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NMR proton relaxation measurements of water associated with high methoxy and low methoxy pectins

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Abstract

Nuclear magnetic resonance was used to study water proton relaxation in high (HMP) and low methoxy pectins (LMP). In solution, on the order of 40% of the water molecules may be affected by interactions with pectin molecules, dependent upon pectin type and concentration. Transverse relaxation rates were greater for HMP than LMP, and were attributed to decreased mobility of water protons and the lesser number of methoxyl groups in LMP. Plots of relaxation rates ($R_2 = 1/T_2$) versus the 90–180° pulse spacing were used to calculate the proton exchange rate (k_b). HMP and LMP has k_b values of 128 and 135 s^{-1} at pH 2.5. For LMP k_b increased to 280 s^{-1} at pH 6.5, but decreased for HMP above pH 6.5. Dry samples were equilibrated to water activity values between 0.11 and 0.75. Proton relaxation was quite rapid in these systems, suggesting limited water mobility. At higher moisture levels, R_2 values were progressively lower and more evenly spread over a wider pulse spacing range, indicating additional populations of more mobile water. Studies on LMP which were gelled with Ca^{2+}, showed a substantial increase in relaxation rates and little dispersion behavior, and this was attributed to the decreased mobility of pectin biopolymers.

Keywords: Pectins; Proton relaxation; Transverse magnetization

1. Introduction

Pectins are used in a wide variety of food products. For many products, native fruit pectins are released by heating and gelled at low pH; additional high methoxyl pectins (HMP) and sugar may be introduced to enhance gelling. Low sugar jams and jellies are made with low methoxyl pectins (LMP) that gel with calcium. Pectins are also used to produce fruit flavored jelly candies (Carr, Sufferling & Poppe, 1995), to enhance the quality of some frozen fruits and ice pops (Wegener, Baer & Rogers, 1951), to improve the mouthfeel of yogurt (Basak & Ramaswamy, 1994), to impart improved cloud characteristics to beverages (El-Shamei & El-Zoghbi, 1994), and to limit lipid migration through edible coatings on confectionery products (Brake & Fennema, 1993).

At the molecular level, pectins are complex polysaccharides with heterogeneous structure and a range of molecular weights. The primary structure consists of D-galacturonic acid and rhamnogalacturonan (Thakur, Singh & Handa, 1997; Towel & Christensen, 1995). Various carboxyl groups of the galacturonic acid may be esterified with methyl groups. Typically, pectins contain both branched and unbranched regions; branched regions contain a higher percentage of rhamnose units, which cause kinks in the chain, and which may carry other neutral sugar side chains (Lau, McNeil, Darvill & Albersheim, 1985; Schols & Voragen, 1994;). Pectins are classified as low methoxyl (LM) or high methoxyl (HM) according to their degree of esterification. The former contain between 25–50% methoxylated carboxyl groups; the latter between 50–80%. The specifics of the molecular structure determine the properties exhibited by various pectin fractions. For example, high methoxy pectins gel in the presence of cosolutes such as sucrose when subjected to heating. In contrast, low methoxy pectins gel in the presence of cations such as calcium.

Pectins are classified as hydrocolloids due to their high molecular weight coupled with the abundance of polar and ionic groups on their sidechains. One of the crucial properties of any food hydrocolloid is its ability to interact with water. The hydrocolloid may bind, immobilize, or otherwise interact with water near its surface. In turn, water acts as a plasticizer for such polymers, that is, it increases the free

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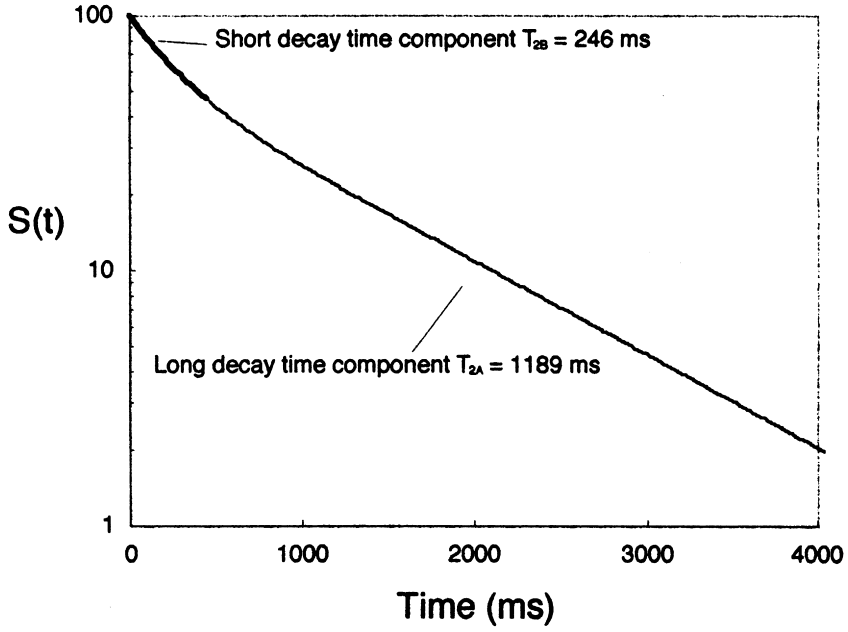


