

^1H - ^{13}C Polarization Transfer Studies of Uronic Acid Polymer Systems

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Solid-state NMR studies were performed on apple cell wall material, citrus pectin, and copper(II) polygalacturonate to determine the degree to which ^1H - ^{13}C polarization transfer and rotating-frame relaxation rates affect quantitation of uronic acids using ^{13}C CPMAS NMR techniques. These experiments were performed because cell wall carbonyl ^1H - ^{13}C polarization transfer rates differed significantly between ripe and unripe tissues. Such differences can be problematic when comparing relative signal response between samples. Cu^{2+} -PGA ^1H - ^{13}C contact time studies indicate that the quantitation of uronic acids in various cell wall systems could be significantly underestimated albeit ESR data indicated a nonrandom distribution of Cu^{2+} that could induce ^{13}C CPMAS NMR spectral distortion. To test this possibility, studies on well-characterized citrus pectin were performed; the results indicated that cell wall carbonyl/anomeric carbon ($T_{1\rho\text{H}}/T_{\text{CH}}$) ratios were large enough to result in quantitative ^{13}C resonance responses at contact times ranging from 0.8 to 1.0 ms.

INTRODUCTION

Polyuronic acids are significant structural components in the middle lamella-primary cell wall complex of higher plants (Albersheim et al., 1960; Knee, 1974). These acidic polysaccharides, which are an important feature of the amorphous domain of plant tissue systems (Hall, 1981), have substantial ion-exchange capabilities that can affect activity coefficients, transmembrane potential, and, thus, ionic flux (Van Cutsem and Gillet, 1982, 1983). Because pectic polysaccharides are involved in plant tissue structure and physiological function, a better understanding of their structural and compositional characteristics in intact plant tissues is of interest. Unfortunately, most techniques (Voragen et al., 1983) for polyuronide characterization provide little intact structural information and, in fact, could yield spurious results (Darvill et al., 1980) due to the requisite extraction and solubilization processes possibly resulting in morphological alterations. The most common quantitative technique specific for uronic acids (Blumenkrantz and Asboe-Hansen, 1973) requires subjecting samples to acid hydrolysis. Since uronosidic bonds are relatively resistant to acid hydrolysis (Darvill et al., 1980), conditions harsh enough to successfully cleave these bonds could result in some uronide degradation. On the other hand, without some form of hydrolytic cleavage, solubilization of all polyuronides from plant cell walls is virtually impossible.

Cross-polarization and magic-angle spinning NMR spectroscopy (CPMAS NMR; Yannoni, 1982) is one of the few methods available for studying insoluble materials spectroscopically (Havens and Koeing, 1983). The technique allows the examination of specific resonances associated with structural features, as well as certain properties of these domains in bulk heterogeneous materials such as plant tissues. In this report, we provide information concerning the dynamics of the cross-polarization process in intact plant tissue and related materials in order to resolve various questions relating to intact cell wall polyuronide structure and composition.

MATERIALS AND METHODS

Apple fruits (cv. Golden Delicious) were obtained from the Beltsville Agricultural Research Center (Sept 26, 1982). Fruits were picked, randomized, and immediately stored

at 0 °C in 1% (v/v) O_2 . After 1 month, ca. 50 fruit were removed and ripened for 0 and 21 days at 20 °C in a flow-through system with humidified and ethylene-scrubbed air. At each time interval, 10 fruit were randomly selected and tissue firmness was measured (Irwin et al., 1984a). The fruit were peeled, cut up into ca. $5 \times 5 \times 3$ mm sections, and dipped in 0.01 M CaCl_2 to inhibit tissue oxidation. The tissues were immediately vacuum infiltrated 2 h or more consecutively in 20, 40, 60, and 80% ethanol/water. The fixed tissue was then equilibrated in 95% ethanol/water for 1 day with repeated solution replacement. The specimens were further reduced in size to 1-mm sections and equilibrated 1 h each in three changes of absolute ethanol followed by critical point drying. This drying technique should maintain cell wall fiber morphology since the tissues have not been exposed to either the freezing and sublimation boundaries or surface tension forces associated with freeze-drying techniques. Dried samples were stored in vacuo in the presence of dehydrated silica gel.

