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Investigation of the Distribution of Methyl Ester Groups in Pectin by High-Field ^{13}C NMR

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ABSTRACT

Fully methyl-esterified, water-soluble citrus pectin was partially hydrolysed with alkali to different degrees of esterification (DE). High-field ^{13}C-NMR spectroscopy of the products showed that ester carbonyl carbons appeared as four signals due to different influences from neighbouring sugar residues (free or methyl-esterified galactosyluronic acid). Based on the relative peak areas of these signals, it was deduced that alkaline de-esterification occurred randomly. The distribution of methyl ester groups was also found to be random in water-soluble native pectins of tobacco (DE 20) and papaya (DE 70).

INTRODUCTION

Pectic substances or pectins have long been known to include diverse types of polysaccharide structures, based on a main chain of (1→4)-linked α-D-galacturonic acid with occasional interruption by L-rhamnose residues (Stephen, 1983). Pectins are of great importance in the construction of primary cellular tissues and, in this role, may be linked covalently to other plant polymers. Pectins are also found in the middle lamella. The occurrence of methyl ester groups in various proportions and distributions in pectins was recognised early, as was a wide variation in molecular weight and attendant properties such as solubility, viscosity and gelation (Stephen, 1983). The degree of methyl esterification (DE) of the galacturonic acid residues in the main chain varies with, for example, botanical source, stage of maturity and storage or other pretreatments of the sample. Acetic and cinnamic ester groups may also

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be linked to the galacturonic acid residues and influence the important gelling properties.

Some of the methods used for the determination of DE are based on titration (Schultz, 1965), copper complexation of free carboxyl groups (Katan & van de Bovenkamp, 1981) or measurement of methanol released on alkaline treatment, for instance by gas–liquid chromatography (Knee, 1978; Voragen *et al.*, 1980).

Another more direct approach uses NMR spectroscopy (Sun *et al.*, 1987) which, in addition to DE, gives information on random or block-wise ester distribution in the main polysaccharide chain (Grasdalen *et al.*, 1988). The distribution of unesterified carboxyl groups in pectins has been studied utilising differences in their affinity on DEAE-Sephacell (Anger & Dongowski, 1985). The carboxyl groups were found to be randomly distributed in most of the fruit and vegetable preparations studied. Based on the calcium ion activity in solutions of the calcium pectinate, a random distribution was also deduced for a native apple pectin preparation (Kohn & Furda, 1967). Alkaline de-esterification of pectin is considered to cause a random distribution of the ester groups (Taylor, 1982). Recently this has been confirmed by ^{1}H-NMR spectroscopy of de-esterified samples prepared from fully esterified apple pectin (Grasdalen *et al.*, 1988).

The aim of the present study was to evaluate the potential of high-field ^{13}C NMR as a tool to distinguish between block-wise and random ester distribution in pectins.

EXPERIMENTAL

Samples

Commercial citrus pectin was obtained from FLUKA, Buchs, Switzerland. The sample of water-soluble tobacco pectin was from a previous study (Sun *et al.*, 1987). Papaya pectin was isolated by homogenisation of the fresh fruit pulp (113 g, Brazil brand) with hot, 80% aqueous ethanol (150 ml) for 5 min in a Sorvall blender. Next, the residue was refluxed in 80% ethanol (600 ml), and the insoluble residue recovered by centrifugation (1000*g*, 15 min) was vacuum-dried (60°C, 2 h) and further refluxed for 45 min with water (250 ml). After centrifugation, the decanted supernatant was acidified with Dowex 50 (H^+), filtered and kept for 2 h at 4°C after addition of 5 volumes of 95% ethanol. After centrifugation, washing with 80% ethanol, vacuum-drying, dissolution in

water (50 ml) and freeze-drying, the precipitated material yielded the water-soluble papaya pectin (655 mg).