Fig. 1. Transverse ^{1}H NMR relaxation curve for 0.025% solution of high methoxy pectin.

volume for molecular motions. At the macroscopic level, such interactions will influence water binding capacity, juiciness, gelation, and textural properties of the material. Nuclear magnetic resonance (NMR) has been used extensively to characterize the hydration properties of proteins and carbohydrates (Hills, Takacs & Belton, 1990; Schmidt & Lai, 1991). In the latter category, the water associated with simple sugars has been studied, as has that in macromolecules such as starch, maltodextrins, cellulose, glycogen, agarose, agar, and carageenan (Bociek & Franks, 1979; Harvey & Symons, 1978; Hennig & Lechert, 1974; Richardson, Baianu & Steinberg, 1987; Tait, Suggett, Franks, Ablett & Quickenden, 1972). Typically, water properties are probed using ^{1}H, ^{2}H, or ^{17}O NMR relaxation phenomena, including both longitudinal (spin–lattice) T_1 and transverse (spin–spin) T_2 processes.

To date, few NMR studies have been done on water dynamics in pectins. Proton relaxation measurements of pectins have been made by Leung and Steinberg (1979) using continuous wave NMR, and by Leung, Steinberg, Wei and Nelson (1976) using pulsed NMR. These studies purport to calculate bound water, based on NMR signal intensity versus water activity. Modern instrumentation and pulse sequences allow direct measurement of longitudinal and transverse NMR relaxation phenomena. In addition, more recent theories have gone beyond free and bound water interpretations, and allow consideration of chemical exchange and dipolar cross-relaxation mechanisms (Belton, 1990). In this study, we used proton NMR relaxometry to study water relations in pectins, and interpreted the results in terms of current understanding of interfacial phenomena that lead to enhanced relaxation of water protons.

2. Methods

2.1. Sample preparation

Nonstandardized low methoxy and high methoxy pectins were obtained as dried powders from Citrus Colloids Company (Parsippany, NJ). The average degree of esterification was 30% for the LM pectins and 68% for the HM pectins. Low concentration pectin solutions (0.025–1% (w/w)) were prepared by slowly dispersing weighed amounts of pectin into distilled water that was being rapidly mixed on a magnetic stir plate. The pH of the solutions was varied between 2.5 and 8.5 by adding minute amounts of NaOH or HCl. The pH was measured immediately before, and just after, NMR measurements were made on the solutions.

Low moisture systems were prepared by equilibrating pectin powders over saturated salt solutions in sealed containers. The salt solutions and their associated relative vapor pressures included: LiCl (0.11), KCH_3CO_2 (0.22), $MgCl_2$ (0.33), $Zn(NO_3)_2$ (0.40), $Mg(NO_3)_2$ (0.53), and NaCl (0.75). The final moisture content of the samples was determined by the weight difference before and after drying in a vacuum oven at 75°C for 72 h.

Pectin gels were formed from 1% LMP solutions at pH 6.0 by the slow addition of 100 mM $CaCl_2$ to give a final concentration of 25 mg Ca^{2+} per gram of pectin. Samples were allowed to set up in a refrigerator at 4°C for 24 h, then re-equilibrated to room temperature prior to measurement. For HMP gels, 1 g HMP and 5 g sucrose were dispersed in about 90 g water, then heated in a flask held in a boiling water bath. Additional sugar was added to give a concentration of 60% sucrose. Finally, citric acid was added to a

Table 1
T_2 relaxation times of high methoxy (HMP) and low methoxy (LMP) pectins determined by CPMG pulse sequence ($\tau = 10$ ms, RD = 10 s). Relaxation curves were fit by a bi-exponential regression routine of the form $S = P'_A \exp(-t/T'_{2A}) + P'_B \exp(-t/T'_{2B})$. Solution viscosity at 25°C relative to that of water (η/η_0) is also shown (within a column, data with different superscripts are significantly different at $p < 0.05$)

% Pectin	P'_A	T'_{2A} (ms)	P'_B	T'_{2B} (ms)	η/η_0
0.025% HMP	58.6	1189[a]	41.4	246[a]	1.10[a]
0.1% HMP	62.1	1299[b]	37.9	263[b]	1.32[b]
1% HMP	60.7	824[c]	39.3	192[c]	11.85[c]
0.025% LMP	57.5	1102[d]	42.5	235[a]	1.23[d]
0.1% LMP	57.8	1110[e]	42.2	240[a]	1.62[e]
1% LMP	62.1	756[f]	37.9	178[d]	26.73[f]

final concentration of 0.11 M. Gels were set at 4°C for 24 h prior to measurement.

