What's in a Nutshell: An Investigation of Structure by Carbon-13 Cross-Polarization Magic-Angle Spinning Nuclear Magnetic Resonance Spectroscopy

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Solid-state ^{13}C CPMAS NMR spectroscopy was used to characterize the primary organic components of nutshells. These are cellulose, hemicellulose, and lignin, in proportions roughly similar to those found in hardwoods. The lignin is composed mainly of varying proportions of syringyl and guaiacyl units, while some nutshells also had smaller amounts of phenylpropane units and tannins. The fibrous packing tissue inside shells of hazelnut (*Corylus* spp.) was high in condensed tannins, while the packing tissue from pecan (*Carya illinoensis*) shells (a red powder) was entirely a prodelphinidin polymer.

INTRODUCTION

In the past decade, the technique of ^{13}C nuclear magnetic resonance spectroscopy with cross-polarization and magic-angle spinning (^{13}C CPMAS NMR) has been used to elucidate the chemical nature of many plant biopolymers. Many of these materials resist easy structural characterization by other chemical or spectroscopic techniques. They may (i) be highly insoluble due to extensive cross-linking or high molecular weight, (ii) be copolymers or physically linked mixtures of two or more dissimilar components, or (iii) lack an exact stoichiometry, repeating unit, or completely regular structure. Carbon-13 CPMAS NMR has already given us much information on the organic chemistry of complex biopolymers, including wood and lignin, plant cell walls, suberin, and cutin, pollen [a collection of references is given in Preston and Rusk (1990)].

There appears to have been no NMR investigation of another class of plant materials, namely nutshells. Nuts are a type of fruit, the body of which is formed from the carpel(s) of flowering plants (angiosperms) and helps to protect and distribute the seed. Nuts belong to the category of fruits that are dry rather than fleshy, and they are indehiscent or achenial; that is, they do not split and the seed is only released when the fruit wall rots or is otherwise damaged. Nuts have a hard and woody pericarp; in other indehiscent forms the pericarp is leathery or winged.

Nutshells can create a waste disposal problem; for example, over 50 000 tons of pecan (*Carya illinoensis*) shells are produced each year in the United States (Kays and Payne, 1982). However, new uses are being found for these materials, as "lost circulation materials" in oil drilling mud, as glue extenders (finely ground pecan shells) in the plywood industry (Kays, 1979), and as sources of molluscicides and antibacterials from cashew (*Anacardium occidentale*) shells (Himejima and Kubo, 1991; Kubo et al., 1986). In the Fraser Valley of British Columbia, hazelnut shells are used for garden mulch and pathways, home heating, and sandblasting and to give a gritty texture to a locally produced soap (H. Wiggin, BC Nut Growers Association, personal communication). The purpose of this investigation was to examine the potential of solid-state ^{13}C NMR to characterize the main organic components in a variety of nutshells.

MATERIALS AND METHODS

Sample Preparation. Samples of Persian walnuts (*Juglans regia*), almonds (*Prunus dulcis*), pecans, Brazil nuts (*Bertholletia excelsa*), and coconut (*Cocis nucifera*) were obtained from local stores. Another sample of walnuts was obtained locally from a backyard tree in Chemainus, near Victoria. One sample of hazelnuts (*Corylus* spp.) was purchased in a local store. It was from the Fraser Valley in British Columbia, was round in shape, and most likely was the Barcelona cultivar (H. Wiggin, personal communication). Two others, for which the shell had an elongated form and pointed ends, came from gardens in Victoria, and it was not possible to determine the species. (The two Victoria samples were designated L and D after the donors.) The macadamia nuts (*Macadamia integrifolia*) were purchased in Maui, HI.

