Effect of Urea on Molecular and Colloidal Aggregation of Proanthocyanidin Polymers from *Pinus radiata*

SEUNGROK KIM,[1] DON SARATHCHANDRA,[1] and DAVID E. MAINWARING[2,*]

[1] Center for Applied Colloid and BioColloid Science, School of Chemical Sciences, Swinburne University of Technology, Hawthorn, 3122, Australia; [2] Department of Applied Chemistry, Royal Melbourne Institute of Technology, Melbourne, 3000, Australia

SYNOPSIS

The effect of urea on the aggregation behavior of proanthocyanidin polymer from *Pinus radiata* (*P.r.*) extract has been studied by rheol ɔgical measurements and photon correlation spectroscopy (PCS). The urea induced structu ɔl changes in the concentrated extracts that were accompanied by significant reductions of apparent viscosity. The size reduction of proanthocyanidin polymers upon addition of increasing amount of urea could also be observed by PCS. In a concentrated alkali-sequential extract, addition of urea leads to a decrease in both the steady shear viscosity and the dynamic elastic modulus. Since the urea-induced structural changes of proanthocyanidin polymers are based mainly on the intermolecular colloidal association, the overall variation of reduced viscosity in the low concentration range of proanthocyanidin in the presence of various amount of urea was negligible. Thus it is suggested that addition of urea leads to the control of the noncovalent colloidal interactions among proanthocyanidin polymers from *Pinus radiata*. In the absence of urea, concentration of the *P.r.* extract to 40% tannin produced stable near-Newtonian solutions of low apparent viscosity. © 1996 John Wiley & Sons, Inc.

INTRODUCTION

Proanthocyanidin (PA) polymers (condensed polyphenol tannins) occur in the bark of all conifers and hardwoods examined to date, and they are frequently present in the wood itself.[1] PA polymers consist of linear chains of flavan-3-ol units that have undergone varying degrees of condensation,[2,3] as illustrated in Figure 1.

While the majority of PA consist of both procyanidins and prodelphinidins, Czochanska and colleagues[3] have shown that *Pinus radiata* (*P.r.*) bark consists of a ratio of 90 : 10 of these respective analogs. The number average molecular weight (M_n) of isolated *P.r.* proanthocyanidins has been found to be 1740.[3]

One of the important characteristics of the proanthocyanidin polymers in their use as polyphenolic resins is the formation of "phlobaphenes" after

extraction. These substances, although initially soluble in water, become insoluble as they associate with each other. Once formed, their dissolution requires polar solvents such as ethanol and acetone or an aqueous base. Phlobaphenes can be readily precipitated from a water solution of PA polymers by acid-induced condensation reactions. Some phlobaphenes are formed spontaneously and are complex mixtures of high molecular weight PA polymers which have in some instances been found to be associated with carbohydrates.[4] Generally, the polyphenols derived from the hot-water extract (HWE) of a bark or heartwood were considered to consist of about 60–65% proanthocyanidin polymers as measured by a standard hide powder absorption test.[1] The remainder has been considered to be a mixture of sugars, pectin, hemicellulose, and low molecular weight (≤ 300) polyphenols. Carbohydrate impurities are considered extraneous substances for such resin adhesives, thus there has been considerable research effort aimed at identifying particular plant species that yield an extract of high polyphenol content with low carbohydrate "contaminants."
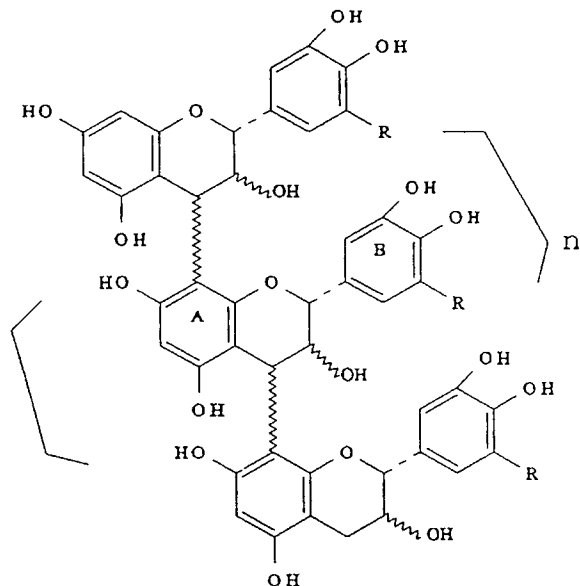
(a) R=H, procyanidin units (PC)

(b) R=OH, prodelphinidin units (PD)

Figure 1   Structure of proanthocyanidins.