Methyl esterification

Citrus pectin (700 mg) was fully esterified by two consecutive treatments with 50 ml of 2M methanolic H_2SO_4 for 120 h at 4°C (Kohn & Furda, 1969). The sulphuric acid was removed from the resulting insoluble residue by thorough washing with methanol, methanol:water (3:1) and methanol (Grasdalen *et al.*, 1988), using centrifugation and decantation until the supernatant was neutral. The residue obtained after air-drying, dissolution in water and freeze-drying (400 mg) was, according to NMR analysis, fully esterified.

Alkaline de-esterification

The fully esterified citrus pectin (130 mg) was dissolved in deuterium oxide (15 ml). After removal of insoluble material by centrifugation, the pectin in the supernatant was partly de-esterified by treatment with different amounts of a 40%-NaOD solution at room temperature. The DE obtained was preliminarily estimated by ^{1}H-NMR analysis and the solution then acidified with 2M HCl, dialysed and freeze-dried. The DE of the final material was determined by ^{13}C NMR.

Complete de-esterification of fully esterified citrus pectin (110 mg) was performed by treatment with 1M NaOH (0·6 ml) in water (10 ml) for 1 h at room temperature with stirring (McCready, 1965). The product was acidified with 2M HCl (0·5 ml), dialysed and freeze-dried.

NMR spectroscopy

400-MHz ^{1}H-NMR and 101-MHz ^{13}C-NMR spectra were recorded at 85°C on a Varian VXR-400 instrument, using sodium 3-(trimethylsilyl)-propionate-d_4 as internal reference. The pulse width was for samples of native papaya and tobacco pectin set at 45°C with a repetition time of 1·5 s. Corresponding parameters for citrus pectins were 67°C and 3·0 s. A waltz modulator was used for broadband decoupling. Freeze-dried samples were analysed as 4–5% solutions in deuterium oxide. For better resolution, line broadening was not applied when the carbonyl resonances in Fig. 2 were plotted.

RESULTS AND DISCUSSION

In the present investigation, water-soluble samples of citrus pectin with different degrees of methyl esterification (DE 0–100), prepared by alkaline hydrolysis of fully methyl-esterified pectin, as well as samples of native papaya (DE 70) and tobacco pectin (DE 20) were studied by high-field ^{13}C NMR. In the spectra of citrus pectin (DE 60), the anomeric carbon, C-1, appeared as two major signals at 102·4 and 102·9 ppm, due to the presence of esterified and unesterified galactopyranosyl uronic acid residues (Fig. 1). In the spectrum of tobacco pectin (DE 20), the corresponding signals appeared at 102·6 and 102·9 ppm. In either case, the mutual assignment of the C-1 signals was based on their intensity ratio and the approximately known DE value. Once the chemical shifts were assigned for these types of anomeric signals, the DE of the sample could be calculated from their intensity ratio.

The resonances of the other galacturonic ring carbons, C-2 to C-5 appearing in the region 70–82 ppm, were assigned by comparison with those observed in fully esterified and de-esterified citrus pectin. Predominant signals in fully esterified citrus pectin were thus found at 102·9 (C-1), 81·5 (C-4), 73·4 (C-5), and 70·9 and 71·0 (C-2 and C-3) ppm, whereas corresponding signals for the fully de-esterified citrus pectin appeared at 102·5 (C-1), 81·0 (C-4), 73·4 (C-5), and 71·1 and 71·4 (C-2 and C-3) ppm.

The spectrum of tobacco pectin, previously investigated by Sun *et al.* (1987), was more complex than that of the citrus pectin since higher amounts of neutral sugar residues were present. Thus, signals for C-1 of galactosyl residues in side chains were observed around 106 ppm. Minor resonances at 19·4 and 19·6 ppm originated from C-6 in rhamnose residues, substituted or unsubstituted with the galactosyl chains. The signal at 55·7 ppm, present in both spectra, originated from the methyl group in esterified glycosyluronic acid residues.