The shells were cracked and the nuts removed for Christmas baking. The internal structure of the walnut shells was removed and discarded, and both walnut and hazelnut shells were thoroughly scraped with a small pairing knife to leave only the hard woody part. The reddish-brown fibrous material surrounding the nut inside hazelnut shells (packing tissue) was collected from one of the locally grown samples. Macadamia shells have a shiny interior, with half being dark brown and the other half almost white. This white material forms a layer about 100 μm thick, and about 750 mg was collected using a small carving chisel. The softer ribs inside the pecan shells were scraped away and discarded, and a sample of the packing tissue (a small amount of red powder) inside the shells was collected separately. Generally, shells were collected from 10 to 30 nuts, with larger numbers used for the smaller ones, such as almonds and hazelnuts. One coconut shell was analyzed.

The broken shells were rinsed with deionized water, dried at 105 °C for 24 h, and then finely ground in a Siebtechnik mill. This grinding took about 2 min and still left a mixture of fine material and coarse fragments up to 0.5 mm in size. These were removed using a 250-μm sieve (ASTM 60), and the fine portion was retained for analysis. The hazelnut lining was ground in a Wiley mill to 30 mesh (600 μm), and the white layer from the macadamias was ground under liquid N_2 with a mortar and pestle to approximately 20 mesh (850 μm).

Chemical Analysis. Samples were analyzed for total carbon by automatic combustion using a Leco Model CR12 carbon determinator. Ash was determined by combustion at 600 °C for 4 h. Due to the low N contents, total N was analyzed using the semimicro Kjeldahl method on 500-mg samples in duplicate as described previously (Preston et al., 1990a).

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NMR Spectroscopy. Solid-state ^{13}C NMR spectra were obtained on a Bruker MSL 100 spectrometer operating at 25.18 MHz for ^{13}C at 2.35 T. Samples were spun at 4 kHz in an aluminum oxide rotor of 7 mm o.d. Most spectra were acquired with 1-ms contact time, 1.5-s recycle time, and 12 000–50 000 scans and were processed using 15-Hz line broadening and baseline correction. (Low molecular weight compounds would not be observed using this rapid recycle time.) Dipolar-dephased spectra were generated by inserting a delay period of 40–100 μs without ^{1}H decoupling between the cross-polarization and acquisition portions of the CPMAS pulse sequence (Opella and Frey, 1979). Chemical shifts are reported relative to tetramethylsilane (TMS) at 0 ppm. Because the spectra were run at low field (2.35 T), there was no distortion of intensity due to generation of satellite lines known as "spinning sidebands".

Spectrum Analysis. Spectra were divided into chemical shift regions corresponding to chemical types of carbon as follows: (*A*) acetate 12–28 ppm; (*B*) methoxyl 50–60 ppm; (*C*) *O*-alkyl 60–96 ppm; (*D*) di-*O*-alkyl and aromatic 96–141 ppm; (*E*) phenolic 141–165 ppm; (*F*) carboxyl 165–185 ppm; and (*G*) aldehyde and ketone 185–210 ppm. Areas of the chemical shift regions were determined from the integral curves and were expressed as percentages of total area ("relative intensity"). For most samples, there was little intensity in the 0–50 ppm region, apart from acetate.

With the spectra divided into these chemical shift regions, proportions of lignin and carbohydrate C were determined according to the procedure outlined below [Preston et al., 1990b, modified from Hemmingson and Newman (1985)]. The relative intensity of the 141–165 ppm region (area *E*) arises almost entirely from the O-substituted carbons C_3 and C_4 in guaiacyl lignin and C_3 and C_5 in syringyl lignin. Therefore, the percent of total C due to the six aromatic carbons of lignin monomer units can be calculated as $3E$ and that due to the three carbons of the lignin side chain (C_α, C_β, C_γ) as $1.5E$. The 50–60 ppm region (area *B*) arises largely from methoxyl C; thus, total lignin C is given by

$$\text{lignin C} = 4.5E + B \quad (1)$$

The 60–96 ppm region (area *C*) arises from carbons 2–6 of cellulose and hemicellulose monomer units as well as from the three side-chain carbons of lignin. Including intensity due to the anomeric C_1, the total contribution of carbohydrate C is then

$$\text{carbohydrate C} = 1.2(C - 1.5E) \quad (2)$$

The ratio of carbohydrate to lignin monomer units (C_m/L_m) is calculated from

$$C_m/L_m = 1.2(C - 1.5E)/3E \quad (3)$$

The ratio of acetyl to carbohydrate monomers was calculated from

$$\frac{A}{0.2(C - 1.5E)} \quad (4)$$

The limitations of this method have been discussed previously (Hemmingson and Newman, 1985; Preston et al., 1990b). These calculations were not done for the coconut and Brazil nut samples, in which the tannin and phenolic lignin signals overlapped and area *E* could not be determined accurately.

