Compositional Heterogeneity in Pectic Polysaccharides: NMR Studies and Statistical Analysis

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SUMMARY: Pectins are a class of heterogeneous polysaccharides used in the food industry as a result of their ability to form gels. They are primarily composed of a (1→4)-α-D-galactopyranosyluronic acid backbone where the carboxylic acid group is methyl esterified at a level which depends on the source of the pectin and processing conditions used to isolate the material. Of considerable interest is the distribution of the free acid and methyl ester groups along this polymer chain. NMR spectroscopy, coupled with statistical analysis, is a powerful technique for the study of sequence distribution of monomers. Experimental conditions (temperature, pD) are reported which are appropriate for the analysis of pectic polysaccharides. Results are presented from a study of sequence distribution in native, modified, and fractionated pectins using ^{1}H and ^{13}C solution NMR methods. The triad sequence information was examined using Bernoullian and Markovian probability models in conjunction with continuous Gaussian distribution and discrete models. Intermolecular heterogeneity in pectins results in triad frequencies which reflect the distribution of acid and ester monomers for native and base saponified pectins. Fractionation of pectin through physicochemical methods and subsequent spectroscopic analysis provide insight into this heterogeneity. Segregation into discrete polymer populations shows a structural diversity best approximated by a 3-component statistical model.

Introduction

Pectins are a class of heterogeneous polysaccharides found in all plants. They are commercially derived primarily from citrus peels and find application in the food industry based on their ability to form gels. Composed of a (1→4)-α-D-galactopyranosyluronic acid (GalA) backbone, pectins may have varying levels of methyl esterification, neutral sugar content, acetylation, and a large polydispersity. The sequence distribution of methyl ester

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groups in the galacturonan backbone is of considerable interest due to the effect it has on the rheological and gel forming properties of pectin[1] Previous studies have focused on assessments of the sequence distribution in pectins by various chemical and chromatographic methods, including degradation, enzyme treatment and calcium binding[2-10]

Nuclear magnetic resonance (NMR) studies have been reported for pectin composition and monomer sequence in the whole polymer, in enzyme-modified pectins, and in oligosaccharides[11-17] These studies have concluded that pectins typically exhibit an approximately random sequence distribution of ester and acid groups in native and alkali saponified samples, while enzyme (higher plant pectin methylesterase)-treated pectins exhibit a blockwise distribution of acid groups. It is important to point out that sequence analysis of whole pectins using NMR represents a statistical average of the acid and methyl ester distributions. Intermolecular and intramolecular heterogeneity inherent in natural pectins[3,8] would be ignored in such analysis. In contrast, fractionation of pectin into discrete components based on molecular weight, charge or affinity, followed by NMR, permits the analysis of intermolecular heterogeneity. The objective of this work is to analyze whole pectins, both native and chemically modified, and pectin fractions, in order to develop a statistical model appropriate for pectin acid and methyl ester distributions. In addition, experimental conditions were studied in order to obtain reproducible spectral acquisition. Pectins are sensitive to pH and temperature, and the selection of improper conditions can lead to erroneous results.

Experimental

Materials: Pectins derived from lemon peel utilized in this work were taken from experimental samples produced by Hercules Incorporated and used without further purification. Several samples were fractionated using a proprietary procedure to generate two fractions differing in the distribution of galacturonic acid methyl ester groups. For example, samples C_1 and C_2 are fractions obtained from the same initial pectin, having similar degrees of esterification (d.e.; 0.72 and 0.68 respectively), but differing in the blockwise distribution of unesterified galacturonic acid residues.

Methyl Esterification of Pectin: A standard high methoxyl pectin (d.e. = 0.67) was methyl esterified to a d.e. = 0.93 following the procedure described by Heri, et al.[18]. A solution containing 10 gm pectin suspended in 118.5 ml 5.43%(v/v) H_2SO_4/CH_3OH was gently stirred for 5 days at 4°C. The pectin was then filtered, resuspended in 45 ml 70% (v/v) isopropanol/water and stirred for 7 min. This latter process was repeated for a total of 3 washings. A final wash in isopropanol was followed by air drying and storage at -20°C.