Christiansen and Gillespie[5] showed that the carbohydrate, essentially in the reducing monosaccharide form, reacted only under acid catalysis with urea in adhesive development within a carbohydrate/urea/phenol/formaldehyde resin system.

In spite of the maturity of studies of plant-derived polyphenols and the increasing number of unequivocal molecular structures being established by NMR studies,[3,6,7] the complexity of the interactions involved in their condensation and aggregation has made it difficult to overcome limitations to their technological application. The facile ability of polyphenols to complex and aggregate with both proteins[8] and carbohydrates[9] is a well-known characteristic. The principal means whereby proteins and polyphenols are considered to reversibly complex and aggregate are via (1) hydrogen bonding and (2) hydrophobic interactions.[10] A recent study by Sealy-Fisher and Pizzi[11] showed that phlobaphene formation and precipitation during sulphite/water extraction of polyphenols from *P.r.* bark was minimized by preventing polyphenol self-condensation via the addition of urea. Urea was found to react preferentially with the reaction intermediates formed by the self-condensation mechanism of the polyphenols, thereby inhibiting the molecular rearrangement leading to phlobaphene precipitates.

The principal aim of this work is to investigate the influence of urea on the molecular aggregation behavior of extracts of *P.r.* bark. The use of additives is a common method to alter the aggregation behavior of macromolecules. Urea also influences the solubility of hydrophobic species, e.g., hydrocarbons in water.[12,13] Two mechanisms were put forward to explain the action of urea in aqueous media: (1) an indirect mechanism, in which urea breaks the hydrogen-bonded structure of water, facilitating the solvation of hydrophobic solutes,[14-16] and (2) a direct mechanism, whereby urea participates in the solvation of hydrophobic solutes in water by replacing some water molecules in the hydration shell of the solute.[17-19] Recent experimental[20,21] and computer simulation studies favor the direct mechanism[22] while recent neutron scattering experiments also show that urea causes no apparent disruption of the water structure even at high concentrations.[23]

EXPERIMENTAL

Sample Preparation

The main isolation and purification methods used to extract the plant materials were similar to those described previously.[24] Distilled water (500 mL) at 100°C was added to 100 g of dried *P.r.* bark powder and stirred vigorously for 10 min, prior to filtering with a Buchner funnel and No. 41 Whatman filter paper to remove insoluble materials and yield the HWE. Varying amounts of urea (BDH Chemicals, Australia), calculated on the basis of the total polyphenol content in the extracts, were added with vigorous mechanical stirring. The dilute extract solutions were concentrated to 20–25% solids in a Buchi rotary evaporator at about 45°C to prevent thermal condensation. The phlobaphene components of the initial extracts were isolated by centrifugation at 4,000 rpm for 30 min at room temperature, and the soluble fractions were examined by dilution capillary viscometry. Storage of these transparent supernatants produced second precipitates by both autocondensation[25] and colloidal or molecular association.[24,26] Thus, dilution capillary viscometry was carried out on the newly extracted fresh samples before the formation of possible molecular clusters. The samples (100 g) of the dried bark residue from 100°C water extraction were subsequently reextracted with 500 mL of boiling water and pH was adjusted to 10.6 with NaOH. The mixture was then stirred vigorously for 10 min in order to isolate hot water-insoluble fractions, prior to another filtering with a Buchner funnel and No. 41 Whatman filter paper. After filtration, concentration of this alkali

soluble sequential extract (ASE) was made under the same procedures as described previously.