Interesting differences, correlated to the DE of the citrus pectins studied, were observed in the region for the resonances of carbonyl groups (carboxyl and ester carbonyls) at 173–177 ppm. In an expanded view of this region for the citrus pectin with different DE values (Fig. 2), a broad signal originating from the carboxyl carbon could be observed. The signal became sharper and moved upfield with decreasing DE. This upfield shift may be due to differences in pH as a result of the increasing carboxyl content of the sample (Deuel & Stutz, 1958; Jaques *et al.*, 1979), as well as in molecular conformation and degree of hydrogen bonding. Further, the different signals of ester carbonyls, observed at 173–174 ppm, indicated that the chemical shifts for C-6 in esterified

residues were affected by different neighbouring sugar residues (free or methyl-esterified acid) in the main pectic chain. It has previously been observed by ^{13}C NMR that some of the individual carbon resonances of mannuronic and guluronic acid residues in alginates are resolved into four lines, reflecting variations in the two immediate neighbouring

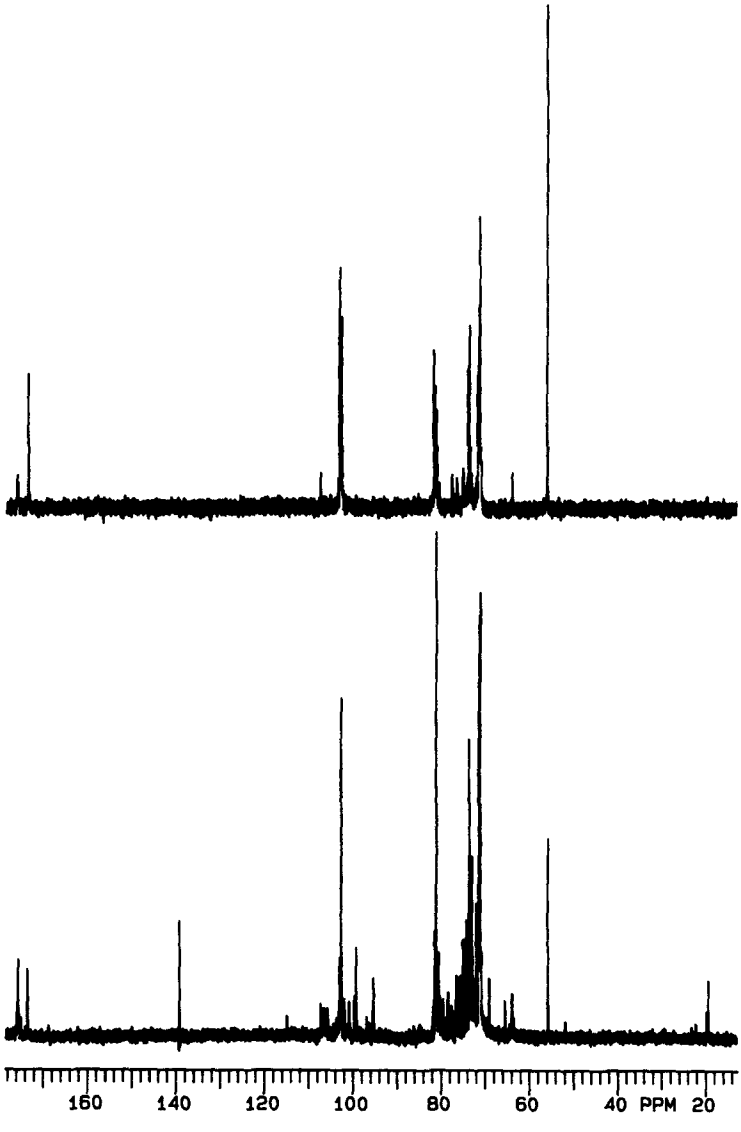

Fig. 1. ^{13}C-NMR spectra of fully methyl-esterified and, thereafter, partly de-esterified citrus pectin (DE 60, upper spectrum) and of native tobacco pectin (DE 20, lower spectrum) in deuterium oxide.

residues (Grasdalen *et al.*, 1981). Thus, an esterified (E) residue in the pectic backbone may be surrounded by esterified residues (EEE), unesterified (UEU) residues or by one esterified and one unesterified residue (EEU and UEE). The effects of DE on the relative amounts of