Deesterification of Pectin: Standard pectins and the fully methyl esterified pectin were saponified following the procedure of Chen and Mort[19]. A 1 gm sample of pectin was suspended in 200 ml of deionized H_2O and adjusted to a final pH of 8 with 1 M NaOH. Random deesterification was initiated by the addition of a specific amount of 0.1 M NaOH. The alkali was added at a rate to bring the solution first to pH 10.5 and then adjusted so as not to exceed this point. The sample was stirred until the pH returned to 8, at which point it was further neutralized to pH 6 with 1 M HCl, followed by dialysis and lyophilization.

Chromatographic Determination of Degree of Esterification: The d.e. of each pectin sample analyzed in this work was determined using an HPLC method for determination of methanol following the procedure of Voragen, et al.[20]. Galacturonic acid content was determined using the *m*-hydroxydiphenyl method of Ahmed and Labavitch[21].

SEC Analysis: Samples of pectin in H_2O (2 % w/w) were prepared and adjusted with a NaOH solution to obtain a final pH of 4. These samples were then placed in a bath thermostatted at 80°C for variable time periods up to 44 hours. After cooling to room temperature, the samples were diluted to a final concentration of 2 mg/ml. A 200-µl sample was then injected on a Waters SEC with a 4-column TSK-Gel column set with a 0.02 M lithium acetate buffer at a pH = 4.8 and a flow rate of 1.0 ml/min. at 40°C. Molecular weights were standardized against PEO/PEG narrow molecular weight standards with THF as the internal standard.

Sample Preparation for NMR: A 0.1 % (w/w) sample of pectin was prepared for ^1H NMR analysis by dissolving and lyophilizing the sample in d_2-H_2O (99.96 %) at pD 4 twice prior to final dissolution in d_2-H_2O (99.996 %). For ^{13}C NMR spectroscopy, samples were prepared at 2 % (w/w) in d_2-H_2O (99.96 %) and adjusted to pD = 4 prior to analysis.

NMR Analysis: ^1H and ^{13}C NMR spectra were acquired at 80°C on Bruker AMX-500 and AMX-400 NMR spectrometers, respectively. Instrumental acquisition parameters were as follows for ^1H NMR spectra, acquired using a 5 m.m. o.d. inverse probe:

sweep width:	6329.12 Hz	acquisition time:	2.59 sec.	number of scans:	128
number of points:	32 k	relaxation delay:	2.4 sec.	pulse angle:	70°

and for ^{13}C NMR spectra, acquired using a 10 m.m. o.d. broadband probe:

sweep width:	25,000 Hz	acquisition time:	1.31 sec.	Number of scans:	12 k.
number of points:	64 k	relaxation delay:	3.5 sec.	pulse angle:	70°
decoupling:	waltz-16 with nOe.				

Chemical shifts were referenced externally to acetone at 2.22 ppm (^1H) and acetonitrile, at 1.30 ppm (^{13}C). Peak assignments used in this study were based on the work of Grasdalen, et al.[11] and Westerlund, et al [14] for ^1H and ^{13}C, respectively, with subsequent experimental con-

firmation in this work. Peak deconvolution was completed by first processing the data on a Windows™ PC using WIN-NMR™ software (Bruker Instruments). The resulting file or portion of the file for analysis was stored as an ascii dataset. These data were then imported into PEAKFIT™ v.4 (SPSS) and fitted to a combination Gauss-Lorentz line profile. The areas obtained for the diad and triad areas were normalized between ^{1}H and ^{13}C spectra by virtue of the peak areas which were used to determine d.e. for the polymers.

Results and Discussion

Of primary concern is the stability of the pectins during analysis under the conditions used for NMR measurements. It was determined through trial and error that the optimal pH for analysis is 4.0. Several factors dictated the selection of this pH value. The methyl ester groups present are subject to saponification at pH values greater than 4.5 at 80°C. Spectra run at pH values above 4.5 showed evidence of methanol generation, indicating saponification was occurring in the short time interval required for a ^{1}H spectrum. Also in the ^{1}H spectrum, at pH values lower than 4, the H5 resonances from unesterified GalA rings shifts downfield and is obscured by signals from H1 protons on esterified rings. In fact, the spectral resolution increases as the pH is increased above 4, but the loss of methyl groups was determined to be unacceptable for this study.

