Comparison of Carbohydrate, Lignin, and Protein Ratios between Grass Species by Cross Polarization-Magic Angle Spinning Carbon-13 Nuclear Magnetic Resonance

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Comparison of the ratios of three fiber constituents (carbohydrate, lignin, and protein) between whole grass samples of Coastal Bermuda grass and Kentucky 31' tall fescue is made by cross polarization-magic angle spinning carbon-13 nuclear magnetic resonance spectroscopy (CP/MAS ^{13}C NMR). The ratios determined by NMR are compared to those determined by near-infrared (NIR) reflectance spectroscopy and wet chemical analysis. The instrumental techniques are shown to relate more closely to each other than to wet chemical analysis. CP/MAS ^{13}C NMR is indicated as a complementary technique to NIR, providing definitive verification of chemical structural information to the rapid NIR analysis technique.

The development of rapid and nondestructive techniques for fiber analysis has received considerable attention within recent years. The most popular of the current techniques is near-infrared (NIR) reflectance spectroscopy (Barton and Burdick, 1981). This method is very rapid and nondestructive (except that the sample must be ground); however, it depends on signals that are often difficult to relate to any single definitive chemical structure. Nuclear magnetic resonance (NMR) spectra, on the other hand, are relatively easly to relate to precise chemical structure. Although not nearly as rapid as NIR, NMR is also nondestructive. The problem with NMR has been that, until recently, it could only be applied to liquid or liquidlike samples the nuclei of which possessed relaxation properties that would give a discernible NMR response. Now with the advent of solid-state NMR (Schaefer and Stejskal, 1979), ^{13}C NMR spectra of rigid materials can be obtained. This development has made possible the use of NMR in the analysis of fibrous materials.

Three important constituents of plant fiber are carbohydrate, lignin, and protein. Solid-state ^{13}C NMR spectra of these constituents have been obtained, separately or combined with one or another, by several researchers in the past 3 years (Atalla et al., 1980; Dixon et al., 1981; Earl and VanderHart, 1980, 1981; Kolodziejski et al., 1982; O'Donnel et al., 1981; Rutar et al., 1980; Schaefer et al., 1981) using cross polarization-magic angle spinning (CP/MAS) (Schaefer and Stejskal, 1979). Several of these researchers have reported that the ^{13}C NMR signals due to protein carbonyls were proportional to values obtained for protein by standard chemical methods.

Extending this concept, we now report the application of CP/MAS ^{13}C NMR to the determination of the ratio of carbohydrate, lignin, and protein between grass species from single spectra of whole plant material.

MATERIALS AND METHODS

Plant Materials. Coastal Bermuda grass [*Cynodon dactylon* (L.) Pers.] and Kentucky 31' tall fescue (*Festuca arundinacea* Schreb.) grasses were cut at a height of about 3 in. above ground from spring growth and 4 weeks of regrowth, respectively, fresh frozen, freeze-dried, ground to 16 mesh in a Wiley mill, and stored in jars excluding light until use. These whole plant materials were further ground to pass through a 1-mm screen in a UDY cyclone grinder immediately prior to NIR analysis. For NMR analysis, all plant materials were ground for 4 days under a nitrogen atmosphere by using a Norton jar mill, yielding an average particle size of approximately 15 μm. Holocellulose was isolated from whole grass according to standard procedures (Phillips et al., 1960). Lignin was obtained from whole grass, after jar milling, as acetal lignin according to procedures previously described (Himmelsbach and Barton, 1980). Chloroplastic protein was obtained by water extraction of a high quality (18.6% protein) Coastal Bermuda grass sample and subsequent fractionating at pH 6 according to procedures previously described by Evans (1982) and contained about 37% crude protein. The purified glycoprotein isolated from this preparation contained about 75% protein and 25% carbohydrate.