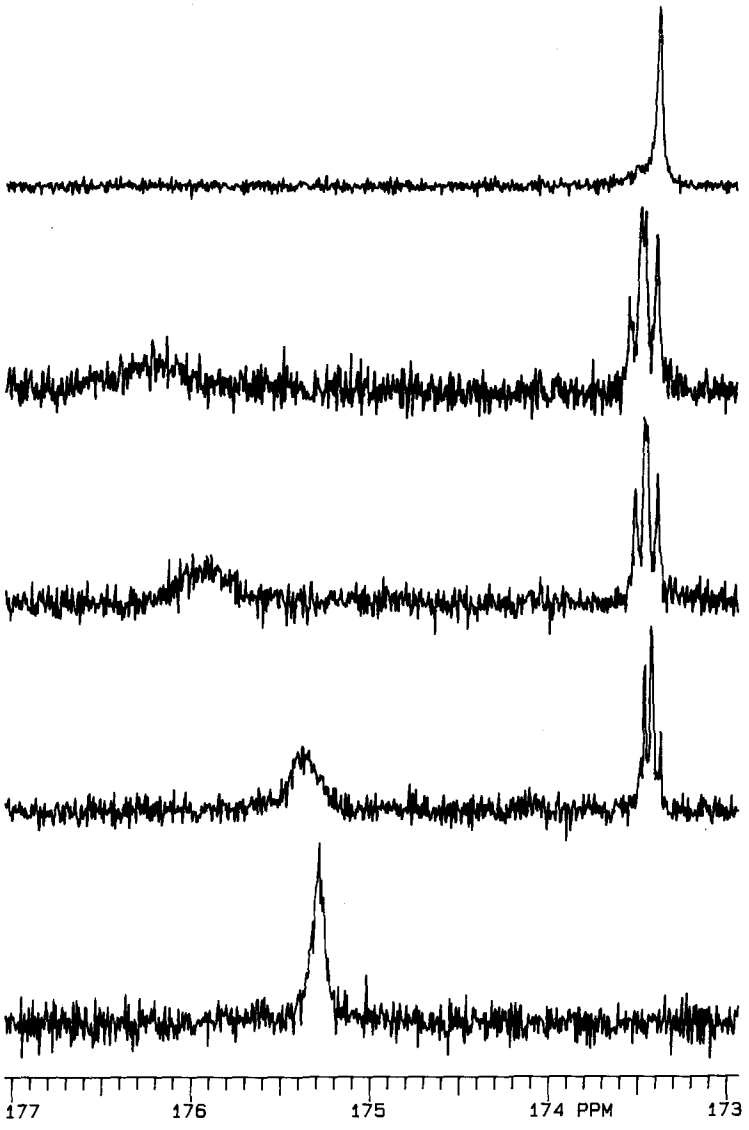

Fig. 2. Carbonyl region of ^{13}C-NMR spectra of fully methyl-esterified and partly de-esterified citrus pectins (DE 100, 60, 50, 40, and 0, shown from the top to the bottom) in deuterium oxide.

these ester combinations, in case of random distribution, were calculated by multiplying the statistical probabilities of finding the two neighbouring residues, and are presented in Fig. 3.

Assignments of the different ester carbonyl resonances were made by comparing the spectra of citrus pectins with different DE values (Fig. 2), taking into account that the relative proportions of these signals are dependent on DE (Fig. 3). The ester carbonyl resonances thus appeared at 173·46 to 173·54 (UEU), 173·42 to 173·46 (UEE or EEU), 173·45 to 173·46 (UEE or EEU) and 173·38 (EEE) ppm. The chemical shifts for an esterified residue linked to one or two unesterified residues were slightly affected by the nature of the more remotely situated residues, as shown by the coalescence of the two middle ester signals (in Fig. 2) and the upfield shift for the left-hand signal with decreasing DE. The relative intensities of these signals, at the DE values investigated, were in close agreement with those values calculated from a random distribution (Fig. 3). These results clearly demonstrate the usefulness of high-field ^{13}C-NMR for studying the distribution of methyl groups in pectin. For the native samples of papaya (DE 70) and tobacco pectin (DE 20), the NMR pattern suggested a random distribution of ester groups (Fig. 4). This was