A second concern was the degradation of molecular weight as a function of time at 80°C. In the ^{1}H case, this loss is minimal. However, the ^{13}C NMR spectra typically require 16 hours of signal averaging to obtain acceptable signal-to-noise ratios. A study of molecular weight versus time at 80°C and pH = 4 was completed to determine the extent of molecular weight degradation during NMR analysis. A plot of M_n versus time at 80°C for a standard high methoxyl, HM pectin is shown in Figure 1. As seen in this graph, the M_n drops by a factor of 2 over the 16 hours required to collect a ^{13}C NMR spectrum. A ^{1}H NMR spectrum was acquired before and after a ^{13}C NMR spectrum was acquired of this pectin to determine the effects on substituent distribution. While the loss in molecular weight is dramatic, the d.e. values determined from ^{1}H and ^{13}C NMR, and subsequently compared with a chromatographic analysis, were all in excellent agreement. This degradation of molecular weight was also noted to increase as pH increased and to occur more slowly at lower pH values.

A ^{13}C NMR spectrum of a citrus pectin with a d.e. 70 is shown in Figure 2. The ^{13}C spectrum contains ester-centered triad sequences exhibited in the ester carbonyl signals at 170 ppm (expansion, E=ester, G=acid) and three different measurements of d.e. from the splitting

FIGURE 1: M_n as a function of sample heating time for an HM pectin at 80°C and pH = 4

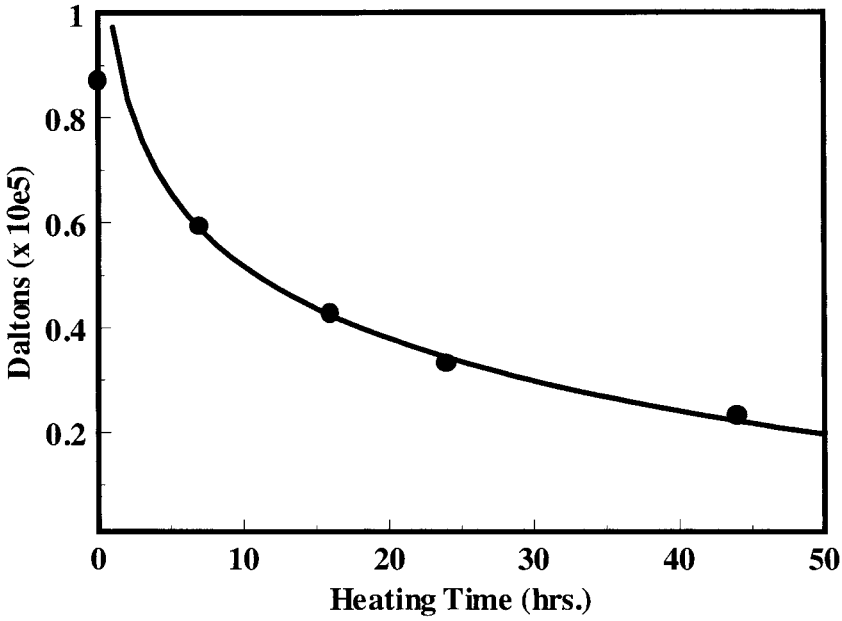
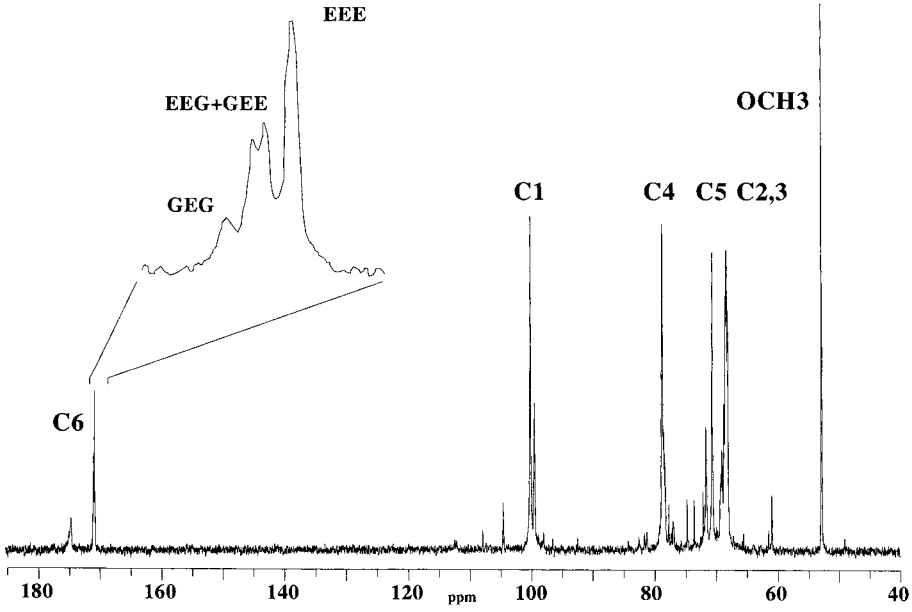