2.2. Viscosity measurements

Kinematic viscosity (η/ρ) was measured using a Canon-Fenske (Kimax 300) capillary viscometer. Samples (10 ml) were placed in the viscometer, which was then held in a circulating water bath to maintain temperature at 25°C. The time t that it took for the liquid to fall between two engraved marks on the viscometer was measured with a stopwatch. All measurements were repeated five times. The capillary constant k was determined using water as a standard and the kinematic viscosity of the solutions was calculated from $\eta/\rho = kt$.

2.3. NMR relaxation measurements

Proton relaxation measurements were made with a 20 MHz NMR spectrometer (Resonance Instruments, Whitney, UK). All measurements were made at 25°C. Transverse (T_2) relaxation times were made using the Carr–Purcell–Meiboom–Gill (CPMG) pulse sequence: $90°_x - [\tau - 180°_y - \tau - \text{echo}]_n$ (Meiboom & Gill, 1958). To determine relaxation dispersion behavior, τ was varied between 25 and 10 ms. For all experiments, the data were averaged over eight scans with a recycle delay time of 10 s. Triplicate samples were measured at each condition. SAS software (SAS Institute, Inc., Cary, NC) was used to analyze data for differences between treatments. The general linear model ANOVA was utilized to test for effects of treatments. Correlation coefficients between measurements were also calculated and compared. The level of significance was defined as $p < 0.05$.

3. Results and discussion

3.1. T_2 relaxation measurements

A typical plot of transverse magnetization over time is shown in Fig. 1. The relaxation curves were generated using the CPMG sequence with $\tau = 10$ ms and a relaxation delay of 10 s. The resulting curves were analyzed using a multi-exponential regression routine. In general, a single exponential curve did not describe the data well, where as a bi-exponential curve did ($\chi^2 < 0.001$). Higher order models did not improve the fit. The bi-exponential model used to fit the data was of the form:

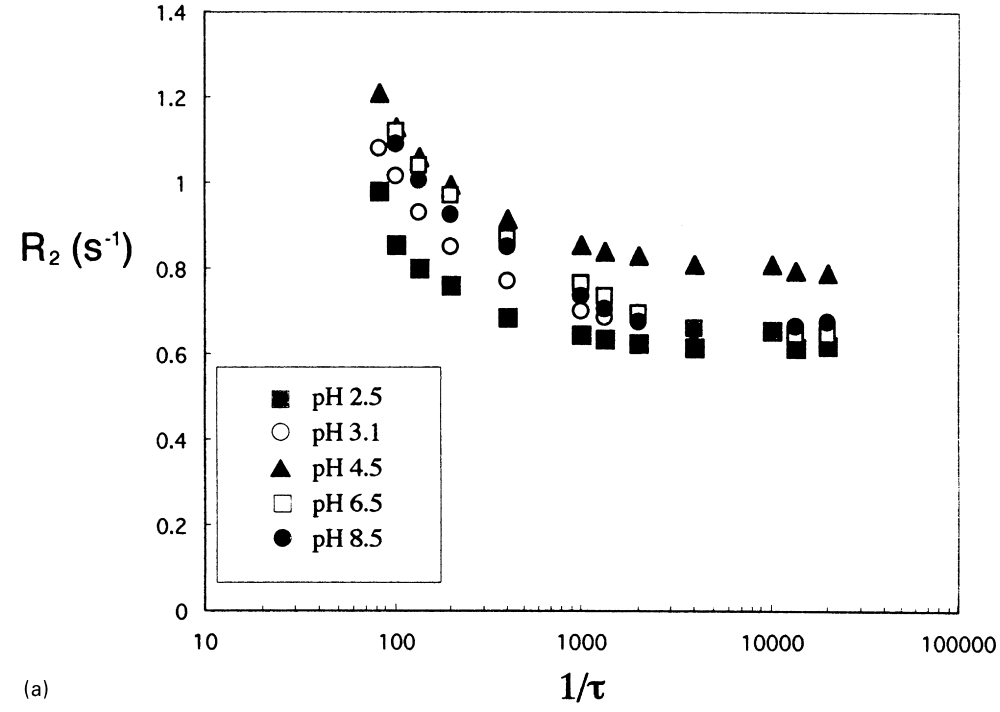
$$S(t) = P'_A \exp\left(\frac{-t}{T'_{2A}}\right) + P'_B \exp\left(\frac{-t}{T'_{2B}}\right) \quad (1)$$

where P'_A and P'_B are the proportions of, and T'_{2A} and T'_{2B} are the transverse relaxation times of components A and B. Table 1 shows tabulated results of T_2 measurements for several pectin solutions of concentrations up to 1% by weight.

In general, the T_2 relaxation curves could be resolved into short time constant (T'_{2B}:178–263 ms) and long time constant (T'_{2A}:756–1299 ms) components. These may be associated with water in differing physical environments. According to this interpretation, populations of water molecules away from the macromolecule have relatively slow relaxation, as the rapid rotation of molecules limits dephasing of spins. In contrast, molecules near macromolecular surfaces have restricted rotational motions or engage in intermolecular interactions, and thus are more able to exchange energy through spin–spin processes. It should be noted that with current understanding, assignment of the terms "bound" water (associated with short T_2) and "free" water (associated with long T_2) should be done only with great caution. Although binding of water to macromolecule sites may very well occur, other factors also influence the apparent relaxation times. In particular, diffusive exchange of aqueous protons between separated regions, or chemical exchange of protons in narrow regions, may occur (Belton, 1990; Hills et al., 1990).