Characterization

Capillary Viscometry

Viscometric measurements were carried out on the soluble fractions after filtration with a 8.0 μm Millipore filter using an Ubbelohde dilution viscometer. A capillary of 0.64 mm diameter was used and the viscometer and sample were maintained at a temperature of 20 $\pm$ 0.1°C. Shear rate effects were ignored, as our previous examination[24] of PA samples with capillaries of different sizes showed a negligible effect. The efflux time was measured for a set volume to flow through the capillary and repeated at least 5 times. The flow time for conductivity water was 159.4 s and the kinetic energy correction was therefore also neglected. The capillary viscometer was filled with nitrogen gas during the viscosity measurements. It is notable that Eliassaf[27] observed increasing efflux times with dilute solutions of polymethacrylic acid in the presence of urea. However, the efflux times of the PA polymers from P.r. in the presence of urea studied here did not change significantly with time over the measurement period.

Quasi-Elastic Light Scattering (QELS) Measurements

All QELS measurements were carried out in photon correlation spectroscopy (PCS) mode using a Model N4 MD analyzer (Coulter Electronics). The photomultiplier assembly was positioned at 90 degrees to the incident laser beam. A helium–neon laser operating at 632.8 nm was used as the scattering source. An incident intensity of 4 mW was employed. Diffusion coefficients were extracted from the autocorrelation functions by use of the regularization program CONTIN.[28] Hydrodynamic radii were then calculated by applying the Stokes–Einstein relationship:

$$r = k_B T / 6\pi\eta_o D_z$$

where r is the Stokes radius, k_B is the Boltzmann constant, η_o is the viscosity of the solvent, and D_z is the z-averaged translational diffusion coefficient,[29] determined at the measurement temperature (T) of 20°C.

The model N4 MD system employs a size distribution processor (SDP) system that utilizes CONTIN to calculate particle size distributions. The SDP and CONTIN analysis methods were utilized to characterize the molecular association of the extract solutions as a function of time. Standard Teflon-stoppered spectrophotometric cuvettes were employed as scattering cells and the cell was ultrasonically cleaned and flushed several times with double-distilled water filtered with a 0.22-μm Millipore syringe filter (Sterile Millex-GV) to remove any dust particles. The cell was further rinsed with filtered (0.22-μm Millipore syringe filter) diluent and filled with 3 mL filtered diluent. Approximately 0.025 mL of the appropriate extract solution (2% w/v) was introduced into the cell via an 8-μm Millipore syringe filter (Millipore-SC) to achieve a desired intensity of scattered light. All samples used for PCS were taken from concentrated 30% extracts and each experiment was repeated several times to assure reproducibility.

Rheological Measurements

Rheological parameters were measured at 25 $\pm$ 0.1°C and 40 $\pm$ 0.1°C with a Rheometrics Fluid Spectrometer RFS II (Rheometrics, Piscataway NJ) equipped with a parallel plate geometry (radius: 25 mm). The latter temperature was used in order to prevent precipitation or inhomogeneity during measurements of some samples, and it allowed measurement of the concentrated ASE by reducing the large torque generated from ASE at ambient temperature. Throughout the experiments, the torque sensitivity was varied by a decade by changing the mode of the force–rebalance transducer. Solvent evaporation was avoided by covering the edge of sample with a low viscosity silicon oil. No conversion of apparent viscosity to relative viscosity was made for the concentrated extracts, as the viscosity change of aqueous medium induced by the addition of urea was negligible.