RESULTS AND DISCUSSION

Figure 1a–g shows the ^{13}C CPMAS NMR spectra of shell material from almonds, walnuts (purchased sample), pecans, macadamias, hazelnuts (purchased sample, var. Barcelona), coconut, and Brazil nuts, with a spectrum of fresh heartwood from western hemlock [*Tsuga heterophylla* (Raf.) Sarg.] for comparison (Figure 1h). Virtually identical spectra were obtained for both walnut shell samples, and this is reflected in the data in Table II (discussed later). Similarly, there was little difference between the two types of hazelnut shells (the round Barcelona and the local elongated shape), and this is also reflected in the small differences in Table II. Due to limitations in the amount of NMR time available, it was not possible to examine further the sample-to-sample variation or to establish unequivocally the precision of the NMR method.

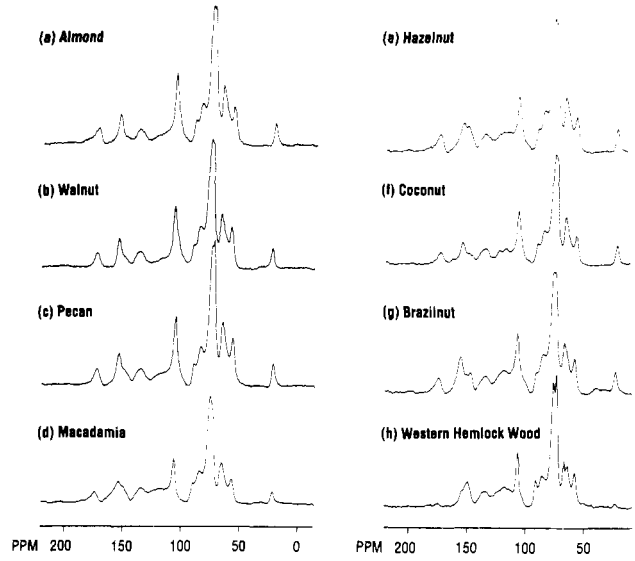

Figure 1. Carbon-13 CPMAS NMR spectra of nutshells (a–g) and western hemlock heartwood (h).

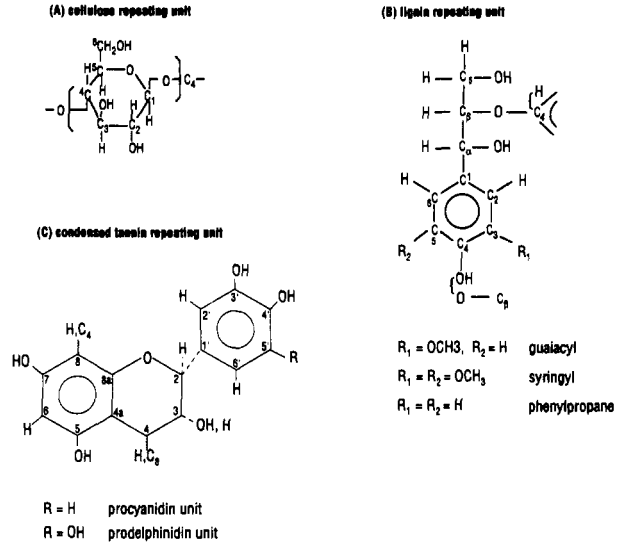

Figure 2. Repeating units for cellulose (A), lignin (B), and condensed tannins (C).

