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Note

Rapid quantification of *O*-acetyl and *O*-methyl residues in pectin extracts

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Abstract

A rapid method for the determination of the degrees of methylation (DM) and acetylation (DA) of pectins was developed. The polymer substitution degree as determined after saponification at 80 °C with NaOD during ^{1}H NMR analysis. Under alkaline conditions, the cleavage of *O*-acetyl and *O*-methyl linkages allows the detection and the integration of the H-4 signal from galacturonic acid residues in the newly unesterified pectins. So, after a 10-min NMR recording, sodium acetate and sodium methanolate can be easily quantified relative to the clearly identified H-4 signal in galacturonic acid residues. Protons signals from pectin neutral sugars do not interfere with H-4. During the analysis, a limited ($< 3\%$) methanol evaporation leading to a weak reduced signal from the methanolate protons was observed. The proposed method allows in few minutes an accurate simultaneous quantification of DM and DA from few mg of pectin extracts, without the need of external standards.

Keywords: Pectins; ^{1}H NMR; Saponification; Methylation; Acetylation

The characterization of pectin extracts includes not only analysis of the molecular weight and sugar composition but also of the degrees of methylation (DM) and acetylation (DA). Several procedures are suitable for the DM and DA determination, however some of them require large amount (30 mg) of material.[1] Chemical procedures for the DM determination requiring less material (100 μg) were described, however they are time consuming since they require the reduction of the esterified galacturonic acid (Gal*p*A) residues before methanolysis, derivatization, and GLC analysis.[2] An alternative methodology for the determination of Gal*p*A in the reduced samples involve colorimetry, but it requires more material (2 or 3 mg).[2] Such procedures, though satisfactory with regard to the weak amount of pectin required, do not allow a one step pectin substitution analysis.

Proton NMR analysis, which is widely used for structural investigation of polysaccharides, was shown to be suitable for the determination of pectins DM.[3] However, pectin DA cannot be determined directly on ^{1}H NMR spectra in spite of well distinct signals from *O*-acetyl protons.[4,5] It was shown for homopolygalacturonic and homopolyglucuronic polysaccharides that ^{1}H NMR analysis allows simultaneously the DA determination and the localization of acetyl residues on Gal*p*A or Glc*p*A residues.[4,5] For pectins, determination of the DA is limited by the absence of well identified signals from Gal*p*A protons which resonate near or overlap signals of protons from α-arabinosyl and β-galactosyl residues. Moreover, methyl esterification of Gal*p*A generates several chemical shifts for H-1 and H-5 of Gal*p*A which may overlap each other according to the DM.[3] So, a specific ^{1}H NMR analysis methodology allowing in few min the determination of pectin DA and DM from low amounts of material was needed and is the subject of this report.

The ^{1}H chemical shifts deduced from 1D and 2D NMR analysis of pectins extracted from stems of growing flax are summarised in Table 1. The 1D ^{1}H NMR

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Table 1
^{1}H chemical shifts of native and saponified pectins from flax and citrus and sodium polygalacturonate at 80 °C

Samples	Chemical shifts (ppm)								
	Protons of substituents				Protons of the galacturonic acid residues				
	Linked acetate	Free acetate	Linked methanol	Free methanol	H-1	H-2	H-3	H-4	H-5
Flax pectins									
Native	2.04 2.09 2.11		3.76		4.70–5.20	ND	ND*	4.40	4.70–5.20
Saponified		1.82		3.28	5.02	3.71	3.89	4.34	4.58
Citrus pectins									
Native			3.76		4.70–5.20	3.71	3.92	4.40	4.70–5.20
Saponified				3.28	5.02	3.69	3.90	4.34	4.58
Sodium polygalacturonate in 100 mM NaOD					5.02	3.69	3.90	4.34	4.58

* ND, not determined.

spectrum (Fig. 1A) clearly revealed 3 signals in the region δ 2.04–2.11 and a signal at δ 3.76 characteristics of protons in *O*-acetyl and *O*-methyl residues respectively. In the low field region (δ 5.20–4.70), no distinct signal was seen on the flax pectin spectrum which contrasted with the spectrum obtained from a commercial methylated citrus pectin (Fig. 1C), devoid of neutral sugars and acetyl residues. Differences between the two spectra were due to acetylation of Gal*p*A residues, and to a high level of neutral sugars (10.8% of arabinose, 16% of galactose, 2.8% of glucose and 4.4% of rhamnose) in the flax pectin extract (data not shown).