Polygalacturonic acid (H^+ form; PGA) was purchased from Sigma Chemical Co. (Lot 112F-0303), St. Louis, MO. For Cu^{2+} experiments, four 500-mg dry PGA samples were added to 25 mL of 10^{-1} , 10^{-2} , 10^{-3} , and 10^{-4} M CuCl_2 solutions in 95% ethanol/water and vortexed. After 7 h at 24 °C, the PGA samples were washed three times with 25 mL of 95% ethanol/water followed by three washes in absolute ethanol. The samples were vacuum dried on sintered-glass filters, and bound Cu^{2+} measurements were obtained via atomic absorption spectrophotometry using standard techniques.

The Na^+ salt of citrus pectin (70% degree of methylation; H. P. Bulmers, Ltd., Hereford, England; Lot 7896) was first prepared by dissolving 2 g in 60 mL of 0.01 M sodium phosphate buffer, pH 6.0, which contained 0.01 M EDTA. After gently stirring overnight at 5 °C, the pectin solution was titrated to pH 7 with 0.1 M NaOH and dialyzed against four changes of distilled water over 48 h. The dialysis tubing had a low molecular weight cutoff of 12000 daltons, with an outside-to-inside volume ratio of ca. 30. The dialyzed pectin solution was centrifuged 1 h at 38000g and the supernatant freeze-dried to a spongy, white solid. Determination of uronic acid content was performed by two independent solution techniques (Food Chemicals Codex, 1981; Blumenkrantz and Asboe-Hansen, 1973). The degree of methyl esterification (DM) was also determined by the former method (e.g., titration; Food Chemicals Codex, 1981).

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Table I. Effect of Bound Cu²⁺ (mol %) on T_{CH} and $T_{1\rho H}$ for Carbonyl and Anomeric Carbon Resonances of Cu²⁺-PGA Complexes

mol % Cu ²⁺ ^a	T_{CH} , μ s		$T_{1\rho H}$, ms	
	C=O		C=O	
12	137 \pm 7 ^b	40 \pm 11	1.3 \pm 0.1	1.2 \pm 0.1
3	136 \pm 5	52 \pm 2	1.9 \pm 0.1	1.5 \pm 0.1
1	148 \pm 9	66 \pm 3	2.0 \pm 0.1	1.6 \pm 0.1
0.05	158 \pm 8	79 \pm 6	2.7 \pm 0.2	2.2 \pm 0.1

^a Mol of Cu²⁺ bound/mol of galacturonic acid in PGA. ^b Plus/minus standard error.

General CPMAS. NMR spectra (8-kHz spectral width) were obtained at a ¹³C frequency of 15 MHz on a JEOL FX-60QS spectrometer with a ¹H decoupling field strength of 11 G. Data points (1K) were sampled and zero-filled to 4K for data acquisition. All chemical shifts were assigned relative to hexamethylbenzene's (HMB) methyl resonance (17.36 ppm) based on the position of Me₄Si. Samples (300–500 mg of dry weight) were spun (2.4 kHz) at the magic angle (54.7°) in a Kel-F bullet rotor. The angle was set with HMB prior to each experiment. All ¹³C CPMAS NMR experiments were performed in the presence of dry nitrogen gas flow. Areas under resonance peaks were determined by triangulating to the base line and taking three planimeter measurements/area. Spectra used for quantitative measurements were acquired by using 0.8–1.0 ms contact periods.