FT-IR Spectroscopy

FT-IR spectra were measured on potassium bromide discs made as follows: 1.0 mg of each sample, previously dried at 40°C, was ground with 200 mg of predried potassium bromide in an agate mortar for 10 min, then pressed at 8 tonnes under vacuum for 5 min. The disc thus obtained (13 mm in diameter) was dried in a vacuum oven at 105°C for 1 day. The dried discs were examined on a Perkin-Elmer 1710 Fourier Transform Infrared Spectrometer. Three scans were made for each run to obtain the spectra.
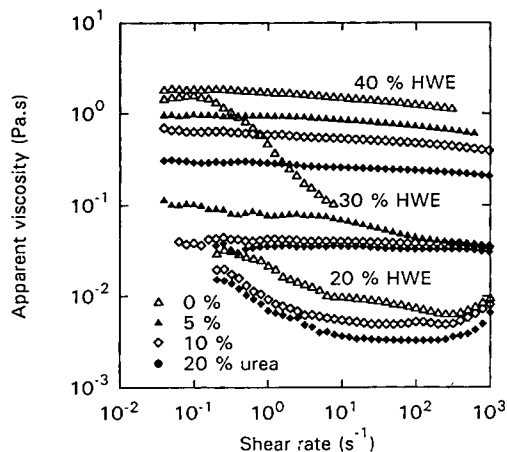
Figure 2 Steady rate sweep data for various concentrations of HWE depending on urea concentration at 25°C.

Figure 3 Steady rate sweep data upon cessation of shear for 30% (w/w) HWE with different urea content at 25°C.

RESULTS AND DISCUSSION

The steady shear apparent viscosity of the HWE was examined initially at various urea concentrations at 25°C, as shown in Figure 2. Hot-water extracts of 20% PA polymer are non-Newtonian shear-thinning extracts that have overall apparent viscosities which progressively reduce with increasing urea content. A clear low-shear Newtonian plateau was not demonstrated by the structured extracts due to instabilities at this low concentration. As the concentration is raised to 30% PA polymer in the absence of urea, a clear low-shear Newtonian plateau is seen prior to the onset of significant shear-thinning at a shear rate of about 10^{-1} s^{-1}. At this PA concentration, 5% urea breaks the microstructure sufficiently to both reduce the apparent viscosity by an order of magnitude and yield a low degree of shear-thinning at higher shear rates. Higher urea concentrations prevent a network microstructure forming and yield almost Newtonian behavior characteristic of independent particle motions. Concentration of the PA polymer to 40% yields a near-Newtonian liquid of apparent viscosity about 3 Pa s, with addition of urea progressively reducing the viscosity of the Newtonian liquids to about 0.5 Pa s. It is important to note that Figure 2 shows the progressive change in flow properties of the HWE without urea addition as the concentration is raised from 20% to 40% tannin. At 20%, low-viscosity solutions with a small degree of shear-thinning indicated limited molecular interactions. As the concentration is raised to 30%, the low shear limit indicates a plateau viscosity and extensive shear-thinning associated with shear-sensitive aggregation and structuring. At a concentration of 40%, a stable
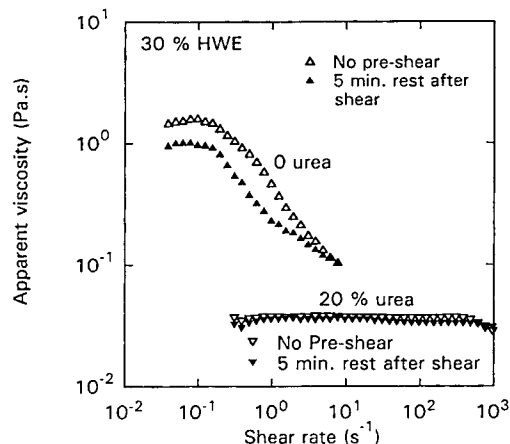
near-Newtonian liquid is formed, with an apparent viscosity of only 3 Pa s. This self-stabilization appears to be a unique feature of the rheology of these PA polymers.

Rate sweep measurements at different time scales were conducted to determine the impact of 20% urea on the disruption of HWE network structure, as shown in Figure 3. Unmodified extract can be seen to shear thin and recover to its original condition upon 5 min of cessation of the previous rate sweep, showing conventional time-dependent thixotropic behaviour. HWE containing 20% urea can be seen to have no recovery over a similar time scale and to remain Newtonian.