FIGURE 2: ^{13}C NMR spectrum of HM pectin with enlargement of methyl ester carbonyl region. (*See text for discussion*)



of the signals due to C1, C4, and C5 at 99.6, 78.6 and 71 ppm, respectively. Depending on the resolution in the NMR spectrum as well as the d.e. of the pectin, diad sequence information could also be determined from C4 and C5 resonances in the ^{13}C spectrum. Also visible is the methyl ester resonance at 54.3 ppm, and numerous signals from neutral sugars which constitute the hairy region of pectin, primarily galactose, arabinose and rhamnose.

Additional sequence information can be gathered from the ^{1}H NMR spectrum, an example of which is shown in Figure 3. The H1 signal is split into two pairs of lines at 5.15 and 4.95 ppm, representing the acid and ester diads, respectively. In addition, the H5 signal is split into two pairs of multiplets at 5.05 and 4.65 ppm. These signals contain ester- and acid-centered triad information respectively. The H4 resonance, seen at 4.45 ppm, is split into ester and acid signals and is an independent measurement of d.e. Assignment and chemical shift data for pectins is presented in Table I.

FIGURE 3: ^{1}H NMR spectrum of an HM pectin. *(See text for discussion)*

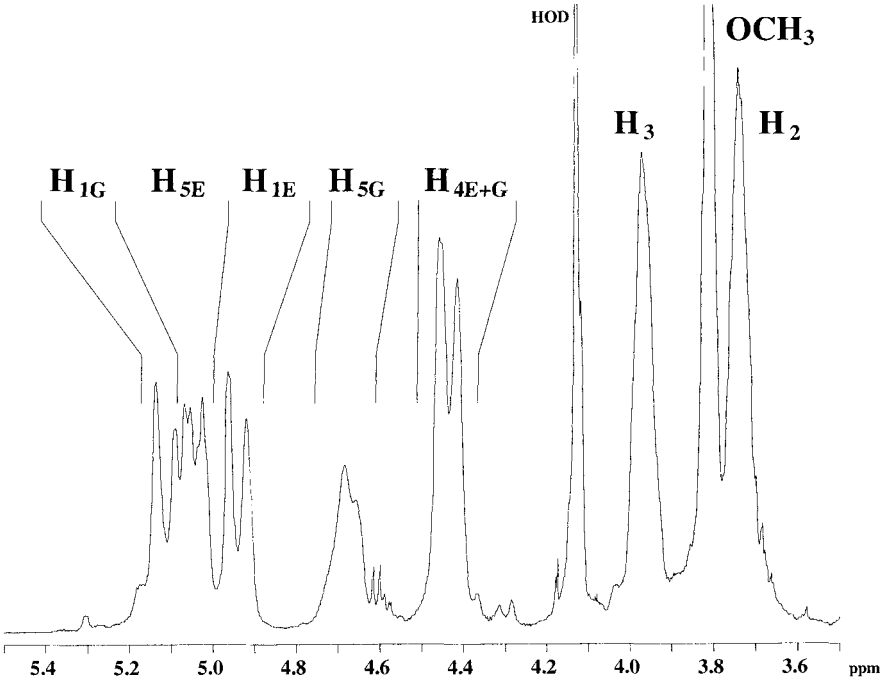


TABLE I: ^{13}C and ^{1}H NMR chemical values and resonance assignments for pectins.

	Position						
	1	2	3	4	5	6	OCH_3
^{13}C assignments:							
E		68.69	68.85	79.20	71.18		53.42
G		69.12	69.47	79.00	71.97		
EE	100.61						
EG	100.49						
GE	99.97						
GG	100.02						
EEE						171.11	
EEG						171.21	
GEE						171.25	
GEG						171.36	
^{1}H assignments:							
E		3.77	4.00	4.49			3.84
G		3.77	4.00	4.44			
EE	5.00						
EG	4.95						
GE	5.17						
GG	5.12						
EEE					5.06		
EEG					5.07		
GEE					5.08		
GEG					5.10		
GGG					4.75		
GGE					4.71		
EGG							
EGE					4.67		

