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CP-MAS NMR of highly mobile hydrated biopolymers: polysaccharides of *Allium* cell walls

Marie-Ann Ha [a], Boyd W. Evans [a], Michael C. Jarvis [a,*], David C. Apperley [b], Alan M. Kenwright [b]

[a] *Chemistry Department, Glasgow University, Glasgow G12 8QQ, Scotland, UK*
[b] *SERC Solid-state NMR Unit, Durham University, South Road, Durham DH1 3LE, England, UK*

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Abstract

The most highly mobile polysaccharides of hydrated *Allium cepa* (onion) cell walls were not visible in cross-polarisation magic-angle spinning (CP-MAS) ^{13}C spectra recorded using a conventional CP contact time of 0.5 ms. These polysaccharides showed very slow CP and thus did not appear in the ^{13}C spectrum until the contact time was extended to 2 ms or more. A spectrum of this slow-CP material was obtained by subtracting the signal intensities obtained in a delayed-contact experiment from those obtained in an experiment with long, variable contact times. It showed motional line-narrowing compared with the more rigid portion of the cell wall and corresponded to a mixture of β(1,4′)-D-galactan and methyl-esterified α(1,4′)-D-galacturonan, polymers known to be flexible in solution. The CP time constant T_{CH} was approximately 1 ms and the proton T_2, measured in a hybrid delayed-contact/long-contact experiment with a variable delay inserted before proton spin-locking, was a few hundred μs. These values were an order of magnitude greater than was observed for the rigid polymers in the same sample. The proton T_2 was intermediate between typical values for the solid and solution states. This approach should be applicable to other soft, hydrated plant and animal tissues. © 1996 Elsevier Science Ltd.

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1. Introduction

The mechanical behaviour of synthetic polymers is becoming better understood at the molecular level, but the presence of water makes biological materials behave very

* Corresponding author.

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differently. Plant cell walls are a good example of a complex, hydrated load-bearing material. The primary cell walls of dicot plants are composites built up from 3–10 nm cellulose fibres, coated with xyloglucans and bonded into a structured matrix of xyloglucan, pectin, and glycoprotein [1,2]. Crystalline cellulose contains relatively little water, and the water present is tightly and specifically bound [3]. The other polymers within the cell wall hydrate unevenly, the most hydrated polymers being the least rigid [4].

Solid-state NMR can be used to probe the molecular rigidity of individual polymers within a biological material such as a cell wall [4–6]. It is important to retain the hydration of the material being measured as this will affect polymer motions [4,6]. Either ^{1}H or ^{13}C mobility can be probed through the relaxation time constants T_1, $T_{1\rho}$ and T_2. T_1 measurements are sensitive to molecular movements on the MHz scale, while $T_{1\rho}$ and T_2 measurements are sensitive to molecular movements on the kHz scale. Much of the MHz motion is of side groups such as methyl esters, not the polymer chain. Side chain motions are less relevant to the mechanical properties of the material [7]. Problems in the determination of the ^{13}C $T_{1\rho}$ [8] and T_2 mean that the most useful measurements are of the proton $T_{1\rho}$ and T_2. The proton $T_{1\rho}$ is spatially averaged over domains a few nm across by spin diffusion [6], whereas the proton T_2 is not.

In experiments using cross polarisation to measure proton mobility with ^{13}C spectral resolution, a problem is that some of the more mobile polymer chains do not appear in the spectrum under normal conditions [9,10]. Nor, in general, are they observable under solution state conditions [11], probably because the complex macromolecules to which they are attached, like most structural biopolymers, are too stiff and elongated in solution to undergo the rapid tumbling motions that allow the spectra of globular proteins of equally high molecular weight to be observed.

We have found that polymer chains that are too rigid for solution state NMR, but too mobile for CP-MAS NMR under normal conditions, can be observed by extending the CP-MAS contact time from 0.5–1 ms to 3–10 ms. During this period, however, the proton magnetisation of the whole sample is decaying by the $T_{1\rho}$ process, and a separate estimation of the rate of $T_{1\rho}$ decay is necessary if data on the most mobile components are to be extracted.

Here we describe a method for obtaining the ^{13}C spectrum of this highly mobile material, and for characterising its proton relaxation and cross-polarisation behaviour.

2. Results and discussion

In a variable-contact experiment on hydrated *Allium* cell walls, the $T_{1\rho}$ measured for protons associated with pectins was comparable with that of the protons associated with cellulose (Table 1), and much longer than the $T_{1\rho}$ of pectic protons in dry cell walls, which was about 3 ms [12,23]. We have obtained similar results in variable-contact experiments on wet cell walls from other species [13,23], with the apparent pectic $T_{1\rho}$ sometimes exceeding that observed for cellulose.

Cellulose is less flexible than pectin, and the difference between them is greater in the presence of water because pectins are much more extensively hydrated to form a gel,

Table 1
Resonance assignments and relaxation kinetics for variable-contact and delayed-contact experiments on hydrated onion cell walls, and the difference between them. All time constants are in ms

Assignment	ppm	Experiment								
		Variable-contact [a]				Delayed-contact [b]			Difference	
		$T_{1\rho}$	s	T_{CHD}	T_{CHR}	r	$T_{1\rho}(1)$	$T_{1\rho}(2)$	$T_{1\rho}$	T_{CH}
Pectic carboxyl, Me ester	171	> 50	1	–	0.69	0.69	1.5	9.2	22	4.2
Cellulose C-1	105	14	0.69	0.014	0.29	0.47	4.1	50	5	1.0
Pectic and other C-1	101	11	0.60	0.050	0.10	0.40	1.0	12	11	1.0
Crystalline cellulose C-4	89	13	0.61	0.004	0.15	0	–	23	–	–
Crystal-surface cellulose C-4	84	16	0.59	0.015	0.14	0	–	17	–	–
Pectin C-4	80	11	0.87	0.004	0.08	0.50	1.7	19	8	0.7
General carbohydrate	72	15	0.60	0.015	0.22	0.25	1.6	19	7	1.6
Pectic C-2, C-3	69	12	0.86	0.024	0.06	0.42	1.3	14	21	0.5
Surface cellulose + galactan C-6	62	16	0.58	0.017	0.37	0.60	4.0	> 50	6	3.7
Pectic methoxyl	54	32	0.90	0.005	0.22	0.84	3.5	> 50	18	6.0
Acetyl CH_3	21	12	0.60	0.2	2.5	0.76	3.3	> 50	7	3.5

[a] The analysis of the variable-contact experiment is based on the equation [14,15,23]: $S = S_0 \exp(-\tau/T_{1\rho})[1 - s\exp(-\tau/T_{CHR}) - (1-s)\exp(-3\tau/2T_{CHR})\exp(-\tau^2/2T_{CHD}^2)]$, where S is the signal intensity at contact time τ, s is the proportion of the cross-polarisation that occurs by the rapid route from covalently bonded protons, and T_{CHD} and T_{CHR} are the time constants for cross-polarisation from covalently bonded and non-bonded protons respectively.

[b] The analysis of the delayed-contact experiment is based on a dual exponential function with time constants $T_{1\rho}(1)$ and $T_{1\rho}(2)$, the corresponding fast and slow phases being in the ratio r:$(1-r)$.

whereas cellulose remains a crystalline solid. In dry and lightly hydrated cell walls, the $T_{1\rho}$ of protons associated with pectic polymers is slightly shorter than the $T_{1\rho}$ of protons associated with crystalline cellulose [13]. It follows that hydration shortens the pectic proton $T_{1\rho}$ relative to that of cellulose. This was not what we observed in the variable-contact experiment, however.

One possible explanation of these findings is that the proton $T_{1\rho}$, if plotted against pectin mobility, passes though a minimum between the dry and wet states. It would then decrease with mobility in dry cell walls and increase with mobility in wet cell walls. The delayed-contact experiment, in which the contact time and the other cross-polarisation conditions were held constant, showed that this explanation is not correct. In the delayed-contact experiment the proton $T_{1\rho}$ relaxation of pectins was much faster than in the variable-contact experiment, and also faster than that of cellulose, as would be expected if the $T_{1\rho}$ decreases with mobility throughout (Table 1). This makes it clear that the long pectic proton $T_{1\rho}$ observed in the variable-contact experiment was an anomaly associated with cross-polarisation from protons to ^{13}C rather than a feature of the proton $T_{1\rho}$ relaxation process.

Anomalous cross-polarisation behaviour suggests an unusual value for the cross-polarisation time constant(s), not for the proton $T_{1\rho}$. However, if the time constant for cross-polarisation is so long that it is comparable with the $T_{1\rho}$, the apparent $T_{1\rho}$ will be distorted. It is known that hydrated cell walls [6] and pectic gels [10] contain a significant proportion of very mobile material that does not appear in conventional

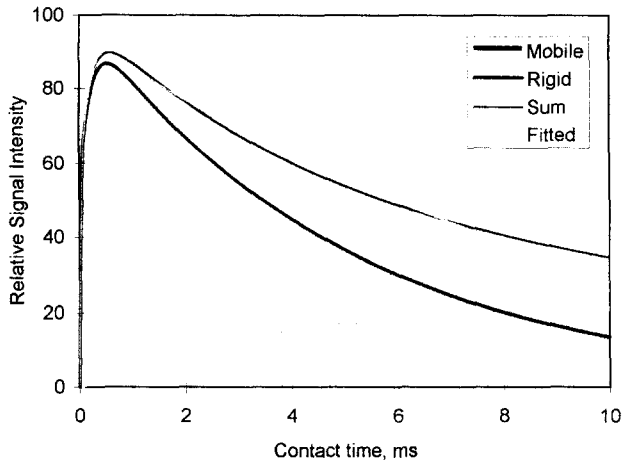

Fig. 1. Modelled contributions of rigid and mobile components, showing fast and slow cross-polarisation respectively, to the evolution of signal intensity with contact time in a CP-MAS experiment.

CP-MAS spectra, although it can be observed by direct polarisation of ^{13}C. Cross-polarisation becomes slower with increasing mobility [13,14]. These observations suggest the following hypothesis.

If a part of the pectic fraction becomes so mobile on hydration that it cross-polarises on the same timescale as the $T_{1\rho}$ relaxation, the signal intensity from that part will still be increasing while the signal intensity from the rest is decaying by the $T_{1\rho}$ process. The total signal may then show a longer $T_{1\rho}$. The mobile part will make little contribution to the signal intensity at the short contact times normally used to record CP-MAS spectra.

A model illustrating this hypothesis is shown in Fig. 1. It incorporates two-phase kinetics for cross-polarisation of the rigid part, as derived theoretically [13] and as shown to give very close fits to the experimental data for dry cell walls ([12]; see Methods section). However, the cross-polarisation of the mobile part is described by a single slow exponential, because we have shown that for mobile groups like rapidly rotating methyls, the initial fast phase of cross-polarisation from covalently-bonded protons is almost eliminated by motion. Fig. 1 shows that the apparent $T_{1\rho}$ decay exhibited by the sum of the rigid and mobile parts of the cell wall remains close to exponential up to a contact time equal to the $T_{1\rho}$, but with a much longer time constant than that of the rigid part alone. The longer of the two cross-polarisation time constants T_{CHR}, corresponding to cross-polarisation of ^{13}C from non-bonded protons, is shortened by the inclusion of the mobile part of the wall. We have observed [12] that for pectic resonances the apparent T_{CHR} commonly decreases on hydration as predicted by the model, the reverse of what might be expected.

The hypothesis suggested above was confirmed by testing three predictions from it. If the hypothesis is correct (1) the difference between the variable-contact and delayed-contact $T_{1\rho}$ curves should give a curve corresponding to the mobile component and should show appropriate kinetics, including a long T_{CH}; (2) the difference spectrum should correspond to types of polymer known to be readily hydrated and very flexible in

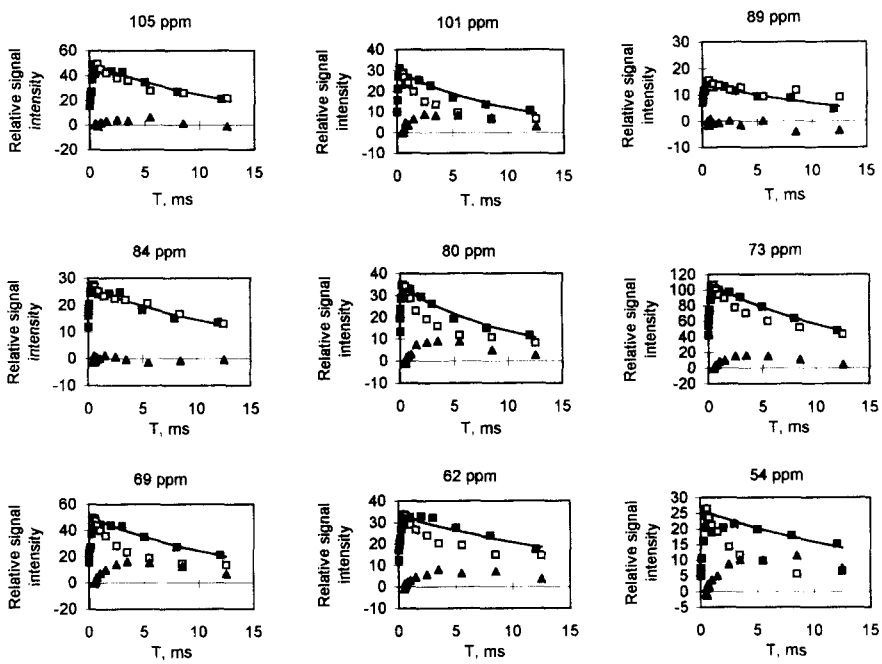

Fig. 2. Difference (▲) between signal intensities in variable-contact (■) and delayed-contact (□) experiments for the principal resonances in the CP-MAS spectrum of onion cell walls. The variable-contact and delayed-contact experiments were normalised to give equal signal intensity at a contact time of 0.5 ms, after correcting for cross-polarisation efficiency.

the hydrated form; (3) there should be independent evidence that the material appearing in the difference spectrum is indeed highly mobile.

Fig. 2 shows the difference between the variable-contact and delayed-contact results for the major peaks in the CP-MAS spectrum. The difference was small for the resonances from cellulose and larger for those of pectic galacturonan and galactan (see Table 1 for resonance assignments). The difference curves for the pectic resonances showed a mean T_{CH} much longer than even the slow cross-polarisation component, although somewhat shorter than the proton $T_{1\rho}$. The apparent $T_{1\rho}$ indicated by the difference curves was longer than was observed for the more rigid pectins in the delayed-contact experiment, but by a similar argument to that followed above, this may reflect a spread of T_{CH} values rather than genuinely slow $T_{1\rho}$ decay.

The difference between the variable-contact and delayed-contact results was greatest at about 3 ms, and extended over contact times up to at least 12 ms (Fig. 2). The difference at various contact times was plotted across the spectrum. The difference spectra showed little variation between 2 ms and 10 ms contact time, except that at 2 ms a small contribution from cellulose remained visible (data not shown). Fig. 3 shows the difference spectrum at 10 ms contact time, i.e. the spectrum corresponding to the putative mobile part of the cell wall. There was considerable motional narrowing of the signals, which were assigned to two polymers: a highly methyl-esterified pectic galactur-

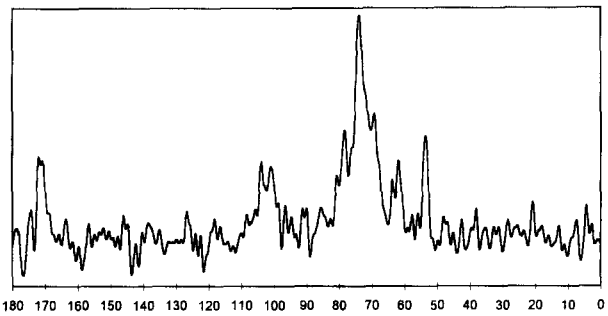

Fig. 3. Difference spectrum corresponding to the highly mobile elements showing slow cross-polarisation in onion cell walls. Each point in the spectrum corresponds to the difference in signal intensity between the corresponding points in (1) a spectrum with 10 ms contact time and (2) a spectrum with 0.5 ms contact time after 9.5 ms $T_{1\rho}$ delay. The variable-contact and delayed-contact experiments from which spectra (1) and (2) were respectively derived were normalised to give equal signal intensity at a contact time of 0.5 ms, after correcting for cross-polarisation efficiency.

onan and a β(1,4)-linked pectic galactan [16,17]. Both of these polymers are relatively flexible in the single-chain form [18,19], and neither is known to form stable aggregates under physiological conditions [20]; both are highly soluble in water. They may therefore be assumed to be amongst the most mobile polymers in the cell wall, as our results imply.

Independent evidence of their mobility was obtained by repeating the construction of the difference spectrum in a simpler experiment and interposing a variable gap after the proton preparation pulse for the proton magnetisation to decay by the T_2 process [21,22]. This experiment (Fig. 4) gave a similar difference spectrum to Fig. 3, decaying with a T_2 (assumed to be exponential) of a few hundred microseconds. This value may be compared with T_2 values of 10–30 μs observed with a contact time of 1 ms for the rigid part of the hydrated cell wall. This confirms the hypothesis suggested above.

These experiments explain why erratic results have been obtained in the measurement of the proton $T_{1\rho}$ by the variable-contact procedure, and confirm the superiority of the delayed-contact experiment for samples that contain polymers varying widely in mobility as is commonly the case with hydrated biological tissues. They also explain why both hydrated tissues and biopolymer gels may contain components that are not observed in ^{13}C NMR spectra under either normal CP-MAS (solid-state) or solution-state conditions [9–11]. The very wide variation in the proton T_2 implies that rates of proton spin diffusion will differ greatly from one part of the cell wall to another, complicating the use of spin-diffusion experiments to determine spatial relationships between the components of the structure [12].

The experiments used to derive the spectra and relaxation characteristics of the mobile part of the cell walls may be of wider applicability to biological materials and tissues, since this range of mobility is not otherwise very accessible by NMR spectrometry. A variety of human and plant biopolymers with lubricating, shock-absorbing or hydrated rubber-elastic properties may be assumed to show mobility of the same order.

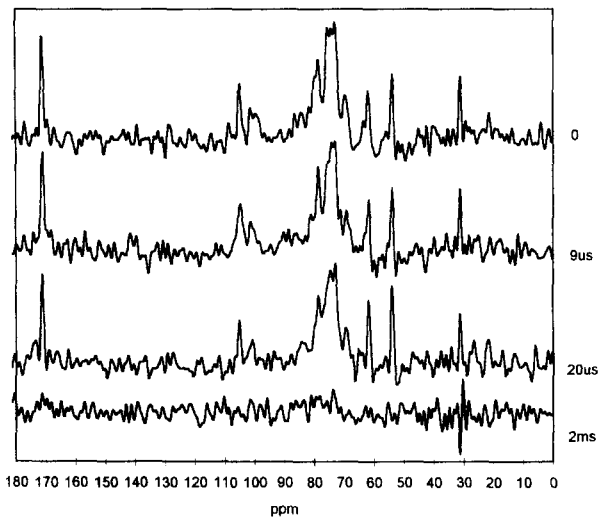

Fig. 4. Influence of variable delay for proton T_2 relaxation on intensity of difference spectrum corresponding to the highly mobile elements of onion cell walls. Each spectrum was obtained by subtracting a spectrum with 0.5 ms contact time after 9.5 ms $T_{1\rho}$ delay from a spectrum with 10 ms contact time and no $T_{1\rho}$ delay. The variable T_2 delay was inserted immediately after the proton preparation pulse, prior to proton spin-locking, in each case.

Significantly, a proton T_2 of a few hundred microseconds is at the lower end of the range accessible by conventional NMR imaging techniques: the combination of imaging and spectrometric techniques has great potential in biology.

3. Methods

Onions, cv. Bobosa (100 g) were homogenised in Triton-X-100 (2 g L^{-1}, 500 mL), and cell walls collected on a sintered glass funnel. The walls were washed with water and the excess of liquid removed by suction. The walls were then stirred for 30 min in 15 mL of phenol-saturated water. The walls were washed extensively with water, cryomilled in liquid nitrogen and dried to a 3.4:1 wall: water ratio prior to use.

NMR experiments were carried out on a Varian VXR-300 spectrometer operating at 75.34 MHz for ^{13}C. MAS rates varied between 3.5 and 4.3 kHz. The proton decoupling field was nominally 35–36 kHz, and the Hartmann–Hahn match was optimised individually for each sample to allow for radiofrequency energy absorption by mobile water protons. The proton rotating-frame relaxation time $T_{1\rho}$ and cross-polarisation rate were measured in both variable-contact and delayed-contact experiments. In the variable-contact experiment 12 values of the contact time, in the range 25 μs–18 ms, were used. The data were analysed by least-squares fitting to the two-phase polarisation transfer model of Wu et al. [14], with single-exponential proton $T_{1\rho}$ decay and the time constant for the

first phase (CP from covalently bonded protons) denoted by T_{CHD} and the time constant for the second phase (CP from more remote protons) denoted by T_{CHR}:

$$S = S_0 \exp(-\tau/T_{1\rho})\left[1 - s\exp(-\tau/T_{CHR}) - (1-s)\exp(-3\tau/2T_{CHR})\exp(-\tau^2/2T_{CHD}^2)\right]. \quad (1)$$

This model has been shown to give close fits to the data for both dry and hydrated cell walls, provided that the ratio $(1-s):s$ between the magnitudes of the fast and slow polarisation transfer phases is not assumed to be 1:1 but is fitted to the data [12]. In the delayed-contact experiment a variable delay, during which proton spin-locking was maintained, was inserted prior to a fixed contact time of 0.5 ms [21,22]. In this experiment a dual-exponential decay function, with time constants denoted by $T_{1\rho}(1)$ and $T_{1\rho}(2)$, was necessary to give satisfactory fits to the data for the pectic resonances.

So that the results from delayed-contact and variable-contact data could be compared, they were normalised to give equal signal intensities at a contact time of 0.5 ms (zero delay in the delayed-contact experiment) after the variable-contact data had been adjusted to allow for the fact that full equilibration of proton and ^{13}C polarisation had not quite been reached after 0.5 ms, even for the relatively rigid cell-wall polymers from which the spectrum at 0.5 ms contact time was derived: the signal intensity was about 95% of the calculated equilibrium level at that point, the exact value depending on the cross-polarisation rate for the resonance concerned. The difference in normalised signal intensity between the variable-contact and delayed-contact experiments, at a given time point τ, is then a measure of the amount of ^{13}C cross-polarising between 0.5 ms and τ.

The proton T_2 of this material was estimated as follows from two experiments: (a) a variable T_2 delay (20 μs to 2 ms) after the proton preparation pulse [21]; then 9.5 ms $T_{1\rho}$ delay (spin-locking for protons only); then 0.5 ms contact. (b) A variable T_2 delay (20 μs to 2 ms) after the proton preparation pulse; then 10 ms contact. Difference spectra (b–a) were obtained for each value of the T_2 delay. The T_2 relaxation was analysed as a single exponential, a close approximation [22] for the mobile polymers contributing to the difference spectrum.

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References

[1] M.C. McCann and K. Roberts, in C.W. Lloyd (Ed.), *The Cytoskeletal Basis of Plant Growth and Form*, Academic Press, New York, 1991, p 109.
[2] N.C. Carpita and D.M. Gibeaut, *Plant J.*, 3 (1993) 1–30.
[3] J. Sugiyama, R. Vuong, and H. Chanzy, *Macromolecules*, 24 (1991) 4168–4175.
[4] A.L. MacKay, J.C. Wallace, K. Sasaki, and I.E.P. Taylor, *Biochemistry*, 27 (1988) 1467–1473.
[5] P. Irwin, P. Pfeffer, W. Gerasimowicz, R. Pressey, and C. Sams, *Phytochemistry*, 23 (1984) 2239–2242.

[6] R.H. Newman, M.A. Ha, and L.D. Melton, *J. Agric. Food Chem.*, 42 (1994) 1402–1406.
[7] A.M. Kenwright and B.J. Say, in R.N. Ibbett (Ed.), *NMR Spectroscopy of Polymers*, Blackie, London, 1993, p 231.
[8] F. Horii, A. Hirai, and R. Kitamaru, *J. Carbohydr. Chem.*, 3 (1984) 641–662.
[9] M.J. Gidley, A.J. McArthur, and D.R. Underwood, *Food Hydrocolloids*, 5 (1994) 129–140.
[10] M.C. Jarvis and D.C. Apperley, *Carbohydr. Res.*, 275 (1995) 131–145.
[11] I. Braccini, C. Herve du Penhoat, V. Michon, R. Goldberg, M. Clochard, M.C. Jarvis, Z.H. Huang, and D.A. Gage, *Carbohydr. Res.*, 276 (1995) 167–181.
[12] M.C. Jarvis, K.M. Fenwick, and D.C. Apperley, *Carbohydr. Res.*, 288 (1996) 1–14.
[13] K.M. Fenwick, M.C. Jarvis, and D.C. Apperley, *Phytochemistry*, 42 (1996) 301–307.
[14] X. Wu, S. Zhang, and X. Wu, *Phys. Rev. B*, 37 (1988) 9827–9829.
[15] L. Muller, A. Kumar, T. Baumann, and R.R. Ernst, *Phys. Rev. Letts.*, 32 (1974) 1402–1406.
[16] M.C. Jarvis, *Carbohydr. Res.*, 201 (1990) 327–333.
[17] M.C. Jarvis and D.C. Apperley, *Plant Physiol.*, 92 (1990) 61–65.
[18] S. Cros, C. Herve du Penhoat, N. Bouchemal, H. Ohassan, A. Imberty, and S. Perez, *Int. J. Biol. Macromol.*, 14 (1992) 313–320.
[19] T.L. Bluhm and A. Sarko, *Carbohydr. Res.*, 54 (1977) 125–138.
[20] M.C. Jarvis, *Plant Cell Environ.*, 7 (1984) 153–164.
[21] P. Tekely and M.R. Vignon, *J. Polym. Sci. C, Polym. Lett.*, 25 (1987) 257–261.
[22] R.H. Newman, *Holzforschung*, 46 (1992) 205–210.
[23] M.A. Ha, M.C. Jarvis, and D.C. Apperley, unpublished.