Further evidence for the effect of urea on the microstructure and rheology was obtained by a study of the apparent viscosity versus shear rate behavior of the HWE at 40°C, as shown in Figure 4. For all urea concentrations, the 30% HWE showed New-
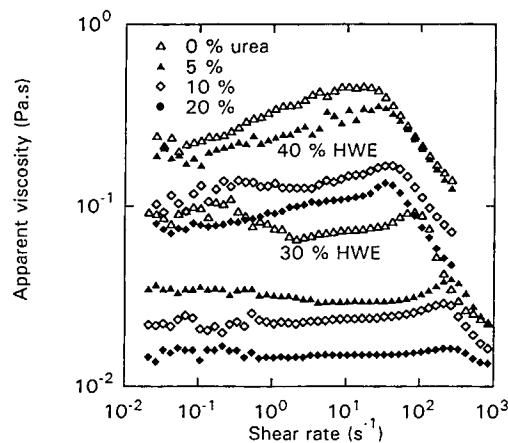

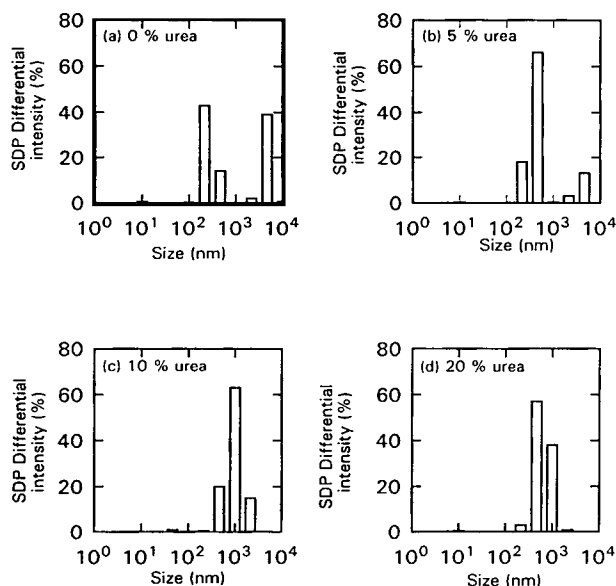**Figure 4** Steady rate sweep data for 30 and 40% HWE with various urea concentrations at 40°C.

Figure 5 Size distribution of HWE at pH 3.5 in the presence of various amounts of urea: (a) no urea; (b) 5% urea; (c) 10%; and (d) 20% urea in solution.

tonian behavior in the low shear rate region, with the onset of pronounced shear-thinning occurring above 100 s⁻¹. The decline in the extent of shear-thinning with increasing urea indicates a decreasing association between many of the individual macromolecules, i.e., less intermolecular interactions and mechanical entanglements with their higher energy dissipation, which are progressively broken down by increasing shear rate. When urea is added to HWE, a significant decrease in the Newtonian plateau viscosity is observed, accompanied by a reduction in the degree of shear-thinning. Disruption of the polymeric extracts by dissociation of intermolecular bonds by urea is necessary to account for this significant reduction of apparent viscosity. It is notable that the onset of shear-thinning in the flow behavior of these extract solutions occurs at higher shear rate with increasing urea content, which together with the reduction in degree of shear-thinning indicates a smaller but less fragile intermolecular association due to the hydrogen bond-breaking action of urea. A more complex flow behavior is shown for 40% HWE in Figure 4, where it can be seen that extracts with different urea contents undergo gradual shear-thickening followed by a significant shear-thinning at about 300 s⁻¹. This gradual shear-thickening flow behavior of the PA polymers was shown by Kim and Mainwaring[24] to be related to the polyelectrolytic character of the HWE through studies at different pH values. Yemelyanov and colleagues,[30] showed that the transition from Newtonian to non-

Newtonian shear-thickening occurred for aqueous polyacrylic acid (PAA) polyelectrolyte solutions as the hydrophobicity of PAA molecules was increased. It was suggested that this transition in flow character was due to the presence of hydrogen bonds of the macromolecule interacting with sites of strong polarity.

