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Carbohydrate Research 339 (2004) 1711–1716
Carbohydrate RESEARCH

On the separation, detection and quantification of pectin derived oligosaccharides by capillary electrophoresis

Anna Ström[a] and Martin A. K. Williams[b,*]

[a]*Unilever R&D Colworth, Sharnbrook, MK44 1LQ Bedford, UK*
[b]*Institute of Fundamental Sciences, Massey University, Private Bag 11-222 Palmerston North, New Zealand*

Received 5 March 2004; received in revised form 4 May 2004; accepted 17 May 2004

Abstract—Having previously reported that capillary electrophoresis can be used as a tool for the analysis of partially methyl-esterified oligogalacturonides we now describe a method that improves the resolution of individual oligomers, and detail a more rigorous quantification scheme that uses an internal standard and takes into account the relative molecular absorbance of different partially methyl-esterified species. The internal consistency of the method is subsequently demonstrated by performing the quantification of an *endo*-polygalacturonase pectin digest before and after de-methylation of the resultant oligomers.

Keywords: Pectin; Polygalacturonase; Polysaccharide fine structure; Oligogalacturonides; Capillary electrophoresis

1. Introduction

While pectin is essentially a linear copolymer of galacturonic acid and its methyl-esterified counterpart, it is arguably the most complex of the plant cell polysaccharides.[1] This complexity, regulated by enzymes capable of modifying pectin fine structure in location and time, gives pectin its utility of function and ensures that the cell may optimally employ each macromolecule. In order to gain insight into structure–function relationships in pectin based systems, both in vivo and in vitro, methods for the assessment of pectin fine structure are crucial.

Recent progress in this area, specifically addressing the intra-molecular distribution of methyl-esterified residues in pectic substrates, has been made using a fragmentation approach in which *endo*-polygalacturonase (*endo*-PG) is used to digest the polysaccharide and the subsequent (methyl-ester sequence dependent) digest pattern is determined. Hence, the separation, detection and quantification of partially methylated oligogalacturonide digest fragments play a key role in the elucidation of the fine structure of pectin. While the bulk of such work to date has been carried out using anion exchange chromatography and mass spectrometry,[2–4] electrophoretic methods have also recently been reported as additional tools in pectic oligosaccharide analysis. These include the use of gel electrophoresis (PACE) coupled with fluorescent labelling,[5,6] and capillary electrophoresis (CE) using UV detection of the unadulterated oligomers.[7] It is the purpose of this paper to report significant recent improvements in the CE methodology since it was initially reported. In particular, experiments have been performed in order to develop a validated quantification scheme that accounts for the dependence of the UV absorbance on both the degree of polymerization and the methyl-ester content of the oligomers.

2. Materials and methods

2.1. Pectic substrate

The pectin substrate was derived from lemon peel and was obtained from CP Kelco ApS, DK 4623 Lille Skensved, Denmark. This sample was manufactured

* Corresponding author. Tel.: +64-6-350-5799x3543; fax: +64-6-350-5682; e-mail: m.williams@massey.ac.nz

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doi:10.1016/j.carres.2004.05.010

from a highly methyl-esterified mother pectin extraction and its degree of methylesterification (DM) was controlled via treatment with a pectinmethylesterase (PME) of fungal origin. This enzyme is believed to generate intra-chain distributions of methylesterification that are close to random. Initial characterization of the DM of the sample was carried out by the manufacturer and the value obtained was found to be 31%. Solutions were prepared by heating in buffer solutions at 60 °C for 20 min or by stirring at room temperature overnight. Repeat experiments revealed no difference between the results obtained with either preparation methodology.