^{13}C NMR Spectroscopy. CP/MAS ^{13}C NMR spectra were obtained by using two different instruments equipped for solid-state NMR (Schaefer and Stejskal, 1979). Initial spectra of all materials were obtained by utilizing a Nicolet NT-150 NMR spectrometer operating at 37.7 MHz for ^{13}C. Subsequent spectra of whole plant materials were obtained by using a JEOL FX-200 NMR spectrometer operating at 50 MHz for ^{13}C. In both cases, samples were packed in bullet-type rotors made of Kel-F having a sample volume of approximately 0.7 cm^3. Samples were spun at a rate between 3.5 and 4 kHz at the "magic angle" of 54.7° relative to the applied field. Single 1-ms cross polarization contacts were made while satisfying the Hartmann-Hahn matching condition (Hartmann and Hahn, 1962). Pulse repetition rates used were 1-3 s. Data were collected over 1024 or 2048 points at 37.7 MHz and 4096 points at 50 MHz with a frequency of data acquisition of 10 kHz. Normally 10000 scans were taken to give an adequate S/N ratio. All spectra were referenced externally to hexamethylbenzene at 132.3 ppm relative to Me_4Si.

NIR Spectroscopy. NIR data were obtained by using a Neotec 6100 monochromator interfaced with a PDP 11/34 computer system employing chemometric procedures previously described (Barton and Coleman, 1981).

Chemical Analysis. Standard chemical analysis procedures were used to analyze for neutral detergent fiber (NDF), permanganate lignin (PML), and crude protein (Barton et al., 1976). Carbohydrate was assumed to be NDF minus PML which approximates holocellulose (cellulose plus hemicellulose). The details of the chemical analysis of these samples have been reported elsewhere (Windham et al., 1983).

RESULTS AND DISCUSSION

CP/MAS ^{13}C NMR spectra of ground whole plant material and isolated fiber constituents from Coastal Bermuda grass (CBG) and Kentucky 31' tall fescue (Ky-31) were obtained at 37.7 MHz. By comparison of these spectra to the spectra of the isolated fiber constituents, specific spectral regions were selected in the composite spectra of

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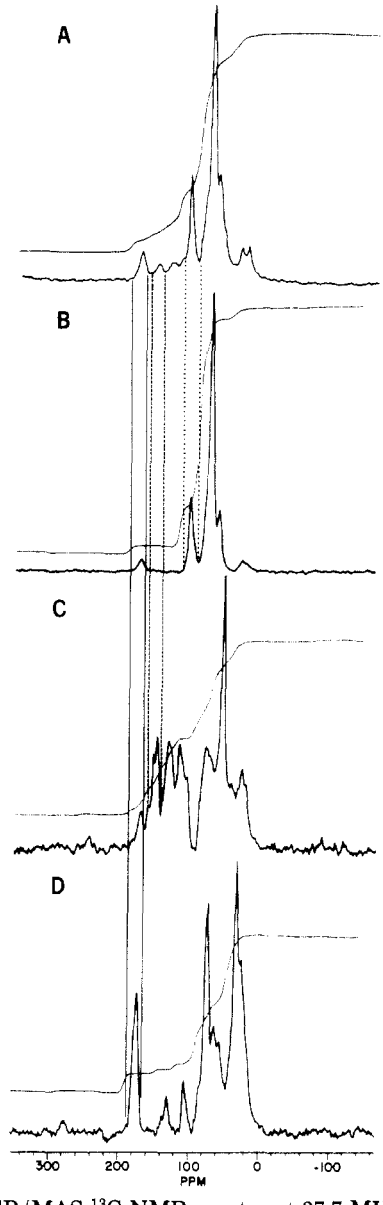

Figure 1. CP/MAS ^{13}C NMR spectra at 37.7 MHz of Coastal Bermuda grass and extracted constituents: (A) whole grass, (B) holocellulose, (C) acetal lignin, and (D) chloroplastic protein. Vertical lines drawn through spectra indicate regions selected to represent the various components [(···) for carbohydrate, (---) for lignin, and (—) for protein].

the whole plant materials from which the relative contribution of each component was determined. Figure 1 shows the spectra that were compared for Coastal Bermuda grass.