The nature of the association formed in the $P.r.$ extracts, responsible for the variation of apparent viscosity shown in Figures 2–4, was examined by PCS at 20°C (Fig. 5). It is clearly observed that urea can degrade molecular clusters which may be linked by either partial self-condensation,[31] electrostatic interaction, and/or molecular entanglement.[24,26]

The effect of urea on the rheology of the concentrated HWE (Figs. 2–4) was further investigated by measuring the reduced specific viscosity of dilute solutions of HWE at various urea concentrations. Figure 6 shows the dependence of the reduced viscosity on the extract concentration with different urea concentrations at 20°C. The reduced viscosity curve is strongly concave upward at lower concentrations, characteristic of a polyelectrolyte, in marked contrast to the behavior of uncharged linear polymers observed previously.[24] A similar behavior upon dilution is shown in the presence of $0.1M$ and $0.5M$ urea; that is, urea does not alter the intrachain expansion due to electrostatic repulsion. The absence of direct evidence of a urea-disordered water structure[14-16] is also consistent with the independence of the reduced viscosity with increasing urea concentration for these dilute PA polymer solutions. The similar shape and value of the reduced viscosity curves upon addition of urea suggests that urea mainly hinders the formation of the intermolecular
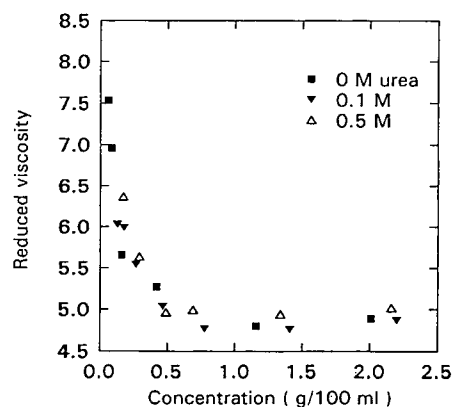

Figure 6 Reduced viscosity of the dilute solution of the HWE versus extract concentration at different urea concentrations.
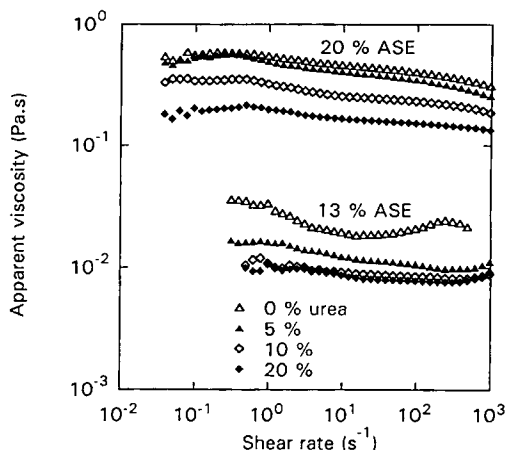
Figure 7 Steady rate sweep data for 13 and 20% ASE with various urea concentrations at 25°C.

Figure 8 Steady rate sweep data for 30 and 40% ASE with various urea concentrations at 40°C.

hydrogen bonds leading to macromolecular or colloidal structuring in more concentrated solutions, but does not influence intramolecular bonds or the individual macromolecular conformation in dilute solution.

The apparent viscosity versus shear-rate behavior of the ASE was also examined at various urea concentrations at 25°C, as shown in Figure 7. Kim and Mainwaring[32] recently demonstrated both the significant viscoelasticity of this ASE fraction and that this viscoelasticity was not shown at any concentration by the hot-water soluble (HWE) fractions. Figure 7 shows a significant increase in overall viscosity compared to the HWE (Fig. 2). This implies that the HWE has relatively smaller molecular flow units or a lower degree of molecular association. When the urea content in the extracts is increased, a decrease in viscosity is again observed.