The spectra have much in common with that of wood and can be assigned on the basis of the many ^{13}C CPMAS NMR studies which have been made of wood, cellulose, and lignin (Barron et al., 1985; Hatcher, 1987; Hemmingson and Newman, 1985; Kolodziejski et al., 1982; Leary et al., 1986; Newman and Hemmingson, 1990; Preston et al., 1990b). The most prominent features may be attributed to cellulose, the structure of which is shown in Figure 2A. This gives rise to peaks at 65 ppm (C_6), at 75 ppm with an unresolved shoulder at ~72 ppm (C_2, C_3, C_5), at 82 and 89 ppm for C_4 in noncrystalline and crystalline forms of cellulose, respectively, and at 105 ppm for the anomeric carbon (C_1). The crystallinity of cellulose is higher in the wood, as can be seen from the sharp signals at 89 and 65 ppm for C_4 and C_6, respectively, in crystalline cellulose. In the nutshells, the peak at 89 ppm is poorly resolved and lower in intensity than that at 82 ppm, and only one broad peak is seen for C_6, although it is at 65 ppm, the characteristic frequency of C_6 in crystalline cellulose. The splitting of the main peak into two components at 72 and 75 ppm and the higher intensity of the 72 ppm component

are also consistent with higher crystallinity in the wood spectrum. In nutshells, the intensity maximum occurs at 75 ppm, with an unresolved shoulder at 72 ppm.

The presence of hemicellulose is indicated by peaks for acetate C at 21 and 171–173 ppm and a shoulder at 102 ppm in the anomeric region. Other peaks of hemicellulose occur in the same region as those of cellulose. In particular, the C_4 of hemicellulose occurs at 84 ppm, and the peak-height ratios of 84 vs 89 ppm have been used to estimate proportions of hemicellulose vs cellulose (Haw et al., 1985) in wood, although there is an overlap with the C_4 peak for noncrystalline cellulose. Separation of hemicellulose and cellulose subspectra can be achieved using a "delayed contact" CPMAS pulse sequence (Newman and Hemmingson, 1990) to determine accurately the degree of crystallinity in wood. This method should also be feasible for the nutshells, which have spectra very similar to those of wood.

Characteristic peaks for lignin are observed at 55 (methoxyl) and 110–160 ppm (aromatic and phenolic). The western hemlock spectrum is typical of that found for softwoods, for which the lignin is almost all based on the guaiacyl structural unit (Figure 2B). For guaiacyl lignin, C_3 occurs at 148 ppm, while the C_4 signal includes the etherified forms (in C_4–O–C_β linkages) at 153 ppm and the nonetherified C_4–OH at 146 ppm. The result for western hemlock is a broad phenolic signal with a maximum at 148 ppm and a shoulder at 153 ppm. The aromatic region has two broad maxima at approximately 132 (C_1) and 115 ppm (C_2, C_5, C_6). The three-carbon side chain of lignin (C_α, C_β, C_γ) occurs in the same region as the *O*-alkyl C of carbohydrate (70–80 ppm).

For syringyl lignin (Figure 2B), C_3 and C_5 occur at 153 ppm with (in wood) a small contribution at 148 ppm from C_3 and C_5 adjacent to nonetherified C_4. C_1 and C_4 occur at 135 ppm and C_2 and C_6 at 106 ppm. Published spectra of hardwoods show a mixture of syringyl and guaiacyl lignin, with generally low or nondetectable levels of *p*-hydroxyphenyl units; these have no methoxyl groups (Figure 2B) and a distinctive C_4 signal at 160–165 ppm (Leary et al., 1986; Manders, 1987).