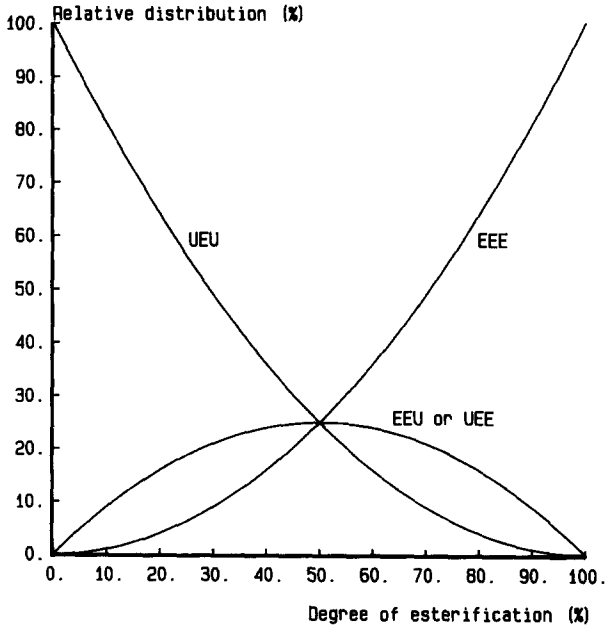

Fig. 3. Calculated relative distribution of methyl-esterified galacturonic acid residues (middle E) linked to different neighbouring residues (esterified residues = E, unesterified residues = U) at different DE values.

shown by the occurrence of more than one ester carbonyl signal, and by the fact that the chemical shifts of the signals were in close agreement with those found for the partly de-esterified citrus pectins of corresponding DE. Also, the relative intensities of these signals were consistent with those expected from a random distribution of esterified sugar residues. The good agreement (within 5%) between calculated and observed values strongly suggests that only minor amounts of block-wise distributed carboxyl groups could be present in the polymers studied. Block-wise arrangement of carboxylic groups could arise from enzymatic hydrolysis of esters (Kohn & Furda, 1968) during the isolation procedure and/or biosynthetical formation. The occurrence of more than one carboxylic signal for the tobacco sample (Fig. 4) might be due to the presence of unbranched and branched rhamnosyl residues (substitution with galactose residues) in the main pectic chain, since this was not observed for the papaya and citrus samples with low contents of rhamnosyl residues.

The present study on the ester distribution in papaya and tobacco pectin was consistent with some previous reports that indicated

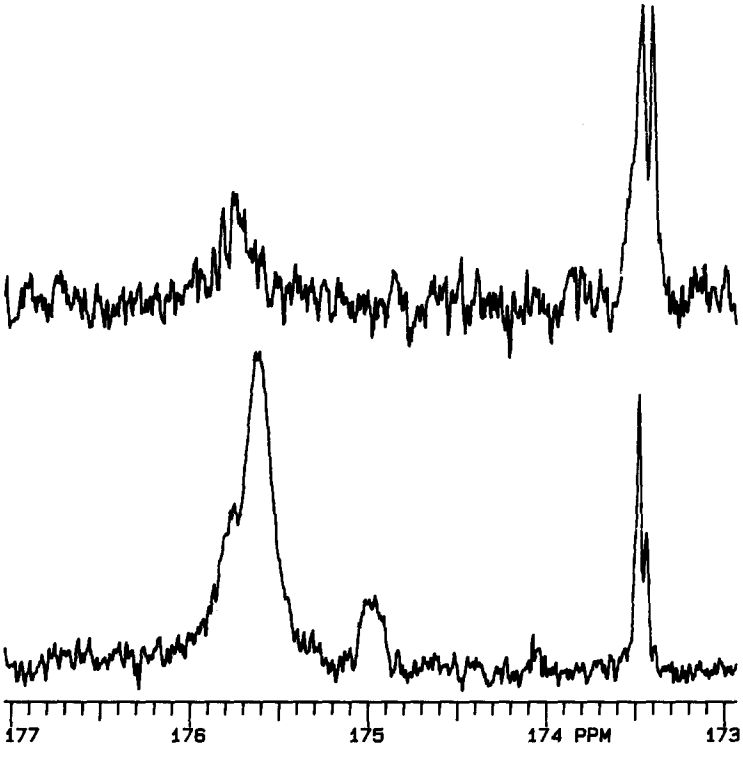

Fig. 4. Carbonyl region of ^{13}C-NMR spectra of native papaya (DE 70, upper spectrum) and tobacco pectins (DE 20, lower spectrum) in deuterium oxide.