CPMAS Contact Time Study (T_{CH} , $T_{1\rho H}$). Peak intensity measurements were used for the calculation of ¹H to ¹³C polarization transfer rate (T_{CH}^{-1}) and decay ($T_{1\rho H}^{-1}$); these values were in agreement with similar measurements using resonance peak areas. Approximately 20-Hz computer line broadening was used for all frequency domain spectra. Recycling times were ca. 6 \times ¹H T_1 's (1–2 s for day 0 and 21 cell wall material, respectively; Irwin et al., 1984a). Initially, as contact times are lengthened, the ¹³C signal amplitude increases and is dependent upon T_{CH} (inverse of the ¹H–¹³C cross-polarization rate). At longer contact times, the amplitude of the ¹³C signals decreases due to ¹H spin-lattice relaxation in the rotating frame ($T_{1\rho H}$). The data were treated by standard literature methods employing least-squares refinement and curve-fitting techniques (Pfeffer et al., 1984). Standard errors were calculated for each measurement.

Cu²⁺ ESR. Approximately 50 mg of dry Cu²⁺-PGA samples was loaded into 30 \times 130 mm quartz ESR tubes that had been previously photobleached under UV lamps to remove trapped electrons. All first-derivative spectra were obtained on a Varian series E-109B spectrometer at 97 K. The experimental parameters were as follows: scan range 1.6 kG; field set 3 kG; microwave frequency 9.1 GHz; microwave power 20.75 dB. Scan times were 4–16 min depending on concentration and gain levels. Time constants were 0.032–0.128 s, respectively. Line widths (ΔH - $[d(H)/dH]_{max}$) were calculated as the difference between the maximum and minimum amplitude of the first-derivative spectra. Distance parameters (d) were derived from Van Vleck's second-moment equation (Table I; Van Vleck, 1948).

RESULTS AND DISCUSSION

Until the advent of proton-enhanced and magic-angle sample spinning NMR, the observation of ¹³C resonance in solids was extremely slow due to their relatively large spin-lattice relaxation times, ¹³C chemical shift anisotropy, and the low natural abundance of the ¹³C nucleus (Pines

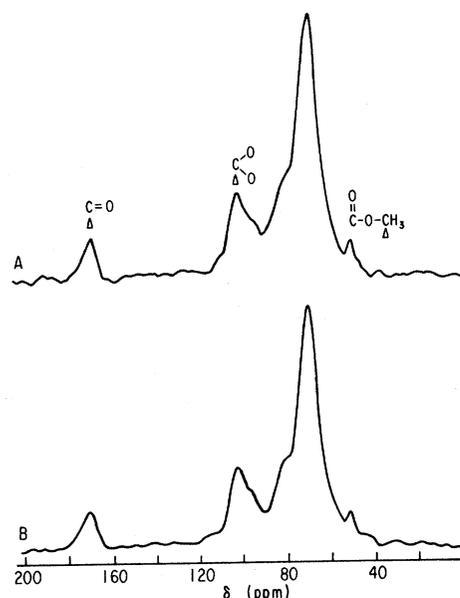


Figure 1. CPMAS NMR spectra of apple tissue ripened 0 (A) and 21 (B) days at 21 °C. Spectra are the result of 8000 transients with a contact time of 0.8 ms and ca. 20 Hz computer line broadening.

et al., 1973). The dynamics of the cross-polarization process can provide certain structural information (Pines et al., 1973). However, quantitation of functional groups (Pfeffer et al., 1984) in heterogeneous bulk materials may be complicated since various chemically distinct polymer domains might experience different polarization transfer rates (T_{CH}^{-1}) and varying degrees of signal loss at comparable contact times due to $T_{1\rho H}$ -mediated processes. These deviations in T_{CH} and $T_{1\rho H}$ are due to differences in polymer correlation times, ¹H–¹³C distances, and various angular terms (Alemany et al., 1983a, 1983b).