Ten pectins produced in various ways were used in this study. Samples A, D and G represented pectins obtained from citrus peel extraction. Samples B, F and I were derived from a fully methyl esterified starting material which was base-saponified to d.e. levels of 0.67, 0.55 and 0.34 respectively and are assumed to be totally random in methyl ester distribution. Sample A was base-deesterified to d.e. values of 0.46 and 0.35 to produce samples E and H respectively. Samples C_1 and C_2 were fractionated from a sample similar to Sample A through a proprietary process to produce a fraction with a blockwise distribution of unesterified GalA units (C_2) and a second fraction which does not contain these blocks (C_1). A stacked plot of the ^{1}H NMR spectra of these samples is shown in Figure 4. Of primary inter-

est in these spectra is the group of resonances at 4.7 ppm which correspond to the H5 proton on unesterified GalA units. These signals represent the acid-centered triads present in pectins and permit the direct observation of GalA block sequences through observation of the GGG triad peak at 4.75 ppm. Also a prominent feature are the changes to the H1 diad signals which are also a valuable indicator of the sequence distribution of ester and acid groups in the materials under analysis.

Diad, triad, and d.e. data from both ^{13}C and ^{1}H spectra were extracted through deconvolution to generate a set of diad and triad intensities as well as the d.e. values for each sample. Triad signals from the acid-centered triads were only observable in the ^{1}H spectra while the ester-entered triads were not always resolvable in the ^{1}H spectrum, but easily accessible in the ^{13}C spectrum. Complete sets of diad and triad intensities were obtained for all the samples examined in this study (Table II).

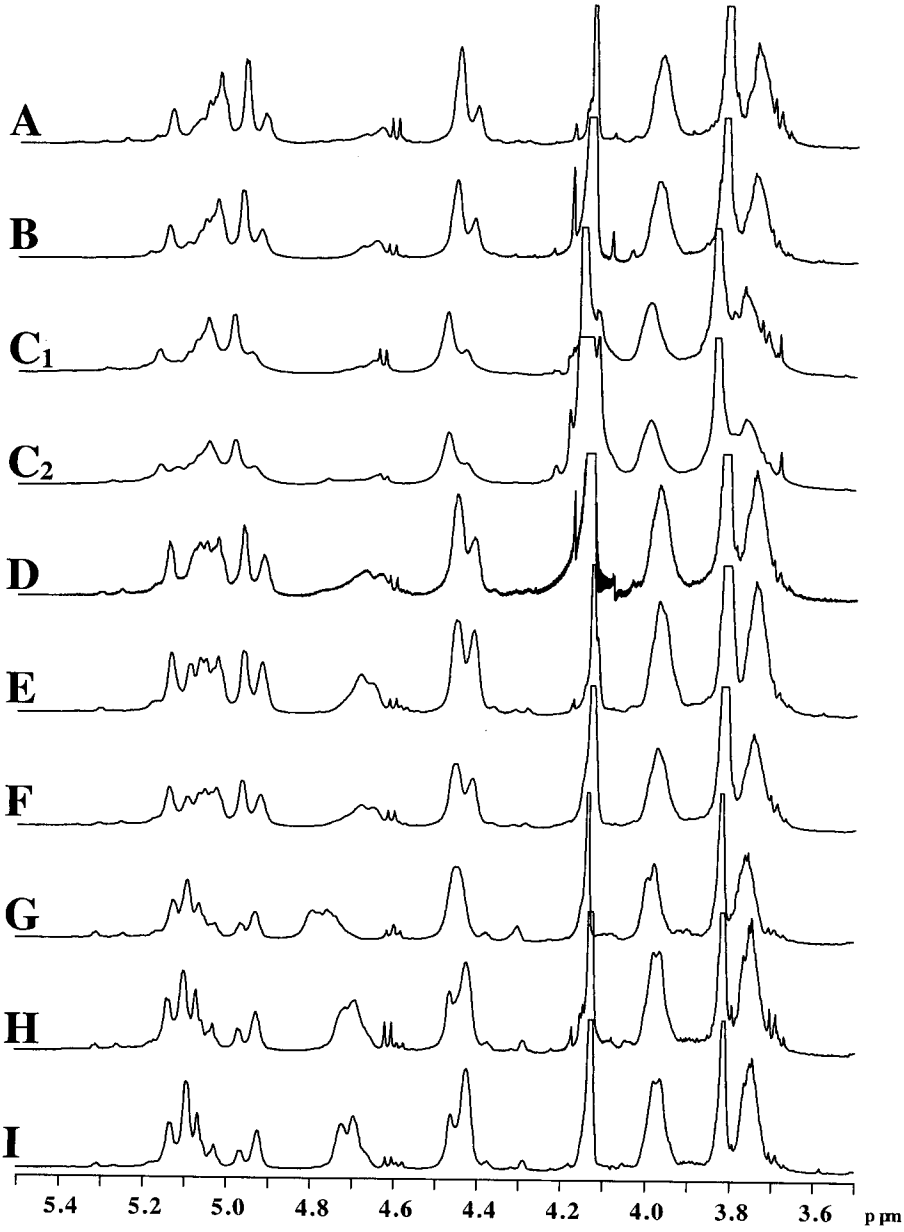
From the data given in Table II, estimates can be made of the average block lengths of E and G sequences: $n_E = 2(E)/(EG)$, and $n_G = 2(G)/(EG)$. These are shown in the last two rows of Table II. Note that these estimates assume that the polymers are homogeneous in composition, and this assumption is a weakness of this simple analysis.