carboxylic groups to be randomly distributed in fruit pectins (Kohn & Furda, 1967; Anger & Dongowski, 1985).

REFERENCES

Anger, H. & Dongowski, G. (1985). Verteilung der freien Carboxylgruppen in nativen Pektinen aus Obst and Gemüse. *Nahrung*, **29**, 397–404.

Deuel, H. & Stutz, E. (1958). Pectic substances and pectic enzymes. In *Advances in Enzymology*, Vol. XX, ed. F. F. Nord. Interscience Publishers, New York, pp. 341–82.

Grasdalen, H., Larsen, B. & Smidsrød, O. (1981). ^{13}C-NMR studies of monomeric composition and sequence in alginate. *Carbohydr. Res.*, **89**, 179–91.

Grasdalen, H., Bakøy, O. E. & Larsen, B. (1988). Determination of the degree of esterification and the distribution of methylated and free carboxyl groups in pectins by ^{1}H-NMR spectroscopy. *Carbohydr. Res.*, **184**, 183–91.

Jacques, L. W., Macaskill, J. B. & Weltner, W. (1979). Electric field theory applied to the hydrogen-1 and carbon-13 nuclear magnetic resonance spectra of carbohydrates. *J. Phys. Chem.*, **83**, 1412–21.

Katan, M. & van de Bovenkamp, P. (1981). Determination of total dietary fiber by difference and of pectin by colorimetry or copper titration. In *The Analysis of Dietary Fibre in Food*, ed. W. P. T. James & O. Theander. Marcel Dekker, New York, pp. 217–39.

Knee, M. (1978). Properties of polygalacturonate and cell cohesion in apple fruit cortical tissue. *Phytochem.*, **17**, 1257–60.

Kohn, R. & Furda, I. (1967). Calcium ion activity in solutions of calcium pectinate. *Collect. Czech. Chem. Commun.*, **32**, 1925–37.

Kohn, R. & Furda, I. (1968). Distribution of free carboxyl groups in the pectin molecule after treatment with pectin esterase. *Collect. Czech. Chem. Commun.*, **33**, 264–9.

Kohn, R. & Furda, I. (1969). Distribution of free carboxyl groups in the molecule of pectin after esterification of pectin and pectinic acid by methanol. *Collect. Czech. Chem. Commun.*, **34**, 641–8.

McCready, R. M. (1965). Pectin and pectic acid. In *Methods in Carbohydrate Chemistry*, Vol. 5, ed. R. L. Whistler. Academic Press, New York, pp. 167–70.

Schultz, T. (1965). Determination of the degree of esterification of pectin. In *Methods in Carbohydrate Chemistry*, Vol. 5, ed. R. L. Whistler. Academic Press, New York, pp. 189–94.

Stephen, A. M. (1983) Other plant polysaccharides. In *The Polysaccharides*, Vol. 2, ed. G. O. Aspinall. Academic Press, New York, pp. 97–193.

Sun, H. H., Wooten, J. B., Ryan, W. S., Bokelman, G. H. & Åman, P. (1987). Structural characterization of a tobacco rhamnogalacturonan. *Carbohydr. Polym.*, **7**, 143–58.

Taylor, A. (1982). Intramolecular distribution of carboxyl groups in low methoxyl pectins. A review. *Carbohydr. Polym.*, **2**, 9–17.

Voragen, A. G. J., Heutnik, R. & Pilnik, W. (1980). Solubilization of apple cell walls with polysaccharide-degrading enzymes. *J. Appl. Biochem.*, **2**, 452–68.