Differences in carbonyl T_{CH} (day 21 carbonyl T_{CH} = 381 \pm 38 μ s, day 0 carbonyl T_{CH} = 251 \pm 14 μ s) values are apparent in the cell wall samples from apple fruits at two levels of ripeness. In apple fruit cell walls about 95% of the carbonyl functionality is due to acid sugars (Knee and Bartley, 1981). Cell walls from the most ripened tissues (day 21) had values about 50% greater than the control whereas $T_{1\rho H}$'s were approximately equivalent (e.g., ca. 4 ms; Irwin et al., 1984a). Pines and co-workers (Pines et al., 1973) have shown that T_{CH} depends upon a correlation time as well as ¹³C–¹H angular (with respect to the static field) and distance parameters; because of this, we cannot specifically ascribe the cell wall T_{CH} differences to any one factor. Nevertheless, assuming that all angular and distance terms are equivalent, a 50% difference in carbonyl carbon T_{CH} values would indicate uronide-containing polymer correlation time or motional differences of the order of 2. These differences do agree with carbonyl-associated $T_{1\rho H}$ and other data (Irwin et al., 1984a) but assume that no ripening-related conformational perturbations occur. Regardless of the mechanism the differences in carbonyl T_{CH} could complicate quantitation of CPMAS NMR. Spectra (Figure 1; 0.8-ms contact time) for the day 0 and 21 cell wall treatments show that the ratio of the carbonyl (174 ppm) to anomeric (105 ppm) resonance areas is nearly equal to 0.3. Assuming a 5% cell wall protein contribution to the carbonyl signal (Knee and Bartley, 1981; Voragen et al., 1983) our apple cell wall samples would have a uronide content of 24% and 25% for the day 0 and 21 treatments, respectively. The degree of methyl esterification (from the 54 ppm resonance) was estimated

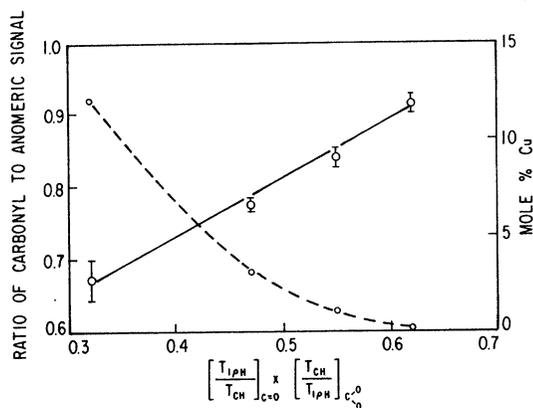


Figure 2. Dependency of the ratio of carbonyl and anomeric carbon resonance integrals (solid line; mean of three measurements with contact times of 0.8 ms and 10000 scans; two standard deviations presented for each value) on the ratio of $T_{1\rho H}/T_{CH}$ for carbonyl and anomeric carbon resonances at different levels of bound Cu^{2+} (broken curve, mol % Cu^{2+} = mol of Cu^{2+} /mol of galacturonate monomer).

to be $54 \pm 5\%$ for both day 0 and 21 tissues. Assuming that the proper conditions for quantitation exist, this finding argues against one proposed mechanism (Knee, 1982; Jarvis, 1984) for polyuronide solubilization in apple fruit during ripening. This mechanism entails changes in the degree of pectin esterification, which could lead to an alteration in intrachain hydrophobic interactions. The ¹³C CPMAS NMR spectra (Figure 1) of cell wall material from ripe (day 21) and unripe (day 0) tissue are virtually identical qualitatively. However, due to differences in T_{CH} such a comparison might be spurious. Proper conditions for quantitation using ¹³C CPMAS NMR can be summarized by the following expression:

$$T_{CH} \ll \text{contact time} \ll T_{1\rho H} \text{ (i.e., } T_{1\rho H}/T_{CH} \gg 1)$$

Clearly, when quantitatively comparing different carbon resonances in a single spectrum, one must satisfy the above expression. However, in the cell wall case, discussed above, we see that the carbonyl functionalities display quite different T_{CH} behavior and, thus, may or may not satisfy this criterion for quantitation. The remaining portion of this paper addresses the issue of quantitative ¹³C signal responses for different functional groups in ¹³C CPMAS experiments under various conditions that can perturb $T_{1\rho H}/T_{CH}$ and can result in signal response changes.