The peak pattern for lignin in the nutshells is similar to that of hardwoods, for which varying proportions of syringyl vs guaiacyl lignin are also observed (Manders, 1987). On the basis of the appearance of the peak at 153 ppm, the ratio *G*/*S* increases in the series almonds, walnuts, pecans, macadamias, hazelnuts. The ratios for coconut and Brazil nut shells (Figure 1f,g) are more difficult to judge because of overlap of a tannin peak (vide infra) but appear to be similar to that of pecan shells. [The broad, weak signal at 37 ppm in Brazil nut shell (1.8% of total area) is most likely due to its tannin component.] For most wood spectra, there is little intensity at 160–165 ppm, but only the walnut shell spectra had this characteristic. For the others, the phenolic peak showed varying degrees of broadening into this region. This was quite obvious for macadamia shells, while a distinct peak is seen at 162 ppm for coconut. Morgan and Newman (1987) attributed this peak in rimu (*Dacrydium cupressinum*) wood to C_4 of etherified *p*-hydroxyphenyl units. The spectrum of the shiny white lining of the macadamia nuts (not shown) was very similar to that of the bulk darker portion of the shell (Figure 1d), except for an additional peak for aliphatic C at 33 ppm with a shoulder at 30 ppm. This amounted to approximately 5% of the total C.

The dipolar-dephased spectrum of pecan shell, shown in Figure 3a, is consistent with the assignments for the lignin and cellulose components. Intensity remains for

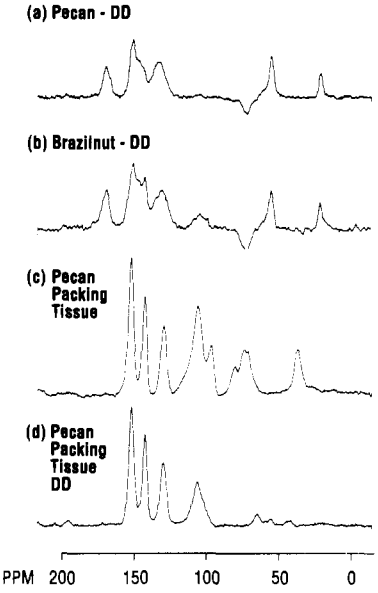

Figure 3. Dipolar-dephased ^{13}C CPMAS NMR spectra of pecan (a) and Brazil nut shell (b) and normal (c) and dipolar-dephased (d) spectra of pecan packing tissue.

Table I. Total C, N, and Ash Concentrations of the Samples of Nutshells

sample	comment	C	N	ash
almond		45.87	0.17	1.1
walnut		46.11	0.12	1.1
walnut	Chemainus	45.66	0.17	0.9
pecan		47.24	0.21	1.8
pecan packing		47.17	0.25	2.1
hazelnut	Barcelona	47.58	0.17	0.8
hazelnut	Victoria L	47.31	0.24	1.3
hazelnut	Victoria D[a]	45.99	0.18	1.0
hazelnut packing	Victoria D	45.13	0.56	–[b]
macadamia		49.06	0.23	0.8
macadamia lining		–	0.51	–
coconut		49.27	0.09	0.7
Brazil		49.14	0.45	1.9

[a] NMR spectrum not run. [b] Not determined, due to small size of sample.

the nonprotonated carbons of lignin, as well as for acetate CH_3 at 21 ppm and methoxyl OCH_3 at 56 ppm, due to the internal motion of methyl groups. All peaks of CH or CH_2 of carbohydrate are at zero intensity, except for the main cellulose peak which has a small residual negative signal. This phenomenon, which has previously been observed for cellulose in wood samples (Preston et al., 1990b), can occur when dipolar interactions are weakened by molecular motion and can also be caused by the magic-angle spinning itself when the spinning is done at high speeds (Newman, 1990). It can result in oscillatory behavior of the peak intensity when either spinning speed or dipolar-dephasing time is varied.

The general similarity of the organic components of nutshells to those of wood is also seen in the total C analyses of the samples (Table I), which were very similar to values found for wood (Preston et al., 1990b), although the total N values are somewhat higher. The nutshells, however, were much harder to grind than wood, and this difference may therefore lie in the three-dimensional arrangement and cross-linking of the components, rather than differences in their organic components.