Experimental triad data were fitted to both Bernoullian and first-order Markovian probability models, using the mean deviation (m.d.) as a measure of the goodness-of-fit[22]. Bernoullian results for all of the samples were found to be unsatisfactory. The results for the

TABLE II: Intensities of the triad sequences for the pectin samples.

	pectin sample									
sequence	A	B	C_1	C_2	D	E	F	G	H	I
(E)	.668	.668	.729	.676	.515	.533	.545	.275	.310	.310
(G)	.332	.332	.271	.324	.485	.467	.455	.725	.690	.690
(EE)	.466	.433	.468	.410	.298	.256	.289	.083	.087	.082
(EG)	.430	.450	.431	.475	.448	.554	.512	.481	.460	.501
(GG)	.104	.117	.101	.115	.254	.189	.199	.436	.454	.417
(EEE)	.324	.286	.447	.335	.142	.099	.170	.021	.043	.016
(EEG)	.280	.313	.280	.283	.253	.268	.289	.104	.123	.140
(GEG)	.064	.068	.033	.087	.147	.175	.109	.132	.152	.167
(EGE)	.130	.189	.166	.106	.171	.109	.126	.054	.078	.085
(GGE)	.140	.112	.051	.121	.213	.281	.232	.262	.348	.311
(GGG)	.062	.030	.054	.097	.101	.071	.097	.410	.264	.294
n_G	1.54	1.48	1.25	1.36	2.17	1.68	1.78	3.01	3.00	2.75
n_E	3.11	2.97	3.38	2.85	2.30	1.92	2.13	1.14	1.35	1.24

FIGURE 4: ^{1}H NMR spectra of samples used in this study



Markovian analysis are presented in Table III. P_{EG} and P_{GE} are the first-order Markovian conditional probabilities; for comparison, P_E, the Bernoullian probablities for ester placement, are

TABLE III: Results of one-component first-order Markovian statistical analysis of triad intensities determined from ^{13}C and ^{1}H NMR assuming absence of compositional heterogeneity.

Sample	P_E	P_{GE}	P_{EG}	$r_E \cdot r_G$	n_E	n_G	m.d.
A	0.687	0.631	0.306	1.3	3.3	1.6	0.6
D	0.517	0.608	0.498	0.7	2.0	1.6	1.2
G	0.253	0.250	0.714	1.2	1.4	4.0	0.4
B	0.666	0.768	0.353	0.6	2.8	1.3	0.6
F	0.550	0.534	0.447	1.1	2.2	1.9	0.5
I	0.338	0.344	0.711	0.8	1.4	2.9	0.4
E	0.461	0.594	0.563	0.5	1.8	1.7	2.5
H	0.349	0.362	0.654	0.9	1.5	2.8	1.6
C_1	0.757	0.837	0.250	0.6	4.0	1.2	1.5
C_2	0.688	0.542	0.292	1.3	3.4	1.9	1.9

also included. The products of the reactivity ratios, $r_E.r_G$, for these samples have values of 0.6 - 1.3, suggesting slightly non-random acid/ester distributions. The average block lengths for E and G sequences (n_E and n_G) have been calculated from parameters of the Markovian model. These calculations again assume that the polymers are homogeneous in composition.