2.2. Enzymes

endo-PG II (EC 3.2.1.15) from *Aspergillus niger* was prepared as described previously.[8] Pectin digests were carried out by incubating 2.5 mL of 2.5 or 5.0 mg mL^{-1} substrate with 1.5 U of this enzyme in 50 mM acetate buffer at pH 4.2, for 16 h at room temperature. *exo*-Polygalacturonase (*exo*-PG) from *Aspergillus tubingensis*, was also obtained as described previously[9] and the digestion of a 1.5 mg mL^{-1} solution of tetragalacturonic acid (4^0) was performed with around 0.01 U *exo*-PG in 50 mM acetate buffer at pH 4.2, at room temperature, for around 5 h. A fungal pectinmethylesterase (PME) from *Aspergillus niger* was used to de-methylated pectin digest fragments and has also been described previously.[10]

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 30, 50 or 90 mM. At pH 7.0 the unmethylated-galacturonic acid residues are fully charged and while the oligomers are susceptible to base-catalyzed β-elimination above pH 4.5, no problems were encountered during the CE runs of some 20 min at room temperature. 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:

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

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).[11] Samples of mono-, di- and tri-galacturonic acid, and L-methionine ethylester hydrochloride, used as a CE standard, were obtained from Sigma–Aldrich Corp., St. Lois, MO, USA.

3. Results and discussion

3.1. Improvements in quantification methodology

3.1.1. Varying the ionic strength of the BGE. Figure 1 shows the effect of the BGE ionic strength on the measured electropherogram obtained from a digest of the 31% methyl-esterified pectin (C31) detailed in Materials and Methods. The result at 50 mM compares favourably with that found previously[7,12] and the peaks are labelled according to the assignments made therein, with n^m representing an n-mer with m methyl-esterified residues. Additionally, it is clear that the effect of the ionic strength on the electrophoretic mobilities is species dependent. This arises from the way in which the buffer salts differentially modify the hydrodynamic friction coefficients of the different fragments. While future work will involve mobility modelling calculations, it is the

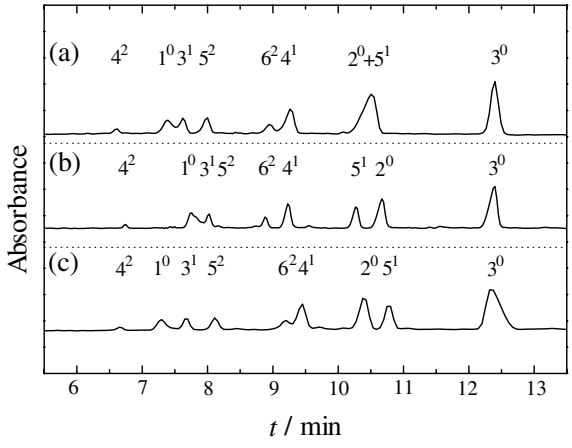

Figure 1. Electropherograms obtained from the injection of an *A. niger endo*-PG II digest of 31% methyl-esterified pectin (C31) in (a) 50 (b) 90 and (c) 30 mM phosphate BGE.

focus of the current study simply to note that better resolution of particular species can be obtained by carrying out experiments at different ionic strengths.

More specifically the 2^0 and 5^1 species that coelute at 50 mM can be seen in Figure 1 to be clearly resolved at a higher ionic strength (Fig. 1b). The cost of this, however, is that the species migrating at shorter times, namely 1^0, 3^1 and 5^2 now display similar mobilities leading to a convolution of the individual peaks and loss of resolution. However, by running an additional separation at a BGE concentration of 30 mM (Fig. 1c), the front part of the electropherogram can be seen to be well resolved, affording better quantification of these peaks even when compared with the previously reported 50 mM conditions. Interestingly, the running order of 2^0 and 5^1 is reversed under these conditions and the 6^2 and 4^1 species are no longer so well resolved. Nevertheless, it is clear that by using a combination of ionic strengths, the separation and quantification of the entire range of peaks of interest is improved.

3.1.2. Use of an internal standard. In previous work[7] quantification was performed using a calibration based on the UV absorbance of known numbers of moles of mono-, di- and tri-galacturonic acid standards. The former is calculated from the measured peak area normalized by the migration time[13] and the latter by knowing the analyte concentrations and the volume of the sample injected, calculated from the injection time and pressure, using Poiseuille's law. Here we introduce an alternative methodology in which L-methionine ethylester hydrochloride is used as an internal standard, thus circumventing the need to know the injection volume and protecting against its run to run variation. This particular compound was selected because it has an absorbance at the digest detection wavelength, 191 nm, and at pH 7 is predominantly positively charged and thus migrates on the opposite site of the neutral marker to the anionic digest itself. Control experiments recording electropherograms of a digest, the chosen standard and a mixture thereof, are shown in Figure 2 and illustrate that there was no interaction between these compounds.