However, water would be expected to closely interact with pectin molecules. Hills, Cano and Belton (1991) have studied a variety of polysaccharides such as dextran, laminaran, schleroglucan, and κ-carageenan. Their results indicate that such polysaccharides influence the state of water around them, and that the transverse proton relaxation is greatly influenced by proton exchange between water and carbohydrate hydroxyl groups. For pectin, each galacturonate or rhamnogalacturonan subunit contains 2–3 hydroxyl groups, depending on pH and degree of esterification, which form dipolar and hydrogen bonds with, or engage in chemical exchange with, water molecules. Binding or chemical exchange may also occur at carboxyl groups. In addition, Walkinshaw and Arnott (1981) have postulated that cages of water molecules surround methyl ester groups in pectin; formation of water clathrates around hydrophobic groups has been evidenced in other systems.

Table 1 shows T_2 times for both HMP and LMP at several concentrations. T_2 times were greater for HMP than LMP at similar concentrations ($p < 0.05$); this was true for both the



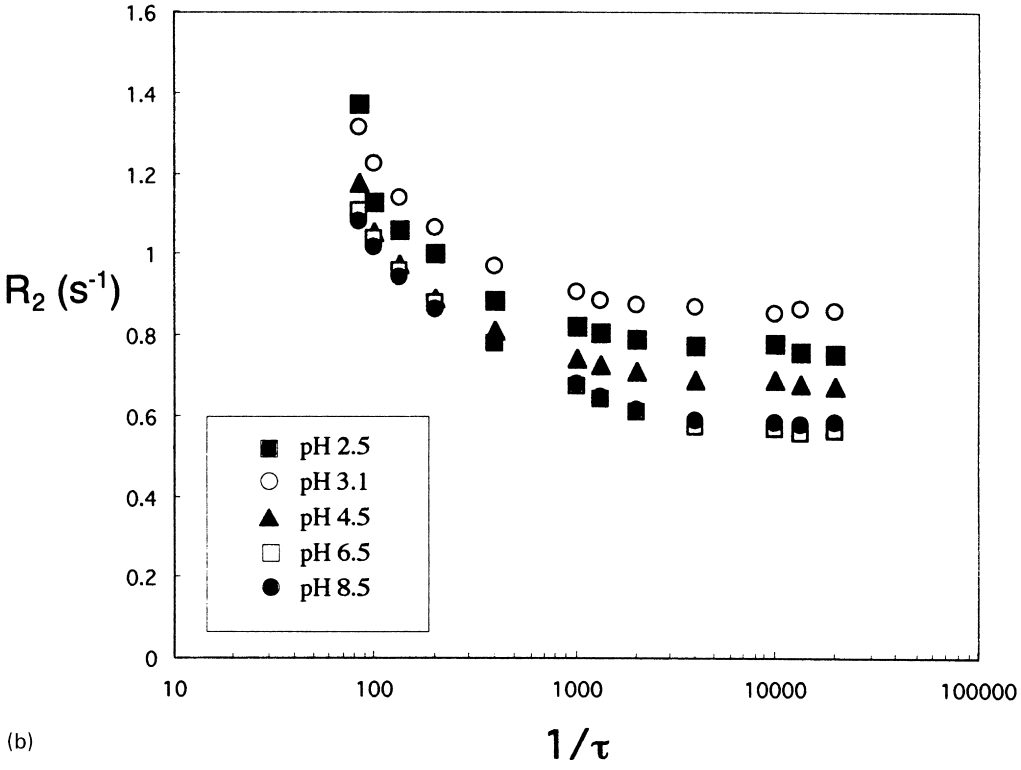


Fig. 2. Transverse relaxation rate ($R_2 = 1/T_2$) versus interpulse spacing ($1/\tau$) for: (a) 1% HMP solutions; and (b) 1% LMP solutions at different pH values.

short time constant component as well as the long time constant component. For example, $T'_{2A} = 1299$ ms for 0.1% HMP, and 1110 ms for 0.1%LMP; similarly, $T'_{2B\_} =$ 263 ms ms for 0.1% HMP and 240 ms for 0.1% LMP. Differences between T_2 values for HMP and LMP were significant at $p < 0.05$ for all similar concentrations, except for $T'_{2B\_}$ values of 0.025% HMP and LMP. The smaller T_2 values for LMP suggests decreased mobility of water

protons. This could be true for bulk water protons, as well as those affected by interactions with pectin molecules. Measurements of solution viscosity support this notion (Table 1). That is, the viscosity was higher in LMP than in HMP at all equivalent concentrations. In general, the correlation time for molecular rotations (τ_c) is increased by higher solution viscosity. The Debye–Stokes theory indicates that τ_c should vary as η/T, where η is viscosity and T the temperature.