In the ^{1}H NMR spectra of pectin citrus, the isolated signal at δ 4.40 attributed to H-4 of 4-linked Gal*p*A residues can be used for the DM determination. So, the average DM of Gal*p*A residues in citrus pectin, as determined by the ratio of the H-4 resonance from Gal*p*A residues to that at 3.76 ppm from *O*-methyl residues, was 70%. On the flax pectin spectrum (Fig. 1A), the closeness of signals at δ 4.40 and 4.56 ($J_{1,2}$ 7.2 Hz), which were attributed respectively to the H-4 and H-1 protons of 4-linked α-Gal*p*A and β-Gal*p* residues, hindered the direct determination of the flax pectins DM and DA values.

Based on the knowledge that *O*-methyl and *O*-acetyl substituents can be cleaved by alkaline treatment, the flax pectin solution in deuterium oxide (10 mg/mL) was supplemented with NaOD (100 mM final) directly in the NMR tube, then the NMR recording (256 scans) was immediately performed at 80 °C (Fig. 1B). In the conditions applied, the spectrum became simpler. A signal at δ 3.28 corresponding to protons of methanol appeared while the peak at δ 3.76 diminished. Simultaneously, the three signals corresponding to the protons of the *O*-acetyl residues disappeared and a single peak corresponding to protons of sodium acetate appeared at δ 1.82. The same NaOD treatment was performed on commercial citrus pectin during NMR recording. As above, the proton signals in *O*-methyl residues disappeared while distinct proton signals from methanol (δ 3.28) and sodium polygalacturonate[6] (Fig. 1D) were clearly identified. The apparition of a low intensity doublet at δ 5.70 ($J_{3,4}$ 3.6 Hz), attributed to H-4 of unsaturated Gal*p*A residues, was significative of a weak pectin degradation by β-elimination during the NaOD treatment.

From the fully saponified flax pectin obtained during ^{1}H NMR analysis 10 min after the NaOD addition, protons signals were compared to those of a standard sodium polygalacturonate ^{1}H NMR spectrum (Table 1). Many signals on the ^{1}H NMR spectrum of the saponified flax pectin were close to each others. Signals for H-2 (δ 3.71) and H-3 (δ 3.89) of Gal*p*A residues in the flax pectin were close to those from protons of galactosyl and arabinosyl residues. The signal for H-5 of Gal*p*A residues (δ 4.58) was near that for H-1 of β-linked galactosyl residues (δ 4.53), this later signal was partially overlapped by the signal for H-1 of rhamnose located 0.2 ppm downfield from that of H-1 of Gal*p*A.[7] In fact, in the studied flax pectin, the rham-

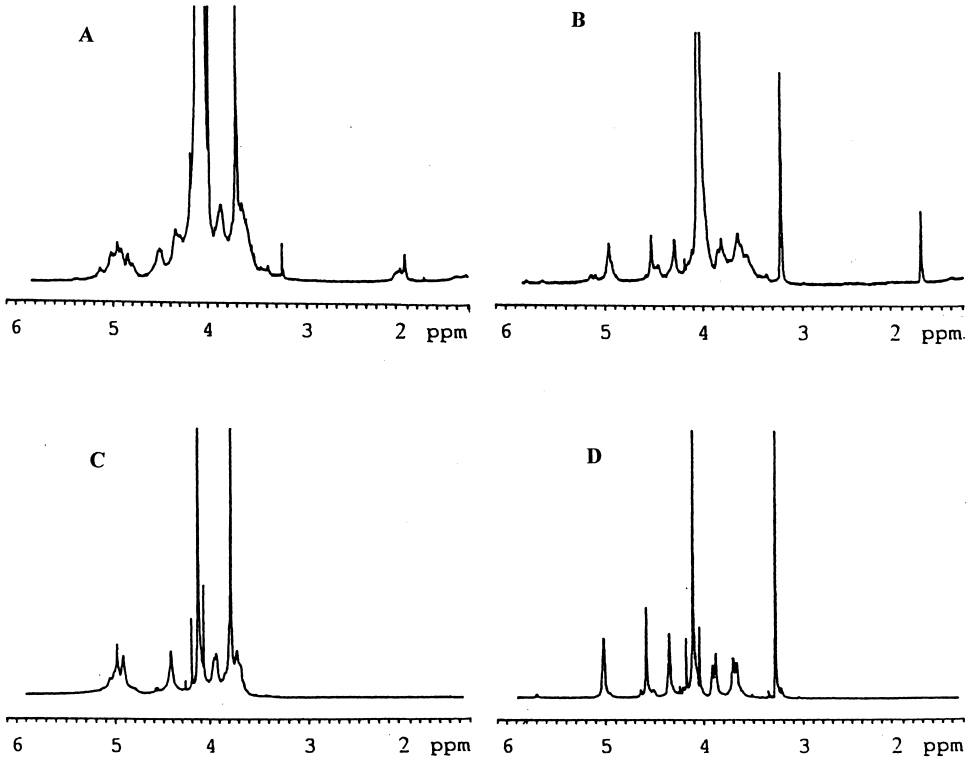

Fig. 1. ^{1}H NMR spectra (300 MHz, 80 °C) of a pectin extract obtained from flax stems (A) and a standard methoxy citrus pectin (C) (5 mg in 500 μL D_2O), and after addition of 1 M NaOD (55 μL) in the NMR tubes containing the flax (B) and citrus (D) pectins. Spectra were recorded (256 scans) immediately after the NaOD addition.

nose content determined by gas chromatography (GC) analysis was about 4% as compared to 66% of Gal*p*A residues.