Turning to the tannin components of the shells, Figure 2C shows the flavan-3-ol structure that forms the basic repeating unit of the condensed tannins. The polymeric procyanidins have two phenolic hydroxyls on the B ring

(C_3', C_4'); the prodelphinidins have three (C_3', C_4', C_5'). The polymers are formed by C_4–C_8 linkages, which can have cis or trans forms, and the monomer units are further distinguished by the stereochemistry at C_3. The stereochemistry is defined in detail in Czochanska et al. (1980).

Most tannin signals are coincident with those of lignin and carbohydrate, but the peak at 145 ppm (for C_3', C_5') can be observed in a region that is otherwise usually clear in wood spectra (Manders, 1987; Morgan and Newman, 1987). Brazil nut shells have the higher tannin content, followed by coconut. Tannins also have a characteristic peak at 108 ppm (the nonprotonated C_{4a}) which can be seen in dipolar-dephased spectra (Wilson and Hatcher, 1988). This is illustrated in Figure 3b for the dipolar-dephased spectrum of Brazil nut shell.

Tannins are more prominent in the material found inside the shells. The ^{13}C CPMAS NMR spectrum of the pecan packing tissue (Figure 3c) was completely consistent with a prodelphinidin polymer (Czochanska et al., 1980). On the basis of their solution ^{13}C NMR spectra of extracts, the spectrum of the pecan packing tissue can be assigned as follows: 154 ppm (C_5, C_7, C_{8a}); 145 ppm (C_3', C_5'); 132 ppm (C_1', C_4'); 108 ppm (C_{4a}, C_8, C_2', C_6'); 99 ppm (C_6); 73, 75, and 82 ppm (C_2 and C_3); 38 ppm (C_4).

The dipolar-dephased spectrum (Figure 3d) shows the nonprotonated carbons at 154, 145, 132, and 108 ppm. The peak at 108 ppm in the normal CPMAS spectrum arises from four carbons (C_{4a}, C_8, C_2', C_6'). Of these, C_2' and C_6' are protonated, C_{4a} is nonprotonated, and C_8, which is a linkage point, is protonated only where it occurs in an end unit. The broad asymmetric peak at 108 ppm in the dipolar-dephased spectrum thus includes both C_{4a} and C_8 in C_4–C_8 linkages. The rather broad line shape may be attributed mainly to a spread of chemical shifts due to packing effects which place more diverse strains on carbons near linkage points. There may also be some contribution from the cis isomer, for which C_{4a} is found at 102 ppm.

The region around 75 ppm is also sensitive to the stereochemistry of the linkages between the heterocyclic rings; C_3 of both cis and trans isomers occurs at 73 ppm, but for C_2 the trans form appears at 84 ppm and the cis form at 77 ppm. In addition, distinct signals for terminal units were observed in the solution spectra at 79 ppm for C_2 and 67–68 ppm for C_3. In the solid-state spectrum, we observe a broad region of intensity with maxima at 73, 75, and 82 ppm.

Kays and Payne (1982) analyzed H_2O-extractable phenols in pecan shells and packing tissue from different cultivars in reference to the production of phenolic plastics and resins from the shelling waste. Levels were very low (up to 1.5% by weight) in the shells but up to 50% in packing tissue. Our results from an unidentified cultivar are consistent with this, as we saw no tannins detectable by NMR in pecan shells, but the packing tissue appeared to be composed in its entirety of a condensed tannin. No other components were detected in the NMR spectrum but could be present at low levels (up to approximately 2%).

A tannin component was also apparent in the fibrous packing tissue from hazelnut shells. The NMR spectrum has a prominent peak at 145 ppm, while the tannin signature peak at approximately 107 ppm can be seen in the DD spectrum (compare parts a and b of Figure 4). The skins of mature peanuts (*Arachis hypogae* L.) were found to contain about 17% by weight procyanidins (Karchesy and Hemingway, 1986). The presence of tannins inside peanut, pecan, and hazelnut shells may protect the nuts from pathogens and herbivores (Zucker, 1983).