The native pectins (A,D,G) can be fitted to the first-order Markovian model although these samples have considerable compositional heterogeneity (*vide infra*). The saponified samples from highly esterified pectins (B,F,I) also appear to fit reasonably well with the first-order Markovian model. However, samples E and H are not satisfactorily represented by either model (m.d.>1.0). These samples were produced by the saponification of sample A. While it is anticipated that base saponification of pectin should be a random occurrence[14], the results for these two samples suggest a relatively non-random distribution of monomers. A possible explanation for this result is that domains of different hydrophobicity exist in the structure of native pectin and are not all equally accessible to the alkali for saponification under the conditions employed in this study.

Triad intensities for fractions C_1 and C_2, obtained by fractionation of a native pectin similar to sample A, are also poorly predicted using a first-order Markovian model. Thus, intermolecular heterogeneity persists in these fractions, even after separating pectin chains into two distinct populations. This chemical heterogeneity cannot be treated with simple one-component Bernoullian or Markovian models. More complex statistical models are needed.

Previously a number of statistical models have been used for synthetic polymers that exhibit compositional heterogeneity[23-27]. In this work both a continuous distribution model[24,25] and a discrete, three-component model[26,27] were used to examine the triad distri-

butions of Fractions C_1 and C_2. In the first case, an exponentially modified Gaussian (EMG) chemical composition distribution (CCD) curve of the form[24]

$$f(t) = \frac{N}{\tau\sigma\sqrt{2\pi}} \int_0^\infty \exp\left[\frac{-(z - P_E - t')^2}{2\sigma^2} - \frac{t'}{\tau}\right] dt'$$

was used in conjunction with the Bernoullian model. Again P_E is the Bernoullian probability, and σ and τ are broadening and asymmetry parameters. Results obtained are given in Table IV and also represented graphically in Figure 5. The values obtained for σ and τ using the modified Gaussian function indicate that Fractions C_1 and C_2 are still substantially heterogeneous in nature with asymmetry in the distribution curves ($\sigma, \tau \neq 0$). For completeness, the analysis of samples E, H, B, F, and I through the EMG function has also been made. As expected, all these samples have relatively narrow CCD curves (small or zero σ and τ values). Interestingly, samples E and B, being the least saponified samples, do not fit the EMG-Bernoullian model well (m.d. > 1.00), perhaps still showing some lingering effects of compositional heterogeneity. However, the EMG-first-order Markovian model does produce much better fit for the two samples (Table IV, footnote).

While the compositional distribution curve can visually demonstrate the extent of heterogeneity for a particular polymer, it does not provide adequate detail on the types of domain structures present in the polymer. Discrete component analysis can produce a more quantitative picture of the ensemble of structural components present in pectin. In the discrete model,

TABLE IV: Results of the analysis of pectin samples by exponentially modified Gaussian (EMG) coupled with Bernoullian model.

Sample	P_E	σ	τ	m.d.
A	0.676	0.116	-0.009	0.36
C_1	0.773	0.090	-0.021	1.05
C_2	0.675	0.152	-0.028	0.72
E	0.502	0.003	0.024	1.98[a]
H	0.302	0.009	0.030	0.72
B	0.680	0.003	-0.021	1.41[b]
F	0.547	0.039	0.000	0.55
I	0.321	0.002	0.009	0.80

[a] Improved goodness-of-fit is obtained using EMG function with first-order Markovian probabilities: P_{EG} = 0.573, P_{GE} = 0.536, σ = 0.019, τ = 0.021, m.d.=1.36.

[b] Improved goodness-of-fit is obtained using EMG function with first-order Markovian probabilities: P_{EG} = 0.346, P_{GE} = 0.762, σ = 0.048, τ = -0.009, m.d.=0.38.

TABLE V: Analysis of samples C_1 and C_2 through discrete Bernoullian components.

Component (κ)	P_E	w_{C1}	w_{C2}	n_E	n_G
1	0.77	0.92	0.29	4.35	1.30
2	0.67	0.04	0.64	3.03	1.49
3	0.02	0.04	0.07	1.02	50

the polymer is assumed to contain several components, and the observed NMR triad intensities are assumed to be the weighted averages of the intensities of the components[26,27]. Since both fractions C_1 and C_2 originate from the same parent sample (A), they can be analyzed simultaneously, thereby providing increased degrees of freedom for the analysis. This discrete model was attempted using Bernoullian and first-order Markovian components. As it turned out, the latter showed only marginal improvements in the mean deviations; thus, the Bernoullian components were used.