In order to evaluate the effect of variable $T_{1\rho H}/T_{CH}$ values on uronide polymer carbonyl and anomeric carbon resonance area ratios, we prepared Cu^{2+} -doped PGA and characterized the samples by CPMAS NMR (Table I; Figure 2) and thus established some predictable relationship between quantitative signal response and corresponding $T_{1\rho H}/T_{CH}$ values. This is possible because paramagnetic ions have a major effect on rotating-frame spin-lattice relaxation behavior ($T_{1\rho H}$; Pfeffer et al., 1984). T_{CH} and $T_{1\rho H}$ measurements for Cu^{2+} -doped PGA samples were calculated (Table I) and demonstrate paramagnetic ion induced fluctuations in the local magnetic field as evidenced by relatively large differences in $T_{1\rho H}$. Surprisingly, T_{CH} also consistently decreased as the mole percent of bound Cu^{2+} diminished. This latter observation should not be due to a paramagnetic effect but could be induced by the effect of Cu^{2+} cross-linking on PGA molecular motion. These contact time experiments also indicate the linear relationship (Figure 2) between the relative carbonyl signal and the carbonyl/anomeric carbon $T_{1\rho H}/T_{CH}$ ratios. However, there is a preferential loss of

Table II. Dependency of Cu^{2+} Line Width ($\Delta H[dI(H)/dH]_{max}$) and Corresponding Nearest-Neighbor Distance (d) on the Concentration of Bound Cu^{2+}

mol % Cu^{2+} ^a	$\Delta H[dI(H)/dH]_{max}$, ^b G	d , ^c Å		
		$\kappa = 1$	$\kappa = 1.4$	$\kappa = 2.92$
0.05	56	16.06	18.01	22.95
1.00	66	11.58	12.99	16.55
3.00	92	8.39	9.41	12.00
12.00	240	5.08	5.69	7.25

^a Mol of Cu^{2+} bound/mol of uronic acid residue X100. ^b Line width of major Cu^{2+} splitting. Van Vleck (1948) has calculated the relationship between the square root of the second moment, $(\Delta v^2)^{1/2}_{av}$, and the distance (r_{ij}) between nearest-neighbor species: $\Delta v[dI(v)/dv]_{max} = 2(\Delta v^2)^{1/2}_{av} = 2 \{^3/4g^4\beta^4h^{-2}[S(S+1)]\sum_j 1/r_{ij}^6\}^{1/2}$ (units Hertz); $\Delta H[dI(H)/dH]_{max} = (24846 \text{ GÅ}^3)\kappa/d^3$ (units = Gauss); g , β , h , and S have their usual values for Cu^{2+} . We have taken the minimum value of $\Delta H[dI(H)/dH]_{max}$ to be 50 G (10^{-4} M Cu^{2+} in 50% glycerol/water at 97 K). ^c For a pair of interacting spins $\kappa = 1$; for a linear array of ions $\kappa = 2^{1/2}$; for a filled matrix $\kappa = 8.5^{1/2}$. The slight change in d could also be due to bound ion-dependent changes in polymer conformation.

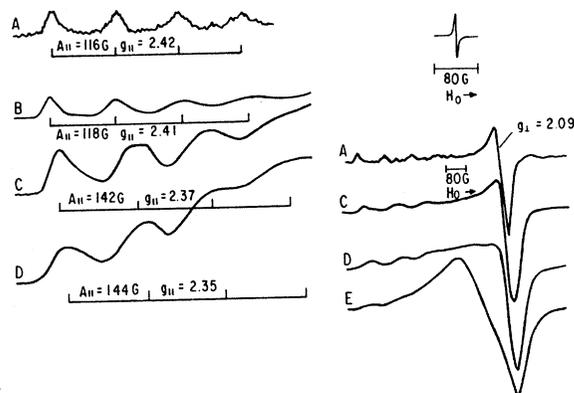
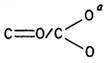


