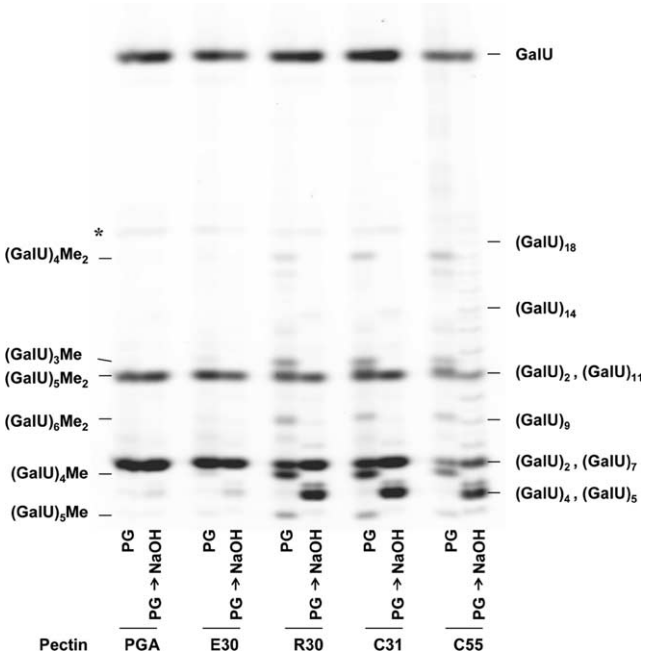

Figure 3. *endo*-PG fingerprint of the five pectic substrates (PGA, E30, R30, C31 and C55) compared with those obtained by NaOH treatment and subsequent neutralisation, before or after the enzymatic treatment. In these conditions, there is co-migration of [$(GalU)_5Me_2$, $(GalU)_2$ and $(GalU)_{11}$], [$(GalU)_3$ and $(GalU)_7$] and [$(GalU)_4$ and $(GalU)_5$]. Other conditions can be used to separate them as previously described.[18] * Unspecific band.

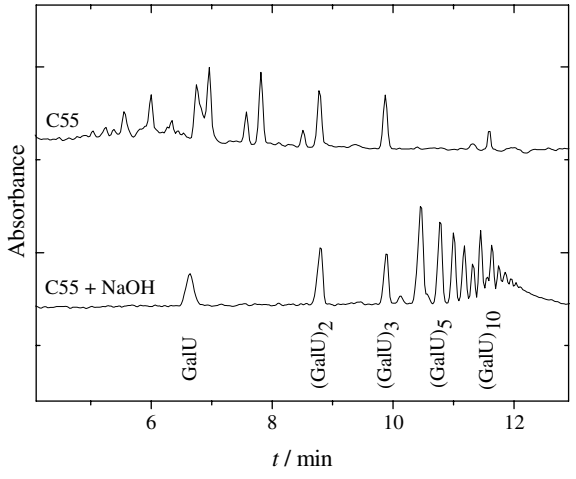

Figure 4. CE electropherograms (in 90 mM BGE) obtained from the *endo*-PG digest of the substrate C55 (as in Fig. 2), and following treatment of the digest with NaOH as described in the text.

3.4. Quantification of pectin digest OGAs by PACE and CE

In addition to the qualitative comparison between the digest fingerprints obtained by both methodologies we sought to present an evaluation of oligogalacturonide composition. In order to illustrate the quantitative comparison of pectin digests via PACE and CE, we selected the digest of R30 (the C55 digest was difficult to quantify in CE owing to the presence of the unresolved higher DP material, and the C31 and R30 digests are particularly similar). The corresponding quantitative data for the R30 digest, obtained from the analysis of the results displayed in Figures 1 and 2, are presented in Table 3.

4. Discussion

It can be seen that, qualitatively, the results obtained from the two techniques are in excellent agreement, not only with each other, but also with what is known about the fine structures of the different selected substrates and the requirements of the *endo*-PG used.[24] The E30 material is expected to contain considerably longer methylester-free blocks in comparison to other substrates of the same degree of methylesterification by virtue of the processivity of the plant PME that was used to perform the de-esterification. This substrate is indeed found to be more extensively degraded to mono-, di- and tri- galacturonic acid than the others, consistent with the increased affinity of *endo*-PG for homogalacturonan, and contains a smaller proportion of detected short partially methylesterified digest fragments (DP < 7).

The R30 and C31 substrates give very similar digest patterns, once again as measured by both techniques, suggesting that the fungal-PME and base-deesterified substrates of similar degree of methylesterification have similar fine structures. Such an observation is of interest as while it has been tacitly assumed that base de-esterification is a spatially random process, there has recently been some debate about the randomness or otherwise of the de-esterification process mediated by fungal-PME.[25]

The C55 sample is observed, again by both the CE and PACE methodologies, to be considerably more resistant to degradation, as evidenced by a substantial decrease in the number of oligomers (DP < 7) being generated compared to the other substrate digests. This is as expected from a fine structure with a statistically reduced chance of significant unmethylesterified runs, and therefore target sites for the binding and successful catalytic action of the *endo*-PG, known to prefer sparsely methylesterified material.[18,24] In line with this reasoning it would be expected to find considerably larger oligogalacturonides in this digest than found with the other substrates, as observed. However, for methylesterified fragments of increasing size there are a large number of possibilities for the degree and pattern of methylesterification yielding complex smears in the PACE and CE experiments that complicate the quantification of identified species. By effectively amalgamating all possible isomeric species into fewer pools dependent only on their DP the post-digest de-esterification process makes it possible to visualise species with higher degrees of polymerisation,

Table 3. Concentration values and relative amounts of partially methylesterified oligogalacturonides measured in the *endo*-PG digest of the pectic substrate, R30, obtained using PACE and CE

Digest Fragment	Concentration/mM		Relative Concentrations/%	
	CE	PACE	CE	PACE
GalU	2.4 ± 0.1	2.6 ± 0.3	25 ± 2	23 ± 1
$(GalU)_2$	1.7 ± 0.2	2.1 ± 0.2	18 ± 1	17 ± 0.5
$(GalU)_3$	2.3 ± 0.2	2.6 ± 0.2	25 ± 1	23 ± 2
$(GalU)_3Me$	0.58 ± 0.04	0.83 ± 0.15	6 ± 1	7 ± 1
$(GalU)_4Me$	1.2 ± 0.1	1.8 ± 0.2	14 ± 1	16 ± 1
$(GalU)_4Me_2$	0.24 ± 0.03	0.18 ± 0.05	2.5 ± 0.6	2 ± 0.4
$(GalU)_5Me$	0.30 ± 0.04	0.48 ± 0.06	3.2 ± 0.4	4.1 ± 0.5
$(GalU)_5Me_2$	0.30 ± 0.02	0.34 ± 0.04	3.2 ± 0.2	2.9 ± 0.2
$(GalU)_6Me_2$	0.34 ± 0.03	0.54 ± 0.19	3.6 ± 0.1	4.6 ± 1.5

All uncertainties presented are standard deviations (SD). The mean relative concentrations and associated standard deviations have been obtained by considering the relative concentrations for each individual dataset.

as seen in Figures 3 and 4, even if the more detailed information regarding the methylester content is lost. Some limited information regarding the maximum possible distance that existed between methyl groups in these larger methylesterified fragments is retained purely by the fact that they are *endo*-PG resistant. It should be noted that, in this CE mode where the natural charge on the galacturonic acid residues is used to mobilise the oligomeric species, then above a DP of ~15 to 20, the migration times of these longer fragments are dependent only on their degree of esterification so that, even in principle, information on chain length is lost.[13] The consideration of whether the amount of material liberated with DP < 7 and in particular, the number of GalU oligomers produced, is consistent with a random distribution of the methylester groups in the C55 substrate forms part of ongoing work.

Further to the successful qualitative comparison between the results obtained using PACE and CE a quantitative comparison was undertaken. In particular, the relative amounts of the different species showed good agreement between the techniques. It is worth stressing again that the relative amount of the different oligo galacturonides generated upon enzymatic digestion contains considerable information about the enzymatic specificity and the polymeric fine structure. The absolute determination of concentration was subject to larger uncertainties but taking these into account and bearing in mind the large difference in the sample treatments and methodologies the agreement was considered to be reasonable, and similar to uncertainties inherent in HPAEC work. As well as cross-validating the methodologies in order to ensure that the most rigorous digest information possible is obtained, the comparison also prompts some discussion of the advantages offered by each of the two techniques.

Though the mechanism of separation is different in the PACE and CE methodologies reported here, being operative in gel or in free solution respectively, both techniques have shown a great utility in optimising separation conditions with changes in experimental parameters such as BGE ionic strength[22] and gel concentration.[18] The major practical implications arising from the choice of technique as reported here are due to the nature of the quantification schemes. While the UV detected CE has the advantage of not requiring labeling steps and thus, for example, enabling real-time kinetic studies to be undertaken more simply, the use of the fluorescent label in the PACE methodology offers its own advantages by increasing the sensitivity of the technique and allowing molar quantification of species with an unknown structure. It should also be noted that the labeling methodology (used here in PACE and previously exploited in CE methods[21]) is much more generally applicable to a whole plethora of polysaccharides as it does not rely on the presence of a naturally occurring charge or chromophore.

Both techniques require relatively small amounts of sample. In CE the order of 10 nL is typically injected in a single run, although in order to practically inject the sample with a commercial instrument between 0.05 and 1 mL of sample would typically be prepared. In PACE typically 0.1 mL of solution is prepared and around 1 μL is loaded in each lane. Although to turn the fluorescence signal into a concentration the molar mass of the oligosaccharide is required it is possible to remove oligosaccharide fragments from the running gel post-separation and subsequently obtain further information using mass spectroscopy. This has been demonstrated in principle for a $(GalU)_4Me$ fragment (Goubet, F.; Quéméner, B.; Dupree, P. Unpublished data). Indeed the CE method used here has the same type of problem, that the DP and DM of a species should be known in order to generate a correct absorbance conversion factor to obtain a concentration, and a similar potential solution by using CE–MS.

Rigorous quantification of pectic fragments will prove invaluable in understanding the links between digest characterisation, enzyme action and substrate fine structure. In the future, we will continue to use both

techniques in order to investigate the relationship between the structure and function of pectin. Using other pectin-acting enzymes that have different tolerances for highly methylesterified pectins[26] could also be an interesting line of enquiry.

Acknowledgements

We thank Syngene (Cambridge, UK) for the gift of the GeneTools software used to quantify the PACE gels. The work using PACE was supported by grants from the BBSRC. The Royal Society of New Zealand is thanked for providing ISAT funding for travel for F.G. and M.A.K.W. and the EU for the award of a Marie Curie fellowship to A.S. The authors would like to acknowledge Naomi Blake for her contribution in the generation of the engineered fine structures and to thank Mike Gidley and Ian Norton for their encouragement and helpful discussions.

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