For Fractions C_1 and C_2, the data can be fitted to three Bernoullian components with d.e. values of 0.77, 0.67, and 0.02, respectively (P_E=d.e. for the Bernoullian model). Components 1 and 2 are random in methyl ester sequence distribution while component 3 is a large homogalacturonan block. Both fractions show a different proportion of these three components, as given in Table V. Fraction C_2 contains a significantly higher amount of component 3 and considerably less of component 1, indicative of a more blocky nature of the unesterified galacturonic acid distribution for this fraction.

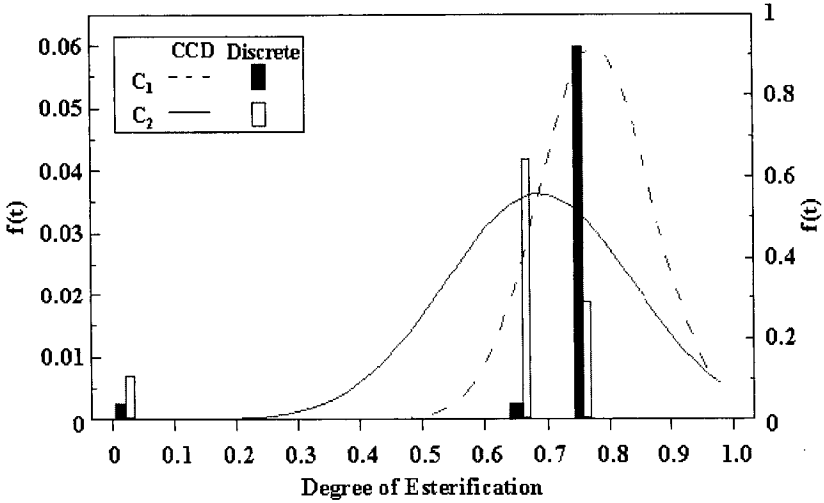
The average block lengths are shown in the last two columns of Table V. From the average block lengths, the overall block length <n> for fractions C_1 and C_2 can be calculated,

$$\text{overall } \langle n \rangle = \Sigma\, w_\kappa\, n_\kappa$$

where κ denotes the component number and ranges from 1 to 3 in this case. Thus, for fraction C_1 the overall $\langle n_E \rangle = 4.16$, and the overall $\langle n_G \rangle = 3.26$. For fraction C_2 the overall $\langle n_E \rangle = 3.27$, and the overall $\langle n_G \rangle = 4.83$. Note that these results are different from the results of direct calculation (Table II) and one-component first-order Markovian analysis (Table III). Thus, an advantage of the more complex models is that they provide more accurate representations of the microstructures of these polymers.

The compositional distributions of the three components for each fraction in the discrete component analysis are also shown in Figure 5 as bargraphs. The two fractions (C_1 and C_2) have rather different compositional distributions. Note that the shift in distribution from C_1 to C_2 is reflected in the different width and asymmetry of the modified Gaussian curves. Although the continuous function and the discrete component approaches are carried out in

FIGURE 5: CCD for Fractions C_1 and C_2 showing both the EMG functions (curves) and the discrete three-components



different ways, the CCD curves and the bargraphs in Figure 5 qualitatively show the same trends, thereby confirming the general validity of the present approaches.

Conclusions

Experimental results and statistical calculations presented in this work provide insight into the heterogeneous nature of methyl esterification patterns in pectins. Superficially, samples isolated from citrus peel appear to exhibit a random distribution of galacturonic acid and its methyl esterified monomers in the polymer. This result belies the fact that there are discrete domains of differing monomer content and distribution within the sample, and attempts to use NMR without further fractionation of the sample tend to produce an average, randomized distribution. NMR has the potential to provide heterogeneity information when coupled with methods for fractionating pectins. A caveat is noted that whereas statistical modelling is useful to analyze data and to understand pectin structure, they still must be considered as hypothetical models. Different models can be fitted to the same data. The models also ignore structural features such as the presence of neutral sugars and their effect on sequence distributions. Continued investigation is required to fully understand the effects of intermolecular (chemical) and intramolecular (sequential) heterogeneity on polymer performance in specific applications.

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