Figure 3. ESR spectra of Cu^{2+} standards in 50% glycerol/water (10^{-4} and 10^{-2} M $CuCl_2$, spectra A and B, respectively) and Cu^{2+} -doped PGA (1, 3, and 12 mol % Cu^{2+} , spectra C-E, respectively) at 97 K.

carbonyl signal since the carbonyl/anomeric carbon ratios do not approach unity even at very low bound Cu^{2+} levels (Figure 2); this latter observation might result from a nonrandom distribution of the paramagnetic ion centers, which could cause a selective loss of carbonyl signal in the regions adjacent to bound paramagnetic ion centers. This hypothesis implies, even at low Cu^{2+} concentrations, that several Cu^{2+} centers exist in a relatively small sample volume (e.g., on the order of 3000 Å^3). To test this possibility, electron spin resonance (ESR; Knowles et al., 1976; Figure 3; Table II) spectroscopic examination of the Cu^{2+} -PGA samples was performed.

ESR first-derivative spectra of Cu^{2+} -doped PGA samples (Figure 3, spectra C and D) demonstrate that Cu^{2+} is bound to the anionic polymer since there was a relatively large decrease in $g_{||}$ values over those observed for Cu^{2+} immobilized in a glassy solid (10^{-4} and 10^{-2} M Cu^{2+} in 50% glycerol/water at 97 K; spectra A and B, respectively). The gradual upfield change in the $g_{||}$ splitting base line is typical for Cu^{2+} coordinated into oxygen-rich electronegative centers. Motschi (1983) has provided an empirical relationship between the relative change in $g_{||}$ and the association constants (K_a) for the bound paramagnetic species. From this relationship, the average $\log K_a$ is 5.88 for the 3 mol % Cu^{2+} sample. Other workers (Deiana et al., 1980, 1983; Van Cutsem and Gillet, 1982, 1983) have demonstrated that Cu^{2+} has a great affinity for various anionic species and results in a loss of the outer ionic hydration shell. Rees and co-workers (1982) proposed that divalent

Table III. Ratio of DM 70 Na⁺ Citrus Pectin Carbonyl/Anomeric and Methoxy/Carbonyl Carbon Resonance Areas as a Function of Variable ¹H-¹³C Contact Periods (τ)

τ , ms		OCH ₃ /C=O
0.5	0.66 ± 0.07 ^b	0.83 ± 0.07
0.8	0.74 ± 0.07	0.81 ± 0.06
1.0 ^c	0.76 ± 0.02	0.67 ± 0.04
1.0 ^d	0.76 ± 0.01	0.71 ± 0.01
1.5	0.84 ± 0.02	0.69 ± 0.07
2.0	0.96 ± 0.01	0.66 ± 0.08

^a C=O, $T_{CH} = 454 \pm 26 \mu\text{s}$ and $T_{1\rho H} = 3.6 \pm 0.20 \text{ ms}$;



$T_{CH} = 128 \pm 42 \mu\text{s}$ and $T_{1\rho H} = 3.69 \pm 0.22 \text{ ms}$. ^b These values are the average of three spectral determinations (10 000 scans, pulse delay 1.5 s). By titration the uronide = 77% and the DM = 74%; by the Blumenkrantz and Asboe-Hansen (1973) technique uronide = 70%. ^c 10 000 transients. ^d 30 000 transients.