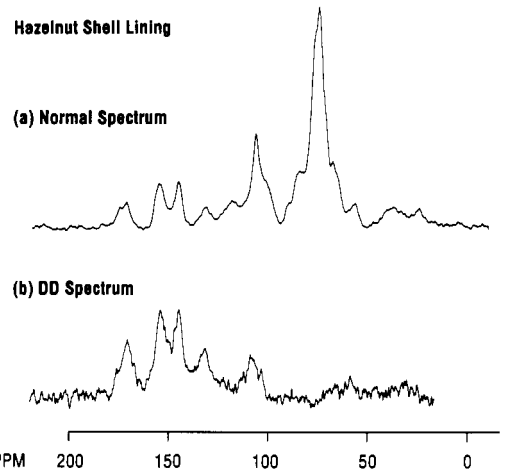

Figure 4. Normal (a) and dipolar-dephased (b) ^{13}C CPMAS NMR spectra of hazelnut packing tissue.

Table II. Proportion of C in Organic Components of Nutshells

sample	% of total C		C_m/L_m	area B/ area E	acetyl C/C_m
	lignin C	carb C			
almond	36.7	54.5	2.6	0.79	0.31
walnut	36.8	58.0	2.9	1.1	0.29
Chemainus	35.7	56.3	2.8	0.82	0.33
pecan	45.8	50.4	2.0	0.82	0.31
hazelnut					
Barcelona	49.7	45.6	1.5	0.47	0.45
Victoria L	52.8	42.5	1.3	0.45	0.46
macadamia	53.3	44.1	1.4	0.41	0.32
lining	43.6	46.1	1.8	0.48	0.50

The proportions of C in carbohydrate and lignin and the ratio of carbohydrate to lignin monomer units are shown in Table II. These values were not calculated for the coconut and Brazil nut shells, in which a tannin peak overlapped the phenolic lignin region. The values for C_m/L_m range from 1.33 to 2.93, again indicating a general compositional similarity to wood. Using the same NMR method, Hemmingson and Newman (1985) reported that C_m/L_m was 2.5 for *Eucalyptus regnans*, and Preston et al. (1990b) found 3.22, 2.22, and 2.24 for heartwood of old-growth Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco.), western hemlock, and western red cedar (*Thuja plicata* Donn.), respectively. The ratio of acetate to carbohydrate monomer units (Table II) ranges from 0.29 to 0.46 for the shells, with the macadamia nut lining a little higher at 0.50. Again, the results indicate similarity to hardwood, for which Hemmingson and Newman (1985) reported a value of 0.3.

We found, in general, that ^{13}C CPMAS NMR spectroscopy clearly elucidated the overall organic composition of nutshells which were composed of cellulose, hemicellulose, and lignin, in proportions roughly similar to those found in hardwoods. Some nutshells and packing tissues also contained tannins; the pecan packing tissue, to our surprise, was entirely tannin. It was not possible in this preliminary general survey to establish the precision and accuracy of the NMR method; this would be more appropriately done for specific applications. However, it is usually found that with good signal-to-noise ratios reproducibility is within 5% for samples such as wood and humus.

It should be noted that the NMR analysis could be taken further; it is possible to determine the relative proportions of syringyl and guaiacyl lignin in wood using ^{13}C CPMAS NMR spectra (Manders, 1987; Morgan and Newman, 1987). This should also be possible for nutshell spectra.

It does, however, require a dipolar-dephased or spin-locked spectrum to show nonprotonated C only for each sample and was beyond the resources or intended scope of this study. Carbon-13 NMR has also been used to determine tannin content in wood, especially for hardwoods with an accuracy comparable to or better than those of traditional wet-chemical methods (Morgan and Newman, 1987), but again using spectra that show only nonprotonated carbon. In this preliminary study we have ranked samples in order of *G*/*S* ratio and tannin content. As mentioned previously, it is also possible to use NMR to determine the degree of crystallinity of cellulose in wood (Newman and Hemmingson, 1990), a procedure that should also be transferrable to nutshells. Applications of solid-state NMR methodology to the analysis of nutshells and packing tissues should be possible in the utilization of nut processing wastes, as well as in studies of their biological functions.

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