3.1.3. Dependence of UV absorbance on DP and methyl-esterification

3.1.3.1. Quantification of galacturonic acid oligomers. Mono-, di- and tri-galacturonic acid were purchased from Sigma–Aldrich, who supplied the purity of the specific batch numbers. Concentration series of these compounds were prepared in de-ionized water containing 0.1% w/w L-methionine ethylester hydrochloride and the samples run in the CE experiment. The normalized peak areas of the galacturonic acids and the standard were calculated from the resultant electropherograms[13]

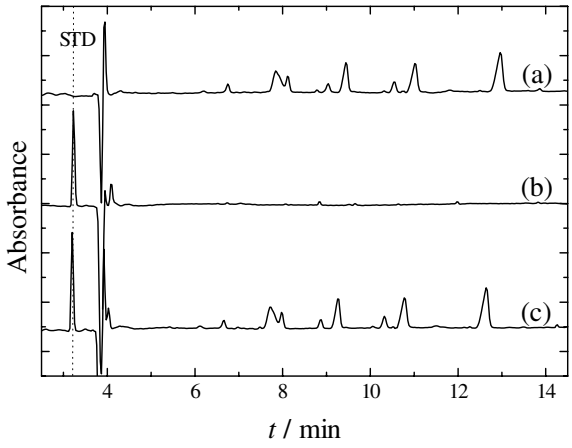

Figure 2. Electropherograms obtained from the injection of (a) an *A. niger endo*-PG II digest of the 31% methyl-esterified pectin (C31) (b) L-methionine ethylester hydrochloride and (c) a mixture of (a) and (b).

and plots of the ratio of these quantities (forthwith referred to as the *relative normalized peak area*) against the concentration of the galacturonic acids were formed. These were found to be linear, with constants of proportionality given in Table 1.

In agreement with previous work[7] we found that while the absorbance of di- and tri-galacturonic acid was, within experimental uncertainty, the same per mole of galacturonic acid residues, monomeric galacturonic acid had a higher absorbance. The data obtained here showed that the absorbance of galacturonic acid was some (1.29 ± 0.07) times larger than that exhibited by the same residue in cases where it was a constituent of a larger oligomer, in good agreement with a ratio of (1.31 ± 0.02) found previously at 50 mM in the absence of an internal standard. As a further illustrative test on using this ratio as a means of 1^0 quantification in digests, tetragalacturonic acid was run in the CE experiment against a standard and subsequently digested to monomers using *exo*-PG, as described in Materials and

Table 1. The constant of proportionality between the mM concentration and relative normalized peak area for the molecular species of interest

Fragment	k/mM
1^0	11.1 ± 0.4*
2^0	7.1 ± 0.2
3^0	4.8 ± 0.2
$n^0_{n>1}$	$(14.2 \pm 0.4)/n$
3^1	5.5 ± 0.2
4^1	4.1 ± 0.2
5^1	3.1 ± 0.1
4^2	4.7 ± 0.2
5^2	3.4 ± 0.1
6^2	2.9 ± 0.2

$[\text{mM}] = k \times$ relative normalized peak area. * This value explicitly tested at 50 and 90 mM; for 30 mM BGE substitute 12.0 ± 0.4. All uncertainties are 95% confidence limits.

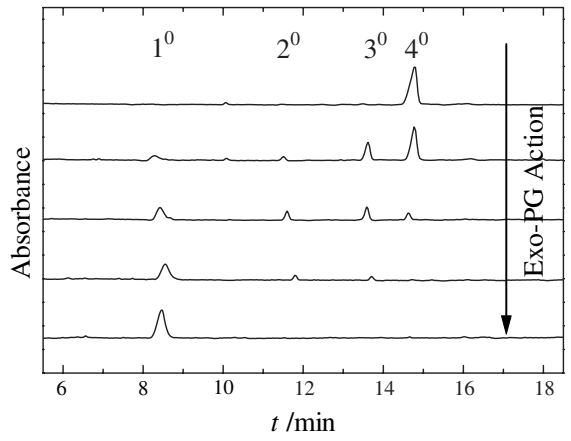

Figure 3. Electropherograms obtained showing the *exo*-PG digestion of 4^0 to 1^0.