cations cooperatively associate with certain polyuronides in solution; in their model, the divalent cations are immobilized between the polymer chains in oxygen-lined cavities under the influence of both ionic and electrostatic interactions. This model is supported (Table II) by the large Cu²⁺-PGA complex spin-spin interactions resulting in significantly broadened g_{\perp} splittings (Figure 3, full spectra insert) even at low bound Cu²⁺ levels (e.g., 1 mol %). From the incremental increase in line width [$\Delta\Delta H - [dI(H)/dH]_{\text{max}}$], relative to randomly dispersed Cu²⁺ in a glassy solid, we estimated (Table II) intercationic spacings in these solid PGA samples as a function of various lattice constants (κ) with the aid of Van Vleck's (1948) second-moment analysis. The lattice constant depends on the arrangement of ions in the fiber matrix and is 1.00 for a two-spin interaction, 1.41 for a linear array, and 2.92 for a cubic lattice of equally spaced spins. As ion-binding sites fill, κ most likely increase from 1.00 (low bound ion concentration) to a maximum of 2.92. From these data (Figure 3; Table II) we propose that the distribution of bound ions is not spatially random, e.g., the ions bind sequentially under the influence of bound nearest-neighbor paramagnetic species, as has been demonstrated elsewhere (Irwin et al., 1984b). The Cu²⁺ distances are reasonably close (1-2 \times) to intrachain carboxylate distances in pectic polysaccharides (Kohn and Larsen, 1972). This concept of cooperative ion association is also supported by the bound ion concentration dependency (Figure 3) of the g_{\perp} 's that indicate that there is a greater affinity for Cu²⁺ as the bound ion concentration increases. Because of this non-random distribution, we propose that up to 10% of the Cu²⁺-PGA carbonyl signal is not observed due to selective broadening even at very low bound Cu²⁺ concentration (Figure 2).

The Cu²⁺-PGA data (Figure 2) indicate that we might be underestimating the cell wall carboxylate functionality (Figure 1) since the ratios of $T_{1\rho H}/T_{CH}$ for the carbonyl and anomeric carbon resonances are in the range of 0.5 ± 0.09 . Assuming that the paramagnetic ion induces changes in T_{CH} and $T_{1\rho H}$ alone (e.g., without preferential line broadening), we could be underestimating the cell wall uronide content by 20-25%. To test this possibility we characterized a known system (the Na⁺ salt of DM 70 citrus pectin), which displayed rotating-frame relaxation behavior (Table III) similar to the cell wall samples. CPMAS NMR of citrus pectin did provide reasonably accurate quantitative results (three replicates at each

contact time of 0.8 and 1.0 ms); from the NMR, the uronic acid content was calculated to be ca. $75 \pm 1\%$ with a DM of $73 \pm 5\%$. The titration method (Food Chemicals Codex, 1981) confirmed these results (77% uronide with a DM of 74%). Since the ratios of $T_{1\rho H}/T_{CH}$ for carbonyl and anomeric resonances in the intact apple tissues are similar to those of pectin and because of the dependency of accurate quantitation on these cross-polarization relaxation times (Figure 2) we could only be slightly underestimating the cell wall uronide content.

CONCLUSIONS

Our NMR and ESR data indicate several significant results with respect to apple cell wall/polyuronide structure and conformation. Since the levels of cell wall polyuronides are similar (Figure 1) in the 0 and 21 day tissues we can speculate that, during the ripening process, uronide-containing polymers are not changing significantly from the standpoint of either total uronide content or overall methyl esterification. This observation does not preclude the possibility of polyuronide turnover or structural alterations associated with changes in pectic neutral polymer composition, size, or placement within cell wall pectin molecules. Indeed, the differences in carbonyl T_{CH} , for cell wall material from ripened tissue, indicate that relatively large changes in structure have occurred and could be due to polymer turnover. However, the relationships shown previously (Irwin et al., 1984a) between uronide-associated T_{1H} , other relaxation times, and the ripening process support the hypothesis that uronide-containing polymers are undergoing considerable structural modifications due to changes in molecular size and argue against overall quantitative alterations in the degree of methylation as a modulator of polyuronide solubilization (Knee, 1982) in apples. The Cu²⁺-PGA and pectin ¹³C CPMAS NMR and ESR experiments indicate that model, paramagnetically perturbed, systems of uronic acid polymers cannot be utilized to determine appropriate cross-polarization conditions for quantitation. Because of the sequential binding properties of Cu²⁺ (Table II) and other paramagnetic divalent cations (Irwin et al., 1984b) to cell wall matrices containing polyuronides, care must be taken when comparing ¹³C CPMAS NMR spectra because of paramagnetically induced spectral distortion (Figure 2). Neither Cu²⁺ nor Mn²⁺ was detectable in our apple cell wall samples.