The isolated signal at δ 4.34 was assigned to H-4 of Gal*p*A residues at the internal position; no significant resonance signal for H-4 of Gal*p*A residues located at the polysaccharide reducing (α and β isomers) and non-reducing ends were detected in the ^{1}H NMR spectrum of the saponified flax pectin (the low intensity signal at δ 5.70, appearing after NAOD treatment and corresponding to unsaturated H-4 of the Gal*p*A non reducing terminus was not considered for further analysis). So, the signal at δ 4.34 was considered as a potential reference for the pectin DM and DA determination.

The boiling point of methanol at 64.9 °C was considered as a possible limit for the determination of the pectin DM by ^{1}H NMR spectroscopy, as analysis was performed at a higher temperature. However, ^{1}H NMR analysis performed at temperatures lower than 64 °C was not suitable as this gave spectra with poor resolution (data not shown). Such results were correlated to the high molecular weight $5 \times 10^4 < Mw < 2 \times 10^6$ (data not shown) of the stem flax pectins leading to viscous solutions at temperatures lower than 64 °C. So, in order to decrease the viscosity in the pectin solution and improve its ^{1}H NMR spectrum resolution, all studies were performed at 80 °C.

In order to determine the yield of methanol evaporation during NMR studies at 80 °C, ^{1}H NMR analyses were performed on a mixture containing methanol (100 mM), acetic acid (100 mM) and NaOD (100 mM) in deuterium oxide (Fig. 2). The recording of 256 scans was first performed at low temperature (20 °C), then the temperature was raised to 80 °C and stabilized inside the spectrometer for further analysis. After heating for 10, 22, 30 and 70 min at 80°, ^{1}H NMR spectra were recorded. Methanol evaporation in the mixture was determined upon integration of signals corresponding to protons from methanol relative to those from sodium acetate used as reference, and compared to ^{1}H NMR results obtained at 20 °C.

We noted a 3% loss of methanol in the assayed mixture after a 20-min incubation at 80 °C. The limited evaporation of methanol observed did not invalidate the proposed method for the DM determination as ^{1}H NMR experiments, including the tuning of the magnetic fields and the recording of spectra (256 scans in the present study) did not exceed 20 min.

The method was validated by the DM determination of a citrus pectin standard (Fig. 1D), where a DM value of $71 \pm 0.6\%$ was obtained. This result was in agreement with that determined directly on the ^{1}H NMR spectrum of this sample.