The role of urea on concentrated ASE has been also examined at the higher temperature of 40°C (Fig. 8). Here, shear-thickening is observed at low shear rates[32] for all 30% ASE solutions, accompanied by a subsequent significant shear-thinning. It has been suggested that this shear-thickening flow behavior of ionic polymers is caused by single-chain elongation as shear causes the breakup of intramolecular ion pair associations and the formation of more intermolecular ion pair associations.[33] It is notable that the onset of pronounced shear-thinning in ASE viscosity occurs at relatively lower shear rates compared to HWE that was characterized by an extensive Newtonian plateau and slight shear-thickening preceding the shear-thinning; thus indicating a more extensive intermolecular association that may be due to greater molecular entanglement or electrostatic interaction. The dominant contri-
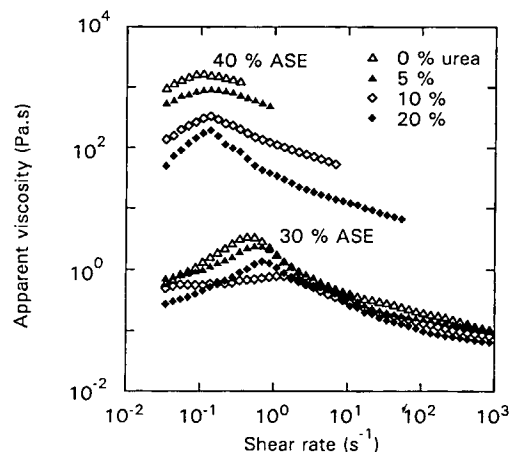
bution of urea to the solution viscosities of ASE is seen again at the higher extract concentration (40%). Increasing the polymer content of the extract to 40% results in a shift of the onset of shear-thinning to much lower shear rates.

Further evidence for the above effect of the phenolic acid fraction (ASE) on the microstructure and rheology of the solutions was obtained from plots of elastic modulus G' and viscous modulus G'' at different urea concentrations. Figure 9 shows the variation of G' and G'' with frequency (ω) for the 26% (w/w) ASE with different urea contents at 40°C. For both extracts examined, G' values dominate G'' and both moduli increase progressively with increasing frequency; i.e., the ASE extracts behave essentially as viscoelastic fluids whereas the corresponding HWE behaved as a viscous liquid, as discussed earlier.[32] This suggests that urea does not
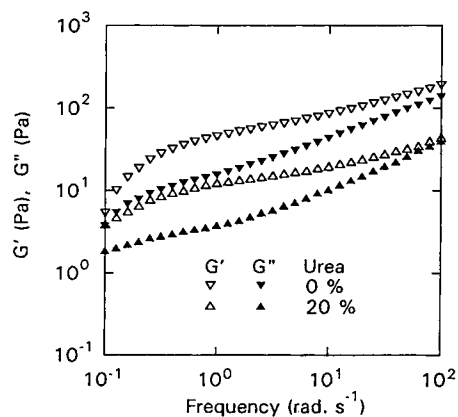

Figure 9 Dynamic frequency sweep data for 26% (w/w) ASE with different urea concentrations at 40°C (strain: 0.5%).

influence the spectrum of relaxation times within the macromolecule, an intramolecular behavior consistent with the reduced viscosity behavior in the presence of urea. However, both the elastic and the viscous moduli decreased in the presence of urea, indicating there is less "connectivity" in the extracts.

The FT-IR spectra of HWE and ASE samples used for the rheological measurements shown in Figures 2 and 7 were similar, as shown in Figure 10, (a) and (c). Consequently the polymers appeared to be basically procyanidin-related compounds, with the difference being caused by the degree of polymerization of the procyanidin unit in the fractions. The IR spectrum of HWE indicated the presence of a conjugated double bond (1611 cm^{-1}), aromatic double bonds (1595 and 1510 cm^{-1}), and a methylene chain (710 cm^{-1}). The FT-IR spectrum of ASE also showed strong broad bands at 3450, 1610, and 1065 cm^{-1} [Fig. 10(c)], indicative of the basic structure of procyanidins. These assignments correspond to the "phenolic acid polymers" of other workers.[34] The spectrum of samples containing urea [Fig. 10(b) and (d)] was very similar to those with no urea, differing only in the carbonyl absorbance at 1630 cm^{-1}.

CONCLUSIONS

The main aim of this work was to investigate the impact of urea, a common hydrogen bond breaker, on aqueous PA polymer solutions from *Pinus radiata*, where urea can be seen to prevent molecular aggregation and to allow the preparation of low-viscosity Newtonian extract solutions. Urea hinders intermolecular association and results in a reduction of viscosity in both the concentrated hot-water extract and alkali-sequential extract. This is supported by the PCS data, where gradual reduction of aggregate size was shown as the concentration of urea was increased. In a concentrated alkali-sequential extract, addition of urea disturbed the association among molecular clusters but did not disturb the molecular conformation in the clusters themselves.

There was no direct evidence from dilution capillary viscometry that urea disordered the water structure, i.e., no change of reduced viscosity upon increase of urea concentration in the presence of dilute PA polymers. Similar shape and similar value of reduced viscosity curves upon the addition of urea to dilute PA suggests that urea mainly hinders the formation of the intermolecular hydrogen bonds on a colloidal cluster basis, indicating no intramolecular bonds were broken by urea. It was shown that upon
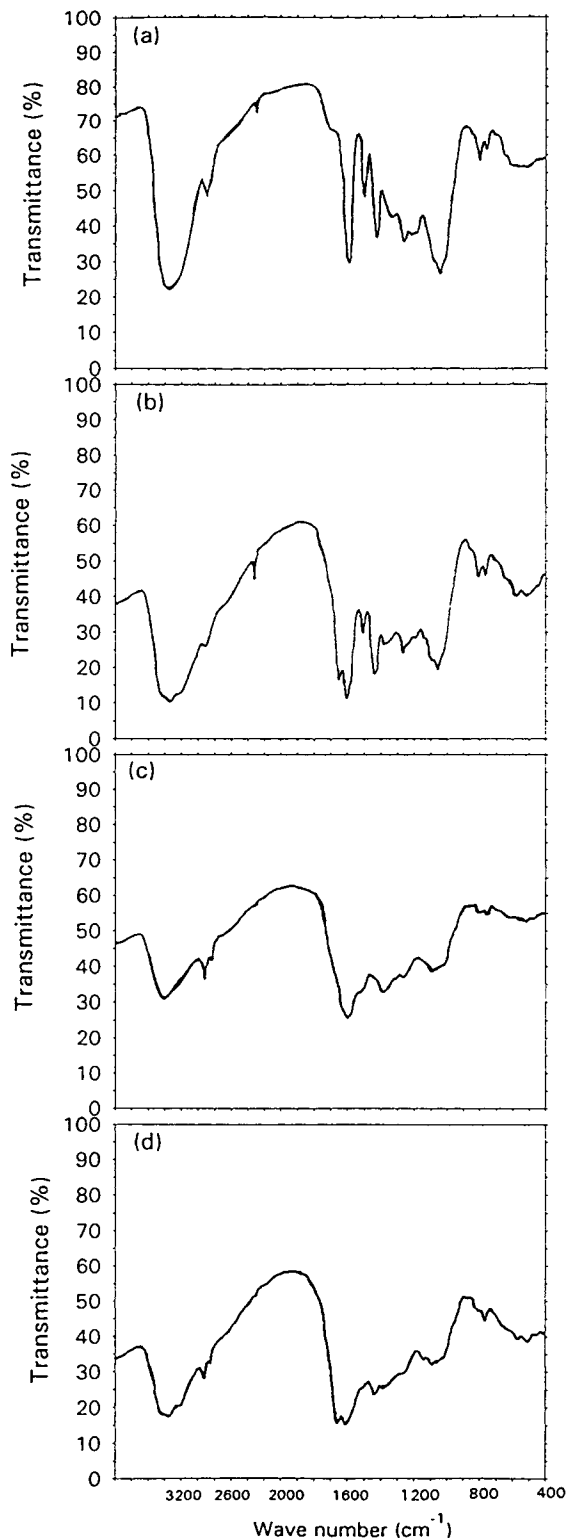
Figure 10 FT-IR spectra of HWE and ASE for (a) HWE: 0% urea; (b) HWE: 20% urea; (c) ASE: 0% urea; and (d) ASE: 20% urea.

concentration to 40% tannin, PA polymers formed stable near-Newtonian solutions with viscosities of only 3 Pa s, by a self-stabilization mechanism within the HWE.

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