methods. The digestion process is illustrated in Figure 3. By taking the normalized peak areas of both the tetramer and the monomeric product, relative to the internal standard, it is indeed clear that the same number of moles of galacturonic acid residues absorb to a greater extent when in monomeric form compared with when they form higher oligomers. From three repeat experiments the ratio of (1.31 ± 0.05) was obtained, once more in good agreement with the proposed ratio.

However while the same ratio (inherent in the constant of proportionality for the monomer in Table 1) has been shown to work well for the quantification of 1^0 in both 90 and 50 mM BGE, it should be noted that strictly it is likely to be a function of ionic strength. Indeed, when prepared in straight de-ionized water the absorbance of a galacturonic acid residue is found to be independent of whether it is in monomeric form or comprising a larger oligomer. The observed lifting of this degeneracy in typical CE conditions is then related to the BGE conditions and its prediction is not facile. Therefore, as it has been proposed advantageous from the perspective enhancing resolution to run experiments at 30 mM, we were careful to repeat the calibration study at this lower ionic strength and further to confirm our findings with spectrophotometry. We confirmed that within experimental uncertainty di- and tri-galacturonic acid, and the L-methionine ethylester hydrochloride standard did not absorb differently over the range of ionic strengths examined (0–90 mM). However, the monomer did indeed appear to absorb less at the lower ionic strength so that an alternative ratio, given in Table 1, is proposed to give the best quantification for samples run in 30 mM BGE.

3.1.3.2. Quantification of the partially methyl-esterified oligogalacturonides. Previous studies assumed for simplicity that the UV absorbance of the methyl-esterified residues was not hugely different from their unesterified counterparts. In order to progress the work herein, experiments have been carried out in order to specifically investigate the effect of the methylesterification on the UV absorbance of the pectin digest fragments. However, while mono-, di- and tri-galacturonic acid can be easily obtained, standard compounds of partially methyl-esterified oligogalacturonides that can be prepared by weighing are not commercially available. In lieu of the possibility of purchasing these materials a procedure has been employed where a sample was first run in CE and its resultant normalized peak area quantified, followed by a de-esterification of the sample and a subsequent re-run of the CE experiment. The unmethylated-daughter sample can then be quantified as described above and hence the concentration of the partially methyl-esterified forbear that yielded the previously recorded normalized peak area is obtained. In essence we use each partially methyl-esterified compound as its own standard by transforming it, post-run, into a quantifiable pure galacturonic acid oligomer.

In order to carry out this work, fractions of single partially methyl-esterified oligogalacturonides were first obtained by fraction collection from pectin digests using HPAEC as described previously.[7] These fragments were then spiked with unmethylated-galacturonic acid oligomers as internal standards and run in the electrophoresis experiment. Single species were also mixed together, as exemplified by Figure 4a, which shows a generated mixture of tetrameric compounds. The normalized peak areas of the partially methyl-esterified fragments were then noted relative to the standard galacturonides. All species in the sample were then de-methylated by treatment with base, and the electrophoresis experiment re-run, with the result that molecules previously running as n^m were transformed into n^0. Figure 4a–c shows the transformation among tetrameric species as the saponification proceeds until de-esterification of the species is

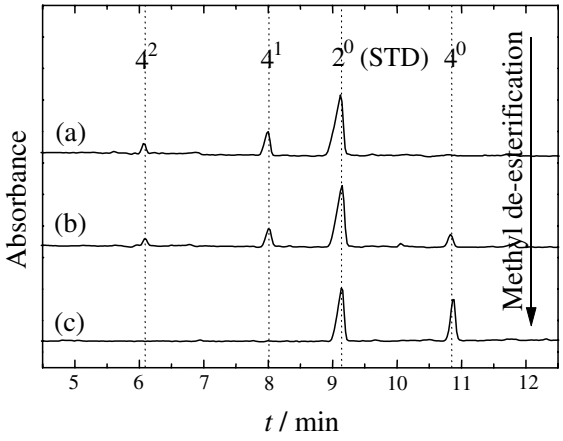

Figure 4. Electropherograms obtained during the base saponification of a mixture of the partially methylated galacturonides, 4^1 and 4^2. Di-galacturonic acid has been added to the starting solution as an internal standard.

completed, as shown in Figure 4c. The normalized peak areas were noted as the de-methylation proceeded. If methylated and unmethylated residues had exactly the same absorbance then the same normalized peak area would be obtained, relative to the internal standard, for both the (pre-base) n^m and the (post-base) n^0 peaks. Such experiments were carried out for a number of fragments and it was found that such an equivalence is not strictly adhered to. From the experimental data, relative factors and hence constants of proportionality between relative normalized peak areas and concentration were constructed for all relevant partially methyl-esterified oligogalcturonides. These are included in Table 1.

These data are also presented in Table 2 as relative molecular absorbances compared with the dimer. In an effort to rationalize the measured values it was assumed that the absorbance of a particular partially methylated oligomer originated from the sum of the absorbances of its constituent residues, and that, in such oligomers, galacturonic acid residues and their methyl-esterified counterparts had their own inherent absorbance (herein denoted A_G and A_M, respectively). While, in detail, the nature of the proximal groups is likely to influence the absorbance of a particular residue, it was felt that this effect was likely to be minor. By making such an assumption, a series of equations were be derived from the experimental data, for example, from experiments on the 5^2 fragments:

$$(3A_G + 2A_M) = (0.270/0.332)5A_G \quad (1)$$

Such equations have been derived for each of the experiments and can be solved together to give $A_M = (0.55 \pm 0.08)A_G$. This result, that methyl-esterified residues do in fact have a significantly lower absorbance at 191 nm than their unesterified counterparts, is in agreement with previous studies that found that the absorbance of low DM pectin was significantly higher at the same concentration than that of highly methyl-esterified samples.[14] The relative molecular absorbances of species of interest obtained based on this solution are shown in Table 2 and can be seen to be in reasonable agreement with the experimentally determined values. Experiments at various ionic strengths indicate that unlike the monomeric galacturonic acid the dependence of the UV absorbance on the methylesterification does not appear to be sensitive to the ionic strength of the BGE.

Table 2. The relative molecular absorbances of oligogalacturonide species of interest, experimental data and figures calculated using $A_M = 0.55A_G$

Fragment	Absorbance per molecule relative to 2^0	Predicted absorbance using $A_M = 0.55A_G$
1^0	0.64 ± 0.03	0.65
2^0	1.00	1.00
3^0	1.50 ± 0.01	1.50
$n^0_{n>1}$	$n/2$	$n/2$
3^1	1.30 ± 0.05	1.28
4^1	1.72 ± 0.05	1.78
5^1	2.28 ± 0.09	2.28
4^2	1.5 ± 0.1	1.55
5^2	2.07 ± 0.08	2.05
6^2	2.42 ± 0.08	2.55

3.2. Digest analysis

3.2.1. Utilizing alkali and/or PME mediated de-esterification. Using the procedures described above the quantification of a pectin digest can be carried out efficiently using CE by running at 90 and 30 mM BGE and using the appropriate conversion factors. Further evidence for the consistency of the proposed quantification scheme has also been sought by comparing the quantification of oligomers before and after de-esterification, brought about by the addition of base or fungal PME. Figure 5 shows electropherograms obtained from a pectin digest and its de-methylated counterpart. Performing such a process directly on digests and running the unadulterated and subsequently de-esterified material in close succession is worthwhile for a number of reasons. Firstly, the relative normalized peak areas of the digest peaks can be used to predict the relative normalized peak areas of each degree of polymerization after de-methylation and these can be compared with the measured values, giving a valuable experimental confirmation of the quantification of peaks DP < 6. Table 3 shows the concentrations of such species liberated from a 31% methyl-esterified pectin substrate by *endo*-PG II calculated as described herein. These values seem eminently reasonable and current work includes developing computer models of the enzymatic degradation process incorporating available biochemical data on the enzyme–substrate interaction, in order to predict the observed digest pattern. Progress in this area will

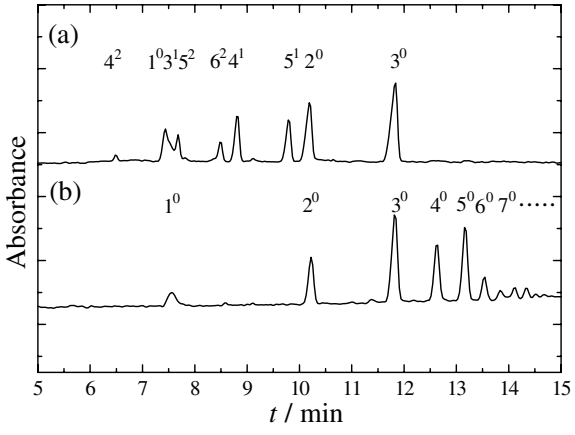

Figure 5. Electropherograms obtained from the injection of (a) an *A. niger endo*-PG II digest of 31% methyl-esterified pectin (C31), and (b) the same digest after base-catalyzed de-esterification.

Table 3. The absolute quantification of the species observed in a digest of C31, calculated according to the method expounded in the text

Fragment	Concentration/mM 90 mM BGE	Concentration/mM 50 mM BGE	Concentration/mM 30 mM BGE	Concentration/ mM post-base	Concentration/mM post-f-PME	$\sum DP_n$ pre-base/mM
1^0		2.4 ± 0.1	2.5 ± 0.2	2.4 ± 0.2	2.4 ± 0.1	
2^0	2.4 ± 0.2		2.4 ± 0.2	2.4 ± 0.2	2.3 ± 0.2	
3^0	1.6 ± 0.1	1.5 ± 0.2	1.6 ± 0.1	2.3 ± 0.1	2.1 ± 0.1	2.2 ± 0.09
3^1		0.59 ± 0.03	0.57 ± 0.03			
4^0				1.15 ± 0.09	1.06 ± 0.09	0.97 ± 0.05
4^1	0.87 ± 0.06	0.81 ± 0.05				
4^2	0.14 ± 0.02	0.13 ± 0.02	0.13 ± 0.02			
5^0				0.92 ± 0.06	0.88 ± 0.07	0.82 ± 0.02
5^1	0.47 ± 0.02		0.47 ± 0.02			
5^2		0.34 ± 0.03	0.36 ± 0.02			
6^0				0.25 ± 0.03	0.20 ± 0.02	0.18 ± 0.02
6^2	0.18 ± 0.02	0.18 ± 0.02				

benefit greatly from the development of a validated method for rapid digest analysis as described in this work, which can provide reliable experimental data, against which theoretical predictions can be tested. Furthermore, the comparison of results such as those described here with those obtained from the same digest by the PACE[5,6] method is currently being undertaken. This will provide an excellent cross-check of both methodologies as the quantification in PACE relies on an entirely different principle that of counting the fluorescence of separated bands generated by labels that are introduced onto the reducing end of each oligomer by a post-digest labelling procedure.

Furthermore, as evidenced by Figure 5, the presence of additional fragments of longer DP is more successfully investigated in this fashion. However, for methylated fragments of increasing size there are a large number of possibilities for the degree and pattern of methylesterification. The consequence of this is that any signal corresponding to these larger methylated oligomers would be smeared into the baseline in the CE experiment. By effectively amalgamating all possible n^m species into fewer n^0 pools the de-esterification process makes it possible to visualize the total amount of species with degrees of polymerization $< \sim$15–20, even if the more detailed information regarding the methyl ester content is lost. Above this DP, however, 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.[15]

Acknowledgements

We would like to thank Jacques Benen for kindly providing enzymes used in this work. Don Farrer is thanked for help with the selection of a suitable standard compound. Members of the Europectin project, and Florence Goubet, Paul Dupree, Ian Norton, Tim Foster, Leif Lundin and Ed Morris are all gratefully acknowledged for helpful and interesting discussions. The EC Commission is thanked for the award of a Marie Curie Fellowship to A.S.

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