The first spectral region considered was that for carbohydrate, the largest fiber constituent. Holocellulose, which as an isolated fibrous fraction most closely approximates total carbohydrate (i.e., cellulose plus hemicellulose), was chosen as the isolated constituent to represent carbohydrate in grasses. The spectrum of holocellulose (Figure 1B) shows that this isolated material contains some protein and possibly some lignin but is primarily characterized by carbohydrate. The carbohydrate moieties involved are glucose (in the form of cellulose) plus arabinose, xylose, and glucose (in the form of hemicellulose). The carbohydrate signals appear in the 60–110-ppm region. By comparison to solution ^{13}C NMR data for carbohydrates (Stothers, 1972), the signals in this region may be assigned. The signal around 62 ppm can be assigned to hydroxymethylenes (carbon 6 in hexopyranoses and carbon 5 in pentofuranoses). The large signal centered at about 75 ppm can be assigned to carbons 2–5 in hexopyranoses and 2–4 in pentofuranoses. Due to a broadening caused by milling and a small downfield shift of this central carbon signal, the signals for carbon 4 are not resolved at 37.7 MHz as they are with purified unmilled cellulose at even lower field strengths (Atalla et al., 1980; Earl and VanderHart, 1980, 1981). The other signal that appears for carbohydrate around 105 ppm can be assigned to carbon 1 in both types of saccharides. This signal, around 105 ppm, was chosen as the signal to represent carbohydrate for comparison to whole plant material. It was chosen because it represents a single carbon type, is well separated from the other carbohydrate signals, and appears as a prominent well-defined signal in the spectra of the whole plant material.

The next spectral region considered was that for lignin. Lignin was chosen to be represented by acetal lignin that had been previously isolated and the solution ^{13}C NMR previously assigned (Himmelsbach and Barton, 1980). The solid-state ^{13}C NMR spectrum of this lignin (Figure 1C) shows two very obviously separated regions, an aliphatic region (0–95 ppm) and an aromatic region (100–160 ppm), plus a signal for carbonyls about 175 ppm. The entire aliphatic region of the spectrum contains signals that overlap either with carbohydrate or protein signals in the whole plant spectra. Even the very sharp signal at about 55 ppm, due to methoxyl carbons, appears as a shoulder on the signal at about 62 ppm, due to carbohydrate, in the whole grass spectra. The aromatic region of the lignin spectrum is relatively free from interfering carbohydrate signals except for the signals at about 108 and 115 ppm. The signal at about 108 ppm is due to carbons 2 and 6 of syringyl units of lignin plus carbon 1 from residual carbohydrates. The signal at about 115 ppm arises from carbons 3 and 5 of *p*-coumaryl units or 2 and 5 of coniferyl units of lignin. These two signals appear as shoulders on the signal at about 105 ppm in the spectrum of the whole plant material at 37.7 MHz (Figure 1A).

A difficult problem was finding signals in the lignin spectrum that did not overlap with signals in the protein spectrum (Figure 1D). Like the lignin spectrum, the protein spectrum also possesses aliphatic and aromatic carbon signals. The spectral region between 140 and 160 ppm seemed to satisfy the requirements of giving a unique response for lignin. Here there were signals due to the phenolic carbons or etherified phenolic carbons in the lignin spectrum that did not have an appreciable counterpart in the protein spectrum of these plant materials. The phenolic carbon signal of L-tyrosine is a potentially interfering amino acid residue signal. However, its total contribution to the protein fraction is only about 4% in these types of plant materials (Evans, 1982). Since total protein levels rarely exceed 20% in grasses, this means that the potential interference resulting from this amino acid residue would amount to only 0.8% at most. This is below the detection limits of most NMR experiments. The fact that this is true is borne out in the spectrum of chloroplastic protein (Figure 1D), where there are essentially no signals in the 140–160-ppm region. Therefore, the signals that appear in this region were chosen to represent lignin in the spectra of the whole plant materials. It was also noticed that the spectra of the acetal lignins, derived from the two plant species considered, showed that there was no significant difference in the integrated area of the 140–160-ppm region due to a difference in lignin structure. Such a difference could seriously affect comparative measurements for lignin. A difference would be expected

Table I. Ratio of Fiber Constituents in CBG[a] vs. Ky-31[b] As Determined by CP/MAS ^{13}C NMR, NIR, and CA[c]

technique	constituent		
	carbohydrate	lignin	protein
NMR			
37.7 MHz	1.27	1.57	0.68
50.0 MHz	1.27	1.66	0.59
NIR[d]	1.27	1.70	0.58
CA[d]	1.33	1.60	0.65

[a] CBG = Coastal Bermuda grass. [b] Ky-31 = Kentucky 31' tall fescue. [c] CA = chemical analysis. [d] Each ratio is the result of at least triplicate replications with a standard deviation of ±1 in percent composition.

in attempting to measure relative amounts of lignin in plant materials of different maturities. More mature plants contain lignin with significantly greater amounts of syringyl units whose spectra have an exceptionally strong NMR signal due to carbons 3 and 5 around 155 ppm. This has been noted in solution ^{13}C NMR spectra of wood lignins (Lüdemann and Nimz, 1973) and is evident in the solid-state ^{13}C NMR spectra of wood lignins (Dixon et al., 1981; Kolodziejski et al., 1982; Schaefer et al., 1981).

The third problem of finding a unique signal to represent protein in the presence of lignin was not possible to solve. Assuming the absence of lignin, as in the case of seed material, the carbonyl carbon signal at about 175 ppm has been used directly to compare relative protein content (O'Donnel et al., 1981; Rutar et al., 1980). This assumption is not valid with the vegetative plant material because the carbonyl carbons in lignin also contribute to this signal (although to a much lesser extent). The contribution of lignin carbonyls, however, can be estimated from the spectrum of acetal lignin. This was done by determining the ratio of the integrated area of the signals in the 140–160-ppm region of the acetal lignin spectrum to those for lignin carbonyls in the 165–190-ppm region. This ratio was approximately 3 to 1. Subtracting $^1/_3$ of the integrated area used to represent lignin (A_L) from the integrated area of the total carbonyl region (A_{CO}) in the whole plant spectrum permitted the determination of the integrated area of the carbonyl signal due to protein (A_P) or $A_P = A_{CO} - A_L/3$. This procedure gave a value related to the amount of protein present in the sample and permitted comparison of relative amounts of protein present in these plant materials. The same result could have been accomplished by utilizing a spectral subtraction routine; however, the point at which the signals in the 140–160-ppm region went to zero would have been hard to judge with much precision, owing to the noise levels in the spectra.

Thus, it became feasible to determine the relative amounts of carbohydrate, lignin, and protein in different grass species by the solid-state NMR technique. Table I shows the results of comparing the relative ratios of the primary fiber constituents in these two plant materials as determined by CP/MAS ^{13}C NMR at two different NMR field strengths utilizing integrated signal areas that had been normalized over the entire spectrum. These results are compared to the ratios obtained by NIR and chemical analysis (CA). NIR gave percent compositions of 74.9 ± 0.2 and 58.3 ± 0.8 for NDF, 4.1 ± −0.2 and 2.4 ± 0.8 for PML, and 10.4 ± 0.3 and 18.0 ± 1.1 for protein in CBG and Ky-31, respectively. CA percent compositions have been reported elsewhere for these samples (Windham et al., 1983).

Fiber constituent ratios determined from CP/MAS ^{13}C NMR spectra at 50.0 MHz showed a considerable improvement over those initially obtained at 37.7 MHz. This

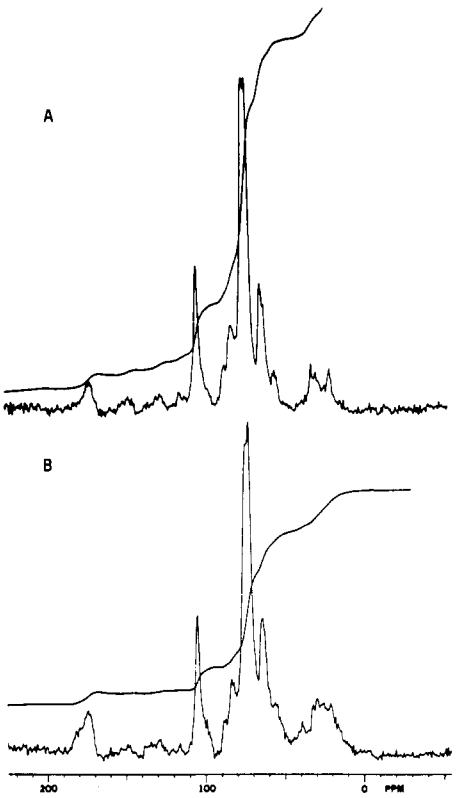

Figure 2. CP/MAS ^{13}C NMR spectra at 50.0 MHz of whole grass material: (A) Coastal Bermuda grass; (B) Kentucky 31' tall fescue.

improvement was probably related to the gain in signal dispersion and S/N at the higher magnetic field. These improvements were evident in the 50.0 MHz spectra of these materials shown in Figure 2.

The data in Table I also shows that the NIR and the higher field NMR methods relate better to each other than either does to the wet chemical data. A probable explanation for this is that the NIR and NMR methods are measuring responses to signals from the constituents in the entire plant material whereas the wet chemical methods are empirical, and measured results may or may not be representative of the entire plant material. In the chemical method, it has been assumed that the plant materials respons to the chemical reagents identically. This, however, may not be the case. For example, more low molecular weight carbohydrates may be washed away as cell solubles from a cool-season grass species such as Ky-31 than from a warm-season grass such as CBG, when using NDF reagent, giving a higher value for the CBG/Ky-31 carbohydrate ratio. This could be caused by structural differences between the plants and not a true difference in carbohydrate content of the intact plant.

Structural differences in lignin could also cause lignin in one species to be more susceptible to chemical treatment. This could be particularly true for "lignin" in a warm-season plant like CBG where *p*-coumaryl units are suspected to occur as side chains on polymeric lignin or even on polysaccharides as α,β-unsaturated esters (Himmelsbach and Barton, 1980). These units would be more susceptible to hydrolysis than would be the polymeric backbones. Prior to the actual determination of lignin by the PML method, some of these units would have been removed in the preparation of acid detergent fiber. This would result in a slighly lower value for the CBG/Ky-31 ratio for "lignin" by the chemical method than was actually present in the plant.

In the case of protein, it is not surprising at all that instrumental techniques might produce a different ratio

of protein value since the standard chemical method of Kjeldahl protein actually determines total reduced nitrogen. The Kjeldahl method could be greatly influenced by the presence of nonprotein nitrogen such as that present as reduced nitrogen in free amino acids or as ammonium ion in ammonium nitrate that had been adsorbed into the plant from fertilization. Both NIR and CP/MAS ^{13}C NMR would tend to discriminate against free amino acids vs protein. NIR discriminates by picking a longer wavelength for protein than amino acids. CP/MAS ^{13}C NMR can be made to discriminate against smaller, and generally more mobile, species (Stejskal and Schaefer, 1975) and would not respond to residual ammonium nitrate.

At this time, it is only valid to compare ratios of constituents measured by solid-state NMR to those measured by NIR and CA. NMR results could be potentially presented as percent composition correlated to wet chemical analysis, as is currently being done with NIR using chemometric techniques (Barton and Coleman, 1981), if a sufficient number of spectra were taken to establish the required prediction equations. This would permit direct comparison of results. However, this would still correlate measurements made on the entire sample (NMR) to measurements that only account for 70–80% of the sample (CA). Greater potential is possible with the NMR method if suitable internal standards can be found and other problems can be avoided to permit direct determination of percent composition of the entire material. NMR has the potential of becoming a means by which to standardize the NIR method, avoiding correlation to wet chