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Determining the degree of methylesterification of pectin by ATR/FT-IR: Methodology optimisation and comparison with theoretical calculations

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ABSTRACT

The application of FT-IR to the study of the structure and interactions of the major plant-cell wall polysaccharide pectin has been reported for many decades. Nevertheless, here we show that the generally reported methodology for one of its most commonly utilised applications, the measurement of the degree of methylesterification (DM), requires careful interpretation and sample handling; including consideration of the moisture content and ionisation state. We propose instead a different methodology based on the assessment of the magnitude of C–H stretches in the methyl groups relative to those in the backbone and demonstrate experimentally the advantage of this method. In addition, we add a theoretical dimension to our work, performing full quantum chemical (DFT) calculations of monomeric-, dimeric-, and trimeric-pectic compounds, in various states of partial methylesterification. These extensive calculations not only confirm the identity of the proposed methyl-band and illustrate its scaling with DM; but also demonstrate the success of the theoretical approach. Thus, DFT calculations are expected to be a valuable tool in the interpretation of IR spectra obtained from more complex systems such as polysaccharide conjugates.

1. Introduction

Amongst the plant cell components, the cell walls, comprised largely of cellulose, hemicelluloses and pectins, have the most important influence on the textural properties of plant tissues. This is manifest, for example, during the storage of fruits, when important physiological and textural changes in the cell wall composition and structure occur, due largely to enzymatic modification of the pectic molecules. While the detailed structure of the pectin macromolecular assembly *in vivo* is still a matter of debate to some extent (Vincken et al., 2003), most commercially available pectin samples can be considered as a collection of polymer chains each consisting of extended regions of homogalacturonan (HG) interspersed sparsely with regions of rhamonogalacturonan I (RGI) (Ralet & Thibault, 2002). HG consists of a linear backbone of (1,4)-linked α-D-galacturonic acid (GalA) residues, commonly partly methylesterified at C-6, while the RG-I backbone consists of [2)-α-L-Rha*p*-(1,4)-α-D-Gal*p*A-(1] repeats with no strong evidence that GalA units in RG-I domains are methylesterified (Voragen, Pilnik, Thibault, Axelos, & Renard, 1995). The rhamnosyl (Rha) residues of RG-I backbone are substituted, mainly at *O*-4, with several types of arabinose (Ara) and galactose (Gal)-containing neutral sugars side-chains (Voragen et al., 1995). Most of the functional uses of pectin are linked to the quantity of methylester groups (as well as their inter- and intra-molecular distribution) and measurement of the degree of methylesterification (DM), defined as the percentage of galacturonic acid residues carrying a methylester group, is a routine procedure in pectin analysis.

Several methods have been developed over the years for the DM determination. Acid–base titration was the first method used (Schulz, 1965) while later alternative methods using alkaline hydrolysis to split the ester linkage and subsequently estimate the quantity of methanol released using HPLC analysis, headspace GC, or NMR, have also been demonstrated (Bédouet, Courtois, & Courtois, 2003; Huisman, Oosterveld, & Schols, 2004; Maness, Ryan, & Mort, 1990; Massiot, Perron, Baron, & Drilleau, 1997; Rosenbohm, Lundt, Christensen, & Young, 2003; Voragen, Schols, & Pilnok, 1986). These methods present certain disadvantages, mainly due to the complexity of the procedures involved and the ensuing sample destruction. Unlike many other natural polysaccharides, pectin has both charge and a UV chromophore (the carboxyl group) which makes Capillary Electrophoresis (CE) a practical analytical tool for its study (Ström, Ralet, Thibault, & Williams, 2005; Ström & Williams, 2004; Zhong, Williams, Goodall,

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& Hansen, 1998; Zhong, Williams, Keenan, Goodall, & Rolin, 1996). This technique provides a simple, rapid and non-destructive method for the quantitative detection and separation of pectins with different degrees of methylation in aqueous solution, with the advantage of gaining information regarding the distribution of the substituents; although its application to low DM samples is limited by counter-ion condensation.

Infrared spectroscopy has also been suggested as a useful and non-destructive tool to distinguish different pectins: (Barros et al., 2002; Haas & Jager, 1986; Monsoor, Kalapathy, & Proctor, 2001; Synytsya, Čopíková, Matějka, & Machovič, 2003). In particular Chatjigakis et al. (1998) and Gnanasambandam and Proctor (2000) used Fourier transform infrared spectroscopy (FT-IR) to determine the degree of pectin methylesterification. They found that absorbance of the ester carbonyl groups ($COOCH_3$) increased with increase in degree of methylesterification and promisingly that the band area seemed linearly related to the DM, with bands occurring between 1730 and 1760 cm^{-1} and between 1620 and 1650 cm^{-1} indicating respectively the esterified carbonyl groups and carboxylic ions (COO^-). However, this technique does have certain disadvantages, in particular if other carboxylates and carbonyl groups are present and, as we will show, is crucially dependant on the presence of water and the pH and ionic conditions.

One objective of this current study was to develop a more robust method for the DM determination using Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR/FT-IR), which, unlike other FT-IR methods does not require the sample to be heated, pressed or ground in order to collect the spectrum; (which takes time and can cause structural changes). It should be noted however that the general method expounded herein is based on spectral interpretation, and as such is applicable to the full range of IR techniques. It was hoped that developing such robust spectroscopy methods might prove of value in validating specific bond formation in proposed coupling reactions involving pectin macromolecules and substrates, which were been carried out in order to facilitate various biophysical experiments. Furthermore, with this in mind, the investigation of the utility of Density Functional Theory (DFT) for calculating features of the IR spectra of such complex polysaccharides was also of interest. While the question of the calculation of saccharide IR spectra has been considered before for single sugar rings of differing substitutions (Korolevich & Zhbankov, 1998; Korolevich & Zhbankov, 1999), the revolutionary development in recent years in available computational facilities and large clusters have made the simulations of comparatively large systems (such as the three sugar rings examined herein) possible. Of late, there have been extensive studies of the IR and Raman properties of other molecular systems using theoretical simulations (Krishnakumar, Keresztury, Sundius, & Ramasamy, 2004; Krishnakumar & Prabhavathi, 2008).

2. Experimental

2.1. Samples

Pectin samples used in the analysis included pectins of DM 30%, 60% and 90% purchased from Sigma (P9311, P9436 and P9561); a sample of DM 78% purchased from Fluka (apple pectin); pectins of DM 35%, 71% and 74% kindly provided by Henk Schols of the University of Wageningen (F, C and X6904); samples of DM 35%, 37% and 48% kindly provided by CP Kelco (randomly distributed methyl groups, LM 12, and blocky samples, 0001-8-F and 0001-8-D, respectively); and samples of DM 58%, 62% and 65% with randomly distributed methyl groups, alkali de-esterified in previous studies (Vincent, Cucheval, Hemar, & Williams, 2009; Vincent & Williams, 2009). Additionally samples of polygalacturonic acid (DM 0) were also obtained from Sigma and Fluka, and mono- and di-galacturonic acid were purchased from Sigma.

All the DMs were provided by the suppliers, with the exception of the homemade de-esterified samples, which had their values determined by Capillary Electrophoresis (CE), as described previously (Williams, Foster, & Schols, 2003). Previously documented data is also considered (Ström, 2006).

2.2. ATR/FT-IR spectroscopy and spectral analysis

Spectral acquisition was performed on solid samples using a Nicolet 5700 FT-IR spectrometer equipped with Omnic software (version 7.1) and a Smart Omni-Sampler (ATR cell with single reflectance germanium crystal). Each recorded spectrum is the average of 32 scans with a spectral resolution of 4 cm^{-1} from 400 to 4000 cm^{-1} on a dried sample, with a background spectrum recorded before each analysis. Three spectra were measured and each one was analyzed and fitted using Origin software (Version 7.5) equipped with Peak-Fitting Module (PFM).

Fitting involved first ensuring that the baseline was averaged to zero in the appropriate spectral range where no bands were detectable (1800–1900 cm^{-1}) followed by a normalisation to the intensity by the largest peak. The spectral region for fitting was then selected (typically from 950 to 1900 cm^{-1}; or 950 to 1500 cm^{-1}). Typically an automated peak-picking algorithm found around 12 main peaks in such a region (peaks could be added or subtracted manually if required). Subsequently, Lorentzian peak shapes were selected and placed at the peak-picked positions whereupon their relative intensities and widths were modified by a fitting algorithm until a best fit was found, as determined by a minimised reduced chi squared. The peak areas of the component Lorentzians, centred at the peak-picked frequencies, are then output. The uncertainty in the repeat measurements performed on the same sample was less than 2%.

2.3. Drying and acidifying

The pectin samples were dissolved (0.5% w/w in MilliQ water) and the resultant solutions were acidified by addition of 0.2 M HCl solution until low pH (≈1–2). They were then dialysed against MilliQ water, freeze-dried and finally dried under reduced pressure in a vacuum oven, at T = 30–40 °C.

2.4. Calculations

Complete Hessian calculations were performed on a monomer, dimer and trimer of α-D-galacturonic acid and their methylesterified analogues using DFT calculations implemented in the *Gamess US* package (Pople & Gordon, 1967). The computational facilities used were an IBM-Bluegene cluster sited at the University of Canterbury, NZ.

Bluegene/L nodes have two processors and two modes of execution are supported: co-processor (CO) and virtual node (VN) modes. In CO mode the first processor runs the program and the second processor handles I/O and communication on behalf of the first processor. In virtual node mode, both processors run the program. In CO mode all node memory is allocated to the first processor, while in the VN mode, the node memory is shared between the two processors. The single rack system we utilised provides a maximum of 1024 processors with 512 MB of RAM each, or 2048 processors with 256 MB of RAM each; with the calculations reported herein exploiting the first option. Initially, the respective systems were placed in standard pyranose $^4C_1$ configurations, completely optimised in the semi-empirical basis AM1, and were then geometry optimised using the B3LYP/6-31G* basis. These ground-states were then subsequently subjected to a complete

optimisation and Hessian calculation using the B3LYP/6-311++G** basis set, (Appell, Strati, Willett, & Momany, 2004; Momany, Appell, Strati, & Willett, 2004), incorporating the diffuse functionals. All simulations were performed at 0 K and in vacuum. The convergence criteria in the energy minimisation for energy differences between cycles of optimisation were less than 1×10^{-6} Hartree, with the gradient set to be less than 1×10^{-4} a.u, as in our previous work (Williams, Marshall, Anjukandi, & Haverkamp, 2007). The scaling and assignment of the vibrational modes was carried out using the *Chemcraft* programe (Zhurko).

3. Results and discussion

Fig. 1(a) shows the ATR/FT-IR spectra obtained from a number of representative pectin samples, with the inset revealing that indeed there appears to be a promising relationship between the DM and the relative contribution of the 2 peaks centred at about 1750 and 1630 cm^{-1}, due respectively to the infrared absorption of the carboxylic ester and the any protonated carboxylic acid groups, and to the infrared absorption of the carboxylic anion as reported previously (Bociek & Welti, 1975; Venyaminov & Kalnin, 1990) and discussed in the introduction. However, although a linear relationship between the DM and the ratio of the area underneath these peaks has been proposed (Chatjigakis et al., 1998) it was found here that when the results from a larger set of samples was studied (with no specific sample treatment protocol) that the relationship between the peak area ratio we obtained from spectral fitting, as described in Section 2, and DM, did not seem to be strongly adhered to (Fig. 1(b)); a linear regression yielding an R^2 value of 0.57. One explanation for this could possibly be interference either from other carboxylates and carbonyl ester groups, such as those from cell wall phenolics, or more concerningly from water (the in-plane deformation band of water δ (H_2O) occurs at 1645 cm^{-1}). As such, samples were extensively dried and while this improved the correlation between the ratio of the suggested band intensities and DM somewhat (data not shown), there were clearly still problems with obtaining accurate DM determinations.

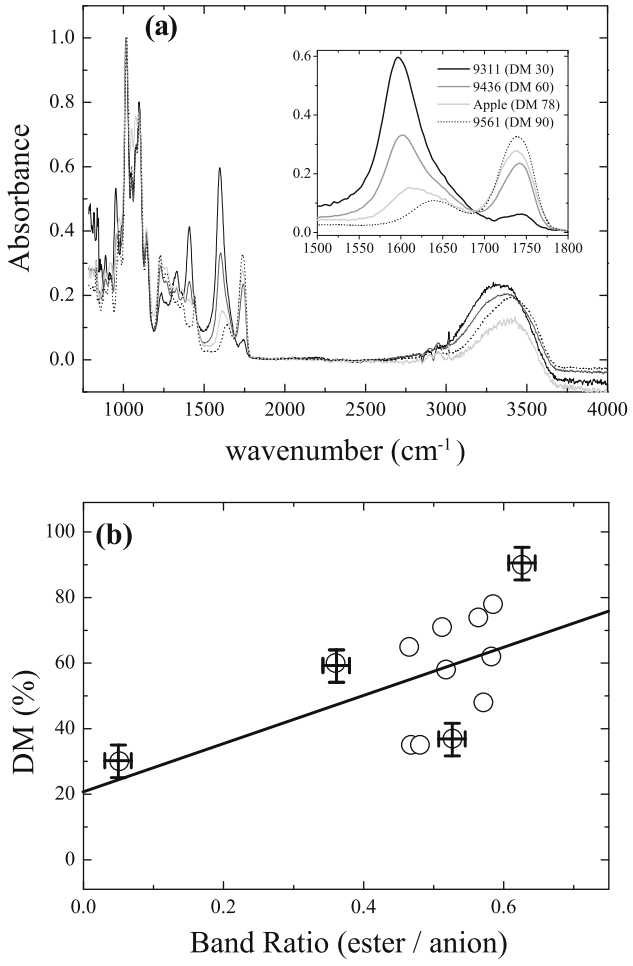

Fig. 1. (a) ATR/FT-IR spectra of pectins with different DM. Inset – the two peaks conventionally used to determine the DM. (b) Regression analysis of suggested band ratio versus DM, carried out on crude samples using the peak areas of these carboxyl bands, at 1630 and 1750 cm^{-1}, obtained by peak fitting as described in Section 2.

Further examination of the results lead us to consider that the intensity of the symmetric (≈1450 cm^{-1}) and asymmetric (≈1630 cm^{-1}) carboxylate stretch modes are in fact very sensitive to the ratio of protonated to ionic carboxylate groups. By carefully precipitating and drying the pectins from acidified solutions, the carboxylic anion band largely disappears, and the band at 1750 cm^{-1} can be seen to increase owing to the presence of the newly formed carboxylic acid groups (Fig. 2). This may seem an unusual procedure in deference to attempting to manipulate the sample conditions in order to obtain *all* charged and no protonated groups, as following this suggested acid treatment the protonated and methylesterified species essentially absorb at the same frequency and the originally proposed method is no longer applicable. However, another alternative now becomes possible, which completely eliminates problems introduced by the presence of the signals from hydration water.

Previous IR studies on pectins have suggested that the esterified CH_3 group presents bands in the 1350–1450 cm^{-1} range, one at 1380 cm^{-1} corresponding to the symmetric stretching of CH_3 and one at around 1440 cm^{-1} corresponding to the asymmetric stretching modes of CH_3 (Synytsya et al., 2003). Therefore, rather than attempting to quantify the bands corresponding to the absorption of the carboxylic ester and the carboxylic acid groups; and to the absorption of the carboxylic anion, we investigated whether the DM could be calculated simply by using the intensity of the asymmetric stretching of CH_3 at 1440 cm^{-1} relative to a backbone vibration at 1010 cm^{-1}. While pectin samples can contain small amounts of neutral sugars (for example in rhamnogalacturonan as described in Section 1), the backbone vibration band is largely due to the homogalacturonan and a reasonably good correlation with the classically measured DM could still be expected.

In fact it was found that in order to perform the reliable quantification of the proposed band over a large DM range, acidification

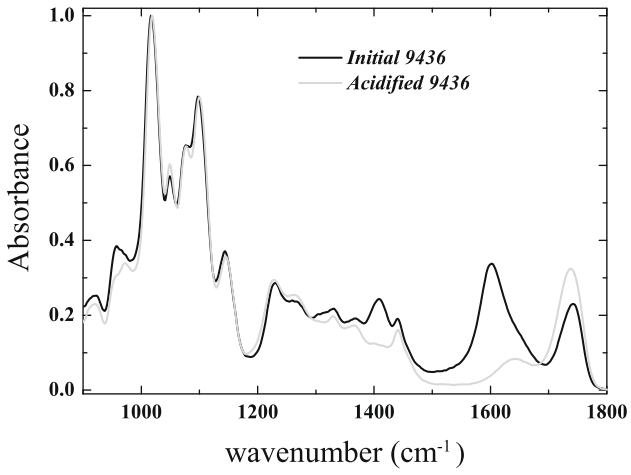

Fig. 2. Effect of drying of pectins from acidified solutions on the measured IR spectrum.

of the sample (which limits the interference of the symmetrical stretching of the carboxylate group (Fig. 2)), and robust peak fitting, as described in Section 2, were both required. While some preliminary progress was made previously by examining the asymmetric stretching modes of CH_3 (Ström, 2006), it is now clear that without properly accounting for the underlying intensity owing to the symmetric modes of any charged carboxylic groups the method struggles to be useful for samples with DM values below 40% (Fig. 3). However, under the conditions suggested herein, employing acidification and robust peak fitting, a linear relationship was established between the degree of methylesterification of the pectin samples and the ratio of the peak areas from bands at 1440 cm^{-1} and at 1010 cm^{-1}, (DM = ((455 ± 20) × ratio) + (2 ± 5)), which yielded a considerably improved R^2 value (0.975) and furthermore within experimental uncertainties a reassuring zero intercept (Fig. 3). Using the given 95% confidence limits in the relationship the DM of an unknown sample can confidently be recovered to around ±7%.

In order to gain further confidence in the proposed methodology, and additionally to investigate the success of DFT in capturing the essential experimental details, a number of calculations were performed. Full quantum chemical calculations were used to generate theoretical FT-IR spectra of the monomer, dimer and trimer of α-D-galacturonic acid and their methylesterified analogues

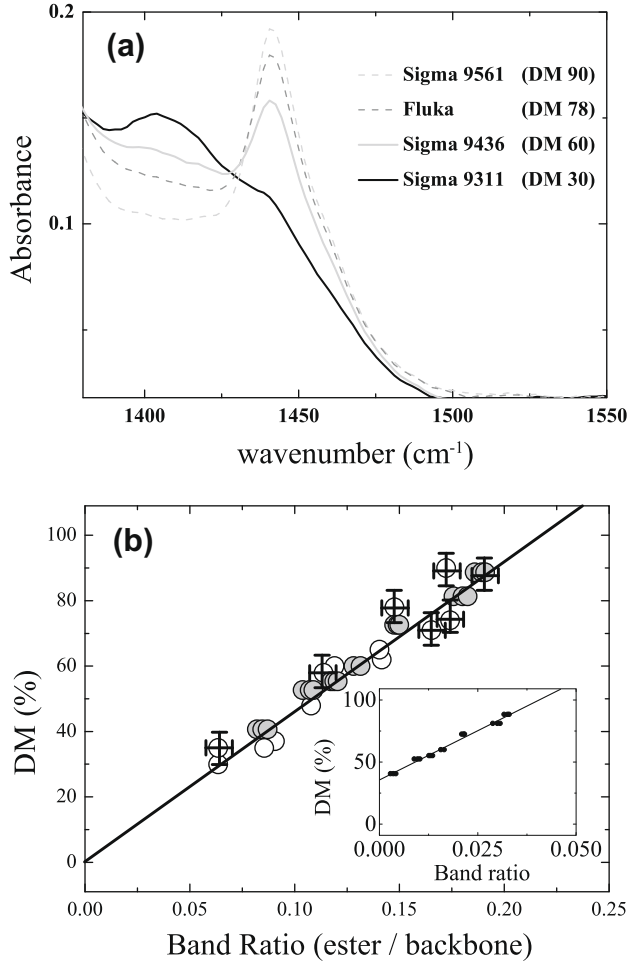

Fig. 3. (a) ATR/FT-IR spectra of pectins with different DM, as in the inset of Fig. 1, but in the region of 1380–1500 cm (b) Regression analysis for dried acidified samples using the ratio of asymmetric CH_3 stretches and the 1010 cm^{-1} backbone vibration band (open circles); also compared with previous work (Ström, 2006) on un-acidified samples (filled circles-inset), and scaled to take into account underlying intensity of the neighbouring band (grey circles).

(Fig. 4 (a), (b) and (c)). The experimental IR spectra of 0% and 90% DM pectin samples and simulated spectra of the monomer, dimer and the trimer of α-D-galacturonic acid and their fully methylesterified counterparts were first compared (Fig. 5).

Fig. 5(a) shows the comparison of the experimental spectra of a 0% DM pectin with the simulated spectra of oligomers of 0% DM of different DPs up to the point at which the calculation become uncomfortably expensive. There is, in general, good accordance between the calculations and the experiment, in particular between the carbonyl group vibrations and the backbone vibration frequencies at (1600–1800 cm^{-1}) and (900–1200 cm^{-1}) respectively. Since the 0% DM pectin samples do not have any methylester groups, we do not find the corresponding vibrations, occurring at around 1430–1490 cm^{-1} as expected; and clearly the water interference in the region at 1600–1650 cm^{-1} in the experimental result is not expected to be present in the calculated spectra. Five bands are specifically highlighted; and their assignments are shown in Table 1. It should noted that such calculations on systems of this size are highly complex, and the fact that the raw calculated frequencies only deviate by some 40 wavenumbers or less from those found experimentally constitutes good agreement. The main reasons for the observed differences result from approximations inherent in the calculations including: they are essentially carried out in vacuum at 0 K, and assume a strictly harmonic form for the relevant potentials. In light of this it is routine to scale the frequency axis by a small amount so as to align major bands with those experimentally observed. The co-alignment of multiple calculated and experimental bands via the same scaling, as seen here, constitutes good agreement.

Fig. 5(b) shows the comparison between the experimental 90% DM data and simulations, again of varying DP substrates, but here with all residues methylesterified. Here, in addition to observing the pectin peaks in the IR spectra as described for the 0% DM oligomers (including the all-important marked backbone vibration), we do indeed find the $-CH_3$ group vibrations at around 1450 cm^{-1} as indicated in the figure.

In order to look at the difference in the $-CH_3$ group vibrations of different DM pectins, a galacturonic acid dimer was used for simulation, as this is the simplest molecule for which it is possible to study at least three different DMs by simulation. By varying the number of methyl groups along the molecule, we could easily generate a 0%, 50% and 100% DM sample for simulation. Comparing the calculated spectra of these 0%, 50% and 100% dimers (Fig. 6 (a)), a clear increase in the peak areas of the $-CH_3$ group vibrations is observed as the DM increases, validating the philosophy behind our proposed methodology. These simulation results should also be compared with the experimentally determined spectra of different DM pectins (0%, 48% and 90%), which are comparable with the ones obtained through the DFT simulations (Fig. 6(b)). Again the simulations and the experimental data agree well.

In all the simulated spectra, when compared with those obtained from experiments on the polymeric samples, we could see that particularly the bands owing to the backbone –CH vibrations (1200–1450 cm^{-1}) are much sharper in simulation than in experiment. In order to investigate the origins of this, which we hypothesised was due to the increased complexity in the polymeric backbone (both in terms of possibilities for coupled vibrations and dynamic averaging of conformations), we took the experimental spectrum of a pure galacturonic acid monomer and dimer (0% DM) and compared them with the polymeric result (Fig. 7). It was indeed observed that the experimental spectra of a sole monomer showed sharp regions in 1200–1450 cm^{-1} accounting for the –CH vibrations; and the experimental and the theoretical spectra (shown in Fig. 5a) were in reasonable accordance with each other. Already some broadening of the peaks is seen experimentally as the dimer spectra is measured, suggesting that the broadening of

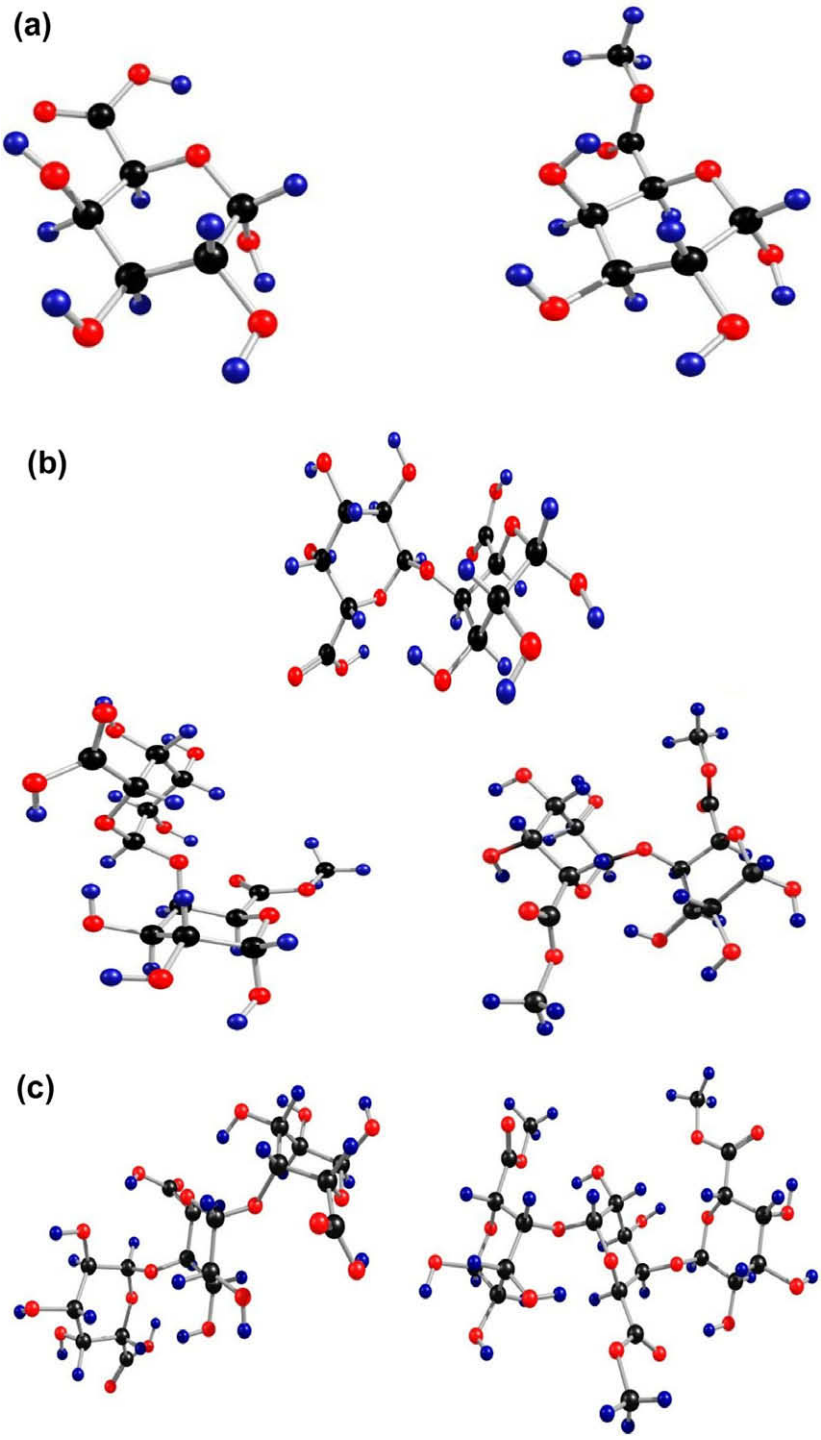

Fig. 4. (a) Pure monomer of α-D-galacturonic acid and its methylester analogue. (b) Galacturonic acid dimers of 0%, 50% and 100% DM depending on the number of the esterified groups in the molecule. (c) Trimers of α-D-galacturonic acid and its completely methylesterified analogue.

the experimental pectic spectra in this regime (1200–1450 cm^{-1}) is indeed a facet of the increasing degree of polymerisation.

4. Conclusion

The FT-IR method described in this study for the determination of the degree of methylesterification of pectins is non-invasive and is significantly more accurate than other reported IR methods; although the expected uncertainty in the measured DM value still stands at around ±7%. In order to get the best results from this method the samples should be treated (precipitated from acidic solution and dried) as described. The ratio of the intensities of the asymmetric vibrations of the CH_3 group and backbone bands can then be used to determine the DM (rather than the commonly used carboxylic bands), which permits the elimination of the interferences from other cell wall components such as water and proteins. In addition, DFT calculations have been shown to reproduce the main features of the experimental spectra, with a clear evolution towards the polymer result as the DP of the saccharide

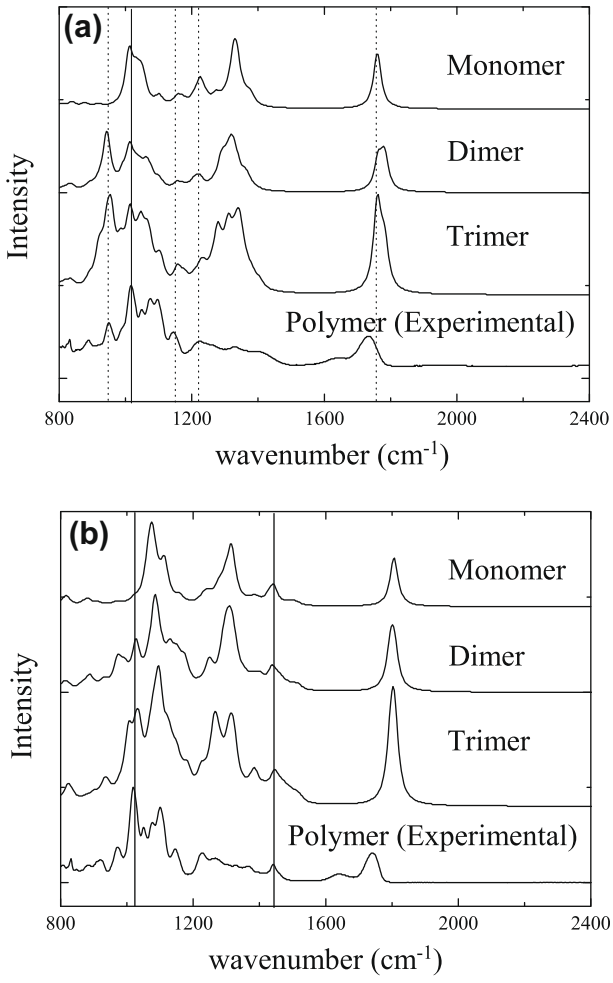

Fig. 5. (a) Comparison of experimental IR spectra of a 0% DM pectin polymer with calculations of monomer, dimer and trimer of α-D-galacturonic acid. Bands indicated by lines are assigned in Table 1; (b) Comparison of experimental IR spectra of a 90% DE pectin polymer with calculations of 100% methylesterified monomer, dimer and trimer of α-D-galacturonic acid .The lines show the position of the asymmetric CH_3 stretches and the backbone ring vibration exploited by the proposed method.

Table 1
The assignment of relevant IR peaks and comparison of the experimentally measured frequencies with those found directly form the DFT calculation expounded herein (PGA, polygalacturonic acid).

Experimental (cm^{-1})	Raw calculated (cm^{-1})	Assignment
1750	1786	ν (C=O), PGA ester
1750	1798	ν (C=O), PGA
1630	–	ν (C=O), PGA ion
1640	–	δ (HOH)
1440	1497	ν_{as} (CH_3), ester CH_3
1380	1442	ν_s (CH_3), ester CH_3
1215	1275	ν (CO), δ(OCH)
1107	1155	ν (CO), ν (CC), Ring.
1010	1051–1062 (acid and ester)	ν (CO), ν (CC), δ(OCH), ring
934	983–1013 (acid and ester)	ν (CO) glycosidic

molecule in the calculation was increased from 1 to 2, and ultimately 3. This spectral elucidation makes FT-IR coupled with DFT an analysis method that has the potential to investigate pectin structure in more complex systems, such as post-coupling reactions. This forms part of ongoing work and will be reported elsewhere.

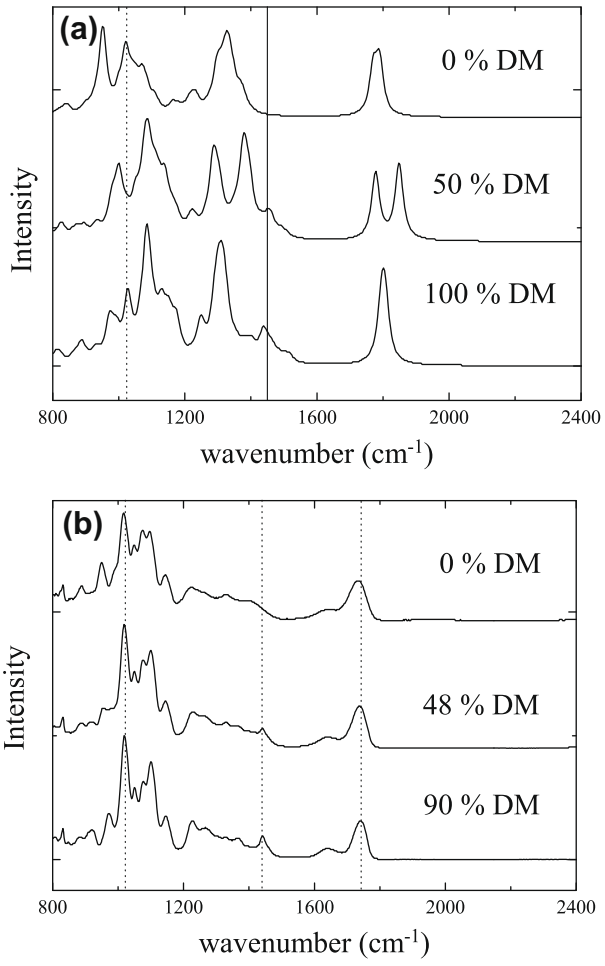

Fig. 6. (a) Simulated IR spectra for galacturonic acid dimers of 0%, 50% and 100% DM; and (b) Experimental IR spectra of pectins of different (comparable to those simulated) DMs. The dotted lines again highlight discussed bands.

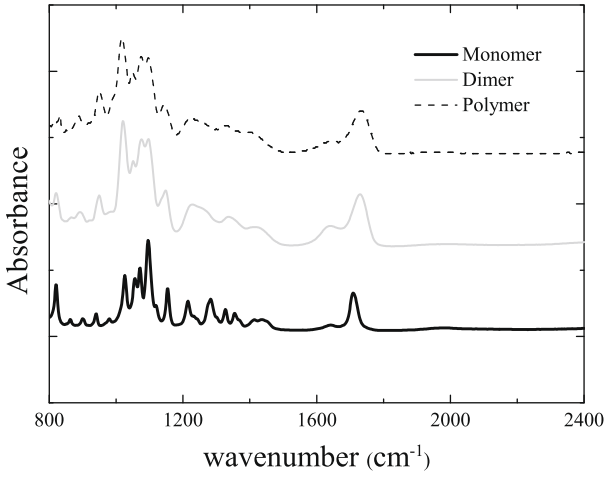

Fig. 7. Comparison of experimental IR spectra recorded for α-D-galacturonic acid monomer, dimer and polymer, illustrating the increasing broadening owing to the increasing degree of polymerisation.

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