An additional explanation for reduced T_2 values in LMP is that the type of interactions at macromolecular surfaces may be slightly different. As previously noted, HMP has a higher degree of esterified carboxyl groups. If one assumes that clathrate like cages are formed around ester groups, then these water molecules would have hindered mobility, but not to the extent that would be realized for dipolar or hydrogen bonded groups. Fullerton, Ord and Cameron (1986) have proposed a similar multiple-state model for hydration of a globular protein. In this view, water existing in a sheath near the macromolecular surface has a mobility that depends upon the nature of the interaction. For example, some water molecules may be bound by a single hydrogen bond but able to rotate, while others may be bound by multiple bonds and unable to rotate. Yet other hydrophobic sites may structure water around them; such water would have a τ_c closer to, yet still faster than, that of bulk water.

The data in Table 1 also show that T_2 values increased slightly as pectin concentration was increased from 0.025 to 0.1%; differences were only significant, however, for T'_{2B} of HMP. As concentration was increased from 0.1 to 1.0%, all calculated T_2 values decreased significantly. This may be due to the substantial increase in viscosity; there may also be additional intermolecular associations at higher concentrations. These could arise because of greater pectin density, as well as decreased electrostatic repulsions due to enhanced shielding by counterions.

The results also indicate that a substantial number of aqueous protons interact with HMP and LMP. Judging from the short T_2 components, between 37.9 and 42.5% of the water protons are affected by interaction with pectin molecules. Such values should be taken only as estimates, however, as the populations P'_A and $P'_{B\_}$ in Eq. (1) may not be exactly equivalent to the true populations of water molecules in different states (Belton, 1990). It is of interest that these values are in keeping with measurements of bound water by other researchers. For example, Leung et al. (1976) used sorption, dehydration, freezing, and NMR techniques to study water binding of pectin, alginate, cornstarch, casein, and cellulose. They found bound water capacities ranging from 14 to 51% depending on the type of food material and the method used to measure it. Again, it should be emphasized that the existence of bound water, and whether two populations of aqueous protons can be assumed is disputed by some (Hills et al., 1990).

In addition, there was not much difference between relative proportions of components with long and short T_2 values. The range of $T'_{B\_}$ for both HMP and LMP was 57.5–62.1%; for P'_A, 37.9–42.5%. The only difference between pectin types was for 0.1% HMP and LMP: P'_B was significantly larger (62.0 versus 57.8%) for HMP, while P'_A was significantly smaller (37.9 versus 42.2%). The effect of increasing concentration on P'_A and P'_B was also not great. Significant differences were only measured between 0.025 and 1% LMP ($P_B = 57.5$ versus 62.1%).

3.2. Relaxation dispersion experiments

3.2.1. Transverse relaxation as a function of pH

Relaxation rates ($R_2 = 1/T_2$) were determined as a function of the 90–180° pulse spacing τ, and are presented in Fig. 2. Measurements were made at 25°C and at various pH levels. All solutions displayed some dispersion of transverse relaxation rates. Such results indicate multiple spin–spin relaxation processes for protons in the system. The observed R_2 is a complex function of these individual processes. In the CPMG pulse sequence, an initial 90° pulse is followed after time τ with a 180° refocusing pulse. For long τ values, all processes have a chance to decay and contribute to the effective relaxation rate. At very short τ values, only rapidly dephasing proton states contribute to the decay. One convenient tool for analyzing such systems is the general two-site model proposed by Carver and Richards (1972). Two cases are of interest. For long pulse spacings ($\tau \gg k_b^{-1}$), then:

$$T_2^{-1} = T_{2A}^{-1} + P_B k_b \left[\frac{T_{2B}^{-2} + T_{2B}^{-1} k_b + (\delta\omega)^2}{(T_{2B}^{-1} + k_b)^2 + (\delta\omega)^2} \right] \quad (2)$$

where T_{2i} is the transverse relaxation time constant associated with site i, k_b the exchange rate between sites, and $\delta\omega$ is the chemical shift between protons at each site. In the limit of short τ, this reduces to

$$T_2^{-1} = P_A T_{2A}^{-1} + P_B/(T_{2B} + k_b^{-1}) \quad (3)$$

Hills et al. (1991) have argued that this approach is useful for distinguishing bulk water protons from protons which are chemically exchanging with macromolecule constituent groups. They indicate that protons that are inhibited by diffusion near the macromolecule surface do not contribute significantly to fast relaxation processes. The data shown in Fig. 2 were fit using Eqs. (2) and (3) to determine k_b and T_b. As the model incorporates four unknowns in two equations, two of these must be estimated beforehand. We chose to specify T_{2A}, $\delta\omega$, and P_B. T_{2A} (for bulk water) was measured as 2 s while the chemical shift was taken as 10 ppm. P_B for 1% (w/w) pectin was estimated assuming two exchangeable OH groups per monomer, and one carboxyl group depending on pH and degree of esterification.

The calculated exchange rate varied with pectin type and pH. At low pH, both LMP and HMP exhibited similar k_b values (128 versus 135 s^{-1}). As pH in the LMP solutions was increased, the general trend was for k_b to increase,

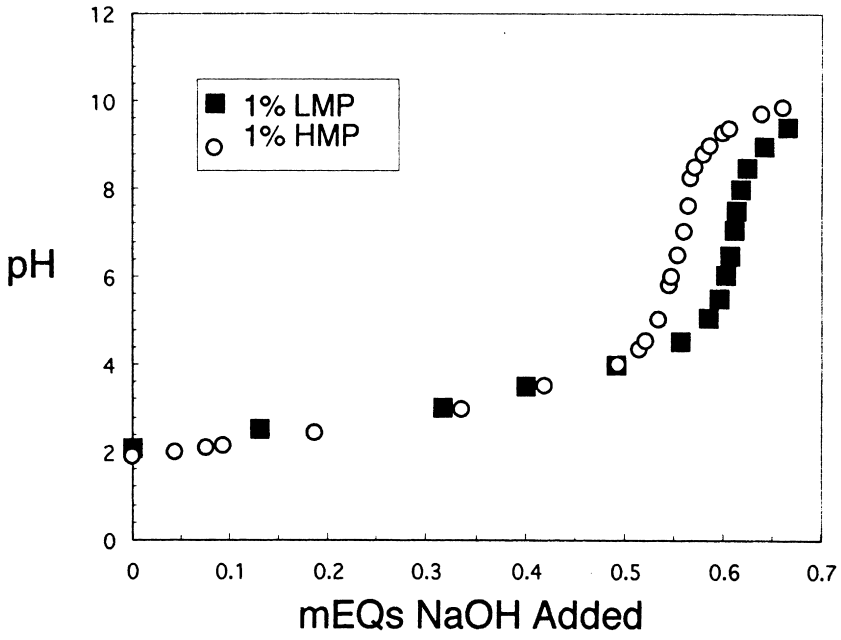


Fig. 3. pH titration curves for high (HMP) and low (LMP) methoxy pectins.

reaching a value of 272 s^{-1} by pH 8.5. Greatest increases were seen as the pH increased above 4.5. These results may due to the acid-base catalysis of proton exchange. Dependence of the proton exchange rate on pH has been observed for a variety of compounds containing labile protons (Rabenstein & Fan, 1986). Here, the system is particularly susceptible to increased rates in basic conditions. These results coincide with results of titration curves (Fig. 3). Such curves indicate that pH increases at a lesser rate per equivalent of base (~1 pH unit per 0.1 mEQ) added at pH levels below 4. At a pH value of 4.5, there is an abrupt increase in the curve to about 1 pH unit per 0.01 mEQ added. This suggests that at least the protons on carboxylic acid groups were more readily removed by OH^- ions at higher pH. The effect of pH on solutions of 1% HMP was more curious. Here, the k_b decreased slightly to 111 s^{-1} at pH 3.1, increased to 192 s^{-1} at pH 4.5, then decreased substantially to 90 s^{-1} by pH 8.5. The exact cause of this behavior is uncertain, but might be postulated to be due to changes in structure or degree of aggregation at high pH. Several researchers have noted that alkaline saponification may occur at pH > 7.5–8.0, and depolymerization at $pH > 8.0$–8.5 (Bemiller, 1986; Rombouts & Thibault, 1986).

3.2.2. Transverse relaxation as a function of A_w

To further investigate the interaction of water with pectins, samples were prepared at several low and intermediate moisture contents. Fig. 4 shows the transverse relaxation rates versus CPMG pulse spacing for HMP and LMP at various A_w levels. As can be seen, apparent relaxation rates were much faster in the low moisture systems, being on the order of 2000–10,000 s^{-1}, as compared to 0.8–1.4 s^{-1} in 1% solutions. T_2 time constants ranged from about 100 μs to 400 ms. This suggests that for systems held at A_w from 0.11 up to 0.75, little if any highly mobile water exists. In other words, there is not a population of water molecules that is separated by large diffusion distances from the pectin molecules. In addition, dispersion curves could not be fit by a simple two-site model; indeed, in some cases the apparent R_2 decreased at longer τ. In general, lower moisture samples had higher R_2 at short pulse spacings. In addition, samples held at 11 and 22% relative humidity had a broader range of R_2 relaxation rates. For example, LMP (Fig. 4b) at 11% RH had $R_2 = 6500\ s^{-1}$ ($T_2 = 153$ ms) at $\tau = 70$ μs, and which had dropped below 100 s^{-1} by $\tau = 100$ μs. This did not seem to indicate the presence of water with a longer time constant, however. In these very low moisture samples the relaxation curves were quite noisy or indeterminate at pulse spacings greater than about 100 μs. Again, comparison with high moisture systems is informative. At long τ values in high moisture systems, both relaxation of bulk water protons and water protons at macromolecule surfaces contribute to a greater apparent relaxation rate; the two-site model predicts lower relaxation rates at shorter τ values (Table 2). In contrast, the low moisture systems do not have two distinct populations. Longer τ values do not lead to higher R_2, as the second population of protons do not exist. At long τ, most protons have re-equilibrated, and little if any signal is left.

At higher moisture levels, R_2 values were progressively lower and more evenly distributed over a wider pulse spacing range. For example, for LMP held at 75% RH, R_2 was approximately 2800 s^{-1} over the τ range 50 μs to 200 ms. One explanation for this phenomena is that additional quantities of water have progressively higher T_2

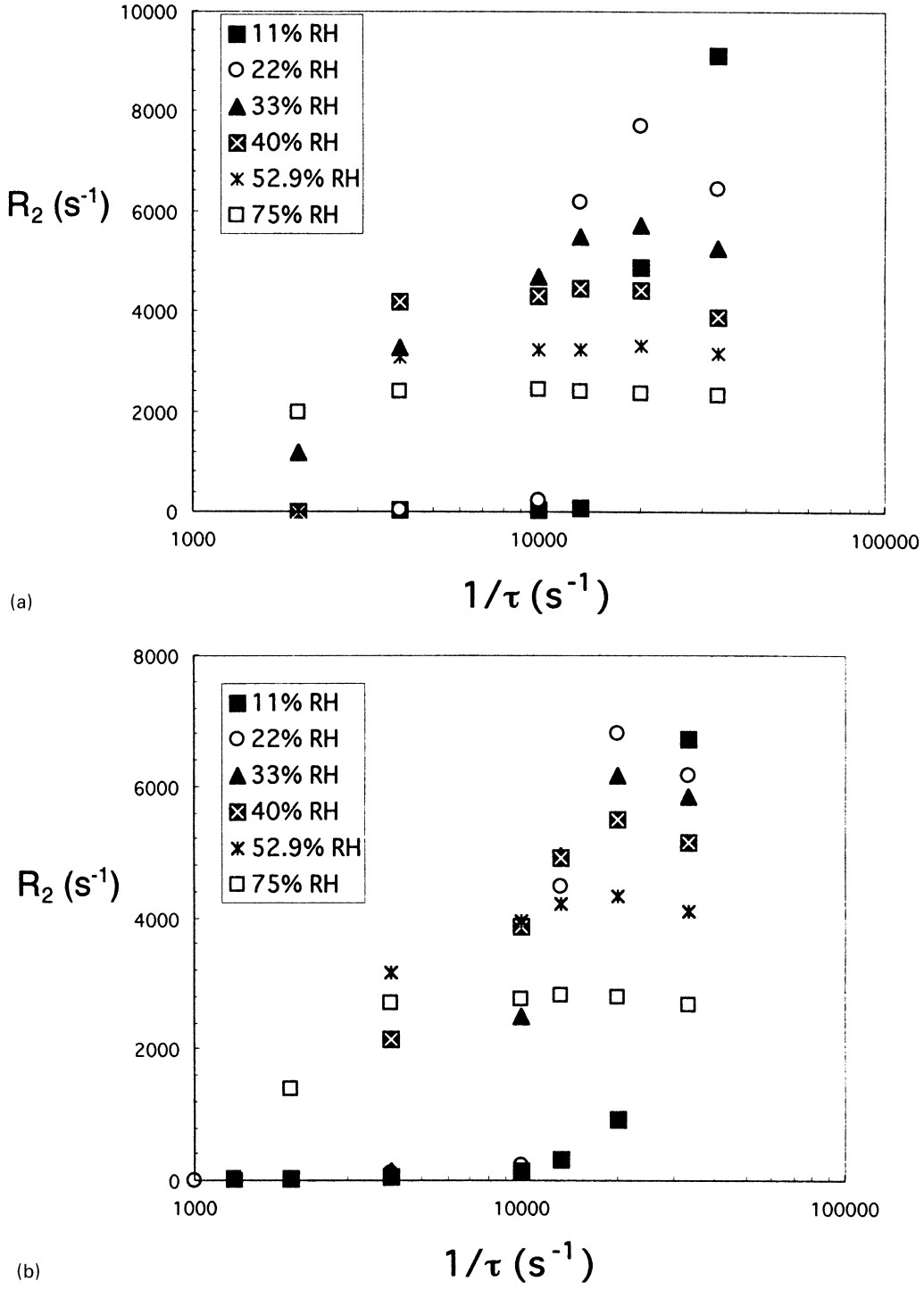


Fig. 4. Transverse relaxation rate ($R_2 = 1/T_2$) versus interpulse spacing ($1/\tau$) as a function of A_w for: (a) HMP; and (b) LMP samples.

values; for example, this water may be more mobile, but not to the extent that bulk water is. This concept is in keeping with the idea of "multilayer" water. That is, a single layer of water exists at the surface, or particular sites on the surface, and is most directly bound or affected by surface phenomena. Adjacent layers are more mobile, but are not as mobile as bulk water. Of course, this system is in fact dynamic; water molecules would be free to move between different layers and bulk water, but on average would be less free to diffuse in regions close to the macromolecular surface.

A simple picture can be derived from the moisture isotherm data (not shown) for HMP and LMP at 25°C. Calculation of the "monolayer" value of water using the BET theory (Peleg & Normand, 1992) gives a monolayer value of $V_m = 0.11$ g H_2O/g dry matter, and corresponding to a A_w of 0.31. We did notice a tendency for broadening of

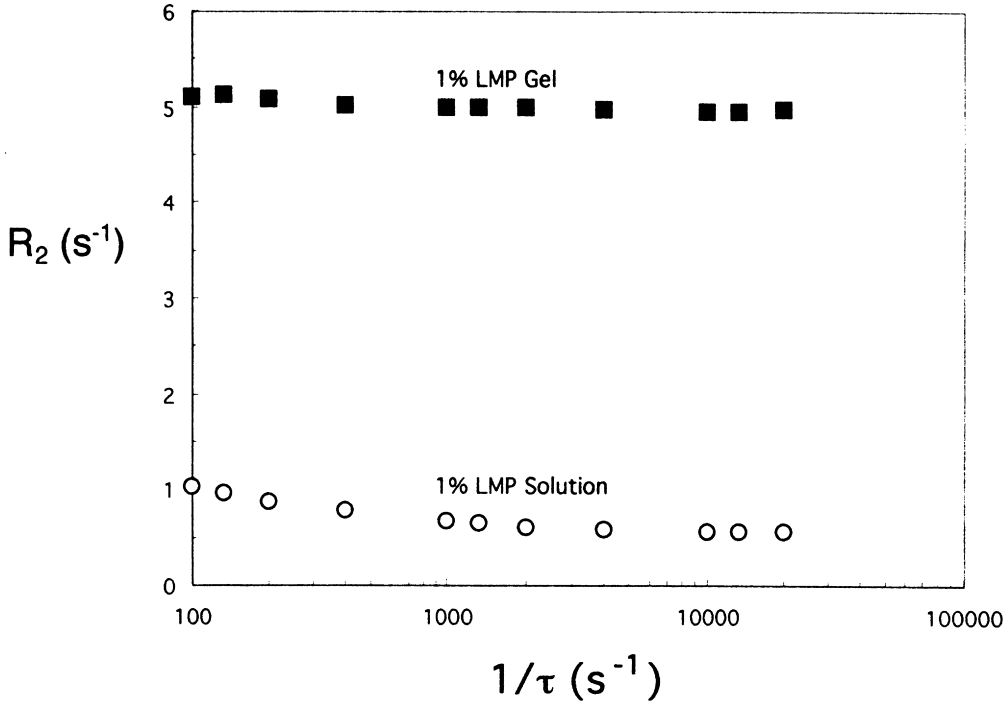


Fig. 5. Transverse relaxation dispersion for 1% LMP solution and 1% LMP gel.

the dispersion curves, and presumably formation of multilayers of water, at A_w values above 0.33–0.40.

3.2.3. Relaxation dispersion upon gel formation

Fig. 5 shows relaxation dispersion curves for a 1% solution of LMP previously adjusted to pH 6.0, as well as 1% LMP that had been gelled by adding 25 mg Ca^{2+}/g pectin. A substantial change in apparent relaxation rates occurs upon gel formation. R_2 increases from around 0.8–1.2 s^{-1} to over 5 s^{-1}. In addition, little if any change in R_2 with pulse spacing was noticed for the gelled system; T_2 for the gel remained around 195–200 ms regardless of τ. Hills (1992) has noted that gels such as schleroglucan or gelatin display flatter CPMG dispersion curves, and often have increased relaxation rates. He attributes this to increased chain rigidity of the biopolymer, due to the formation of junction zones and cross-links. In essence, the rotational correlation time τ_c, roughly the average time for the molecule to rotate, is significantly reduced.

Table 2
Proton exchange rate (k_b) calculated from data of Fig. 2, using two-site exchange model [Eqs. (2) and (3)]

Sample	PH	k_b (s^{-1})
1% LMP	2.5	128
1% LMP	3.1	185
1% LMP	4.5	171
1% LMP	6.5	280
1% LMP	8.5	272
1% HMP	2.5	136
1% HMP	3.1	111
1% HMP	4.5	192
1% HMP	6.5	87
1% HMP	8.5	90

4. Conclusions

The results from these experiments indicate that the way in which water interacts with pectins depends on a variety of factors including degree of esterification, solution pH, moisture content, or presence of a gelled system. In solution, on the order of 40% of the water molecules may be affected by interactions with pectin molecules. The result of this interaction is to reduce the transverse relaxation time of protons. Water molecules may have restricted motion near macromolecular sites due to binding as such and may experience cross-relaxation through dipole–dipole coupling; in addition, water molecules may exchange protons with surface constituent groups. The rate at which this occurs depends on solution pH. In low moisture conditions, most water molecules reside near the macromolecule and are affected by interactions with it. In gel systems, pectin molecules are severely restricted in their motion, thus more rapidly exchange spin energy with surrounding water molecules.

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