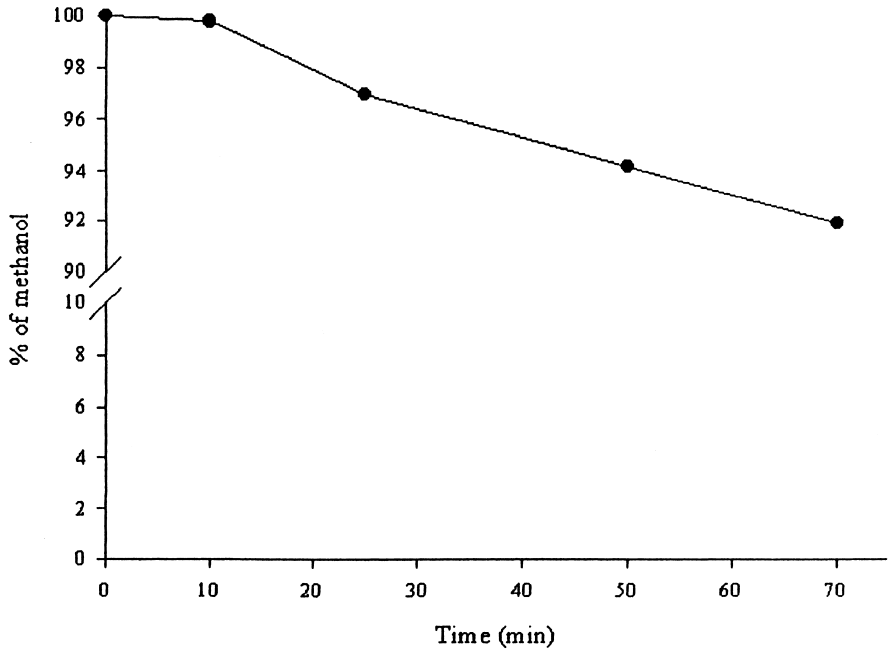

Fig. 2. Determination of methanol evaporation during the NMR analysis at 80 °C of a mixture containing methanol (100 mM) and acetic acid (100 mM). Spectra were recorded 10, 22, 50 and 70 min after addition of 100 mM NaOD in the NMR tube stabilized at 80 °C. Methanol evaporation was determined relative to sodium acetate (used as reference) after six manual integrations of methanol and acetate ions peaks. The T_0 (100%) value was obtained from NMR analyses performed at 20 °C.

Our results show that the determination of pectins DM and DA can be performed in less than 20 min, by saponification of about 5 mg of material during ^{1}H NMR analysis. The method represents an attractive technical opportunity for a rapid control of pectins esterification.

1. Experimental

1.1. Materials

Pectin from citrus (Sigma P-9561) was used as standard. According to chemical analysis, the DM value was about 72%.[2] Sodium polygalacturonate from orange was purchased from Sigma. The tested pectins were from flax (*Linun usitatissimum*) stems. Flax plants were collected during growth, washed with water, then the stems were recovered and dried by lyophilisation.

1.2. Extraction of the flax pectins

Dried flax stems (5 g) were ground for 1 min in 50 mM AcONa buffer (200 mL) at 4 °C with a domestic liquidizer. After filtration (filter Whatmann n°3), the insoluble fraction was washed twice (10 min) with 1:1 $CHCl_3$–MeOH (200 mL). Then, 50 mM HCl (100 mL) was added to the fraction and the mixture was incubated during 10 min at 70 °C with magnetic stirring. Pectin extraction was stopped by cooling the mixture at room temperature and addition of 1 M NH_4OH to bring the solution at a pH value of 5. The pectin extract was centrifuged (10 min, 12,000*g*, 15 °C) and to the supernatant 3 vol of EtOH were added. After 1 night at 4 °C, the pectins were collected upon centrifugation (10 min, 12,000*g*, 4 °C). The pellets were solubilized in water and lyophilised.

1.3. Analytical

The pectin uronic acids (UA) and neutral sugars (NS) contents were assayed with 3-phenylphenol[8] and resorcinol[9] micromethods performed in microtitration plates (Nunc, Maxisorp). L-Arabinose and D-galacturonic acid (Sigma) were used as standards. Quantification of NS was obtained after correction of the interference due to UA. The sugar composition of pectin extracts was determined as follow: the samples in 50 mM pyridine acetate (pH 5) (5 mg/mL) were digested with pectinase (Sigma P-4716) (0.1 U/mg of pectin) for 24 h at 37 °C and dried by lyophilisation.[10] The dried hydrolysates were methanolysed in 0.5 M HCl (Methanolic Instant Kit, Alltech) (10 h, 80 °C), mesoinositol was used as standard. The methanolic solution was evaporated under Ar and the *O*-methylglycosides were derivatized at 4 °C in Py with the BSTFA/TMCS (Alltech) mixture. Silylated derivatives were injected on a HP 1 capillary column (25 m × 200 μm) installed in a HP 6890 chromatograph (Hewlett-Packard).

1.4. Size exclusion chromatography

Pectins were characterised by size exclusion chromatography according to the conditions previously described.[11]

1.5. NMR spectroscopy

^{1}H NMR experiments were recorded on a Bruker Avance 300 spectrometer operating at 300.13 MHz. Dry pectin samples (5 mg) were dissolved in D_2O (Aldrich, 99.9% D) (10 mg/mL). The proton chemical shifts were determined using the conventional proton pulse sequence provided by Bruker with a 30° impulsion. The spectral window was 3000 Hz for 8 k data points with a pulse of 7 μs, an acquisition time of 1.36 s and a relaxation delay of 1 s, 256 scans were recorded. The 2 D-COSY experiment was performed using a (90°)-τ_1-(90°) sequence with a *z* gradient probe. The spectral width in F_1 and F_2 was 1795 Hz, the number of data points in F_2 was 2048, 256 increments were recorded. The chemical shifts were assigned relative to the HOD resonance.[12]

1.6. ^{1}H NMR determination of the DS of pectins upon saponification

After a first ^{1}H NMR experiment at 80 °C on the native pectin (10 mg/mL) in order to check the presence of free MeOH and AcONa, 55 μL of NaOD (1 M, Sigma) in D_2O were added in the NMR tube and 256 scans were further recorded. The DS value was the result of three distinct analyses.

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