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LITERATURE CITED

- Albersheim, P.; Muhlethaler, K.; Frey-Wyssling, A. *J. Biophys. Biochem. Cytol.* **1960**, *8*, 501.
 Alemany, L. B.; Grant, D. M.; Pugmire, R. J.; Alger, T. D.; Zilm, K. W. *J. Am. Chem. Soc.* **1983a**, *105*, 2133.
 Alemany, L. B.; Grant, D. M.; Pugmire, R. J.; Alger, T. D.; Zilm, K. W. *J. Am. Chem. Soc.* **1983b**, *105*, 2142.
 Blumenkrantz, N.; Asboe-Hansen, G. *An. Biochem.* **1973**, *54*, 484.
 Darvill, A.; McNeil, M.; Albersheim, P.; Delmer, D. "The Biochemistry of Plants"; Academic Press: New York, 1980; Vol. I, pp 91-162.
 Deiana, S.; Erre, L.; Micera, G.; Piu, P.; Gessa, C. *Inorg. Chim. Acta* **1980**, *46*, 249.
 Deiana, S.; Micera, G.; Muggioli, G.; Gessa, C.; Pusino, A. *Colloids Surf.* **1983**, *6*, 17.
 "Food Chemicals Codex", 3rd ed.; National Academy Press: Washington, DC, 1981; pp 215-217.
 Hall, M. A. 7th Long Ashton Symposium on Quality in Stored and Processed Vegetables and Fruits; Academic Press: London, 1981; pp 53-64.

- Havens, J. R.; Koenig, J. L. *Appl. Spectrosc.* **1983**, *37*, 226.
- Irwin, P. L.; Pfeffer, P.; Gerasimowicz, W. V.; Pressey, R., Sams, C. *Phytochem.* **1984a**, *23*, 2239.
- Irwin, P. L.; Sevilla, M. D.; Shieh, J. J. *Biochim. Biophys. Acta* **1984b**, *805*, 186.
- Jarvis, M. C. *Plant Cell Environ.* **1984**, *7*, 153.
- Knee, M. Colloques Internationaux C.N.R.S. No. 238, Facteurs et Regulation de la Maturation des Fruits, 1974, pp 341-345.
- Knee, M. *J. Exp. Bot.* **1982**, *No. 33*, 1263.
- Knee, M.; Bartley, I. M. "Recent Advances in the Biochemistry of Fruits and Vegetables"; Academic Press: London, 1981; pp 133-148.
- Knowles, P. F.; Marsh, D.; Rattle, H. W. E. "Magnetic Resonance of Biomolecules"; Wiley: London, 1976; pp 1-29.
- Kohn, R.; Larsen, B. *Acta Chem. Scand.* **1972**, *26*, 2455.
- Motschi, H. *Naturwissenschaften* **1983**, *70*, 519.
- Pfeffer, P. E.; Gerasimowicz, W. V.; Piotrowski, E. G. *Anal. Chem.* **1984**, *56*, 734.
- Pines, A.; Gibby, M. g.; Waugh, J. S. *J. Chem. Phys.* **1973**, *59*, 569.
- Rees, D. A.; Morris, E. R.; Thom, D.; Madden, J. K. "The Polysaccharides"; Academic Press: New York, 1982; Vol. I, pp 195-290.
- Van Cutsem, P.; Gillet, C. *J. Exp. Bot.* **1982**, *33*, 847.
- Van Cutsem, P.; Gillet, C. *Plant Physiol.* **1983**, *73*, 865.
- Van Vleck, J. H. *Phys. Rev.* **1948**, *74*, 1168.
- Voragen, F. G. J.; Timmers, J. P. J.; Linssen, J. P. H.; Schols, H. A.; Pilnik, W. *Z. Lebensm.-Unters.-Forsch.* **1983**, *117*, 251.
- Yannoni, C. S. *Acc. Chem. Res.* **1982**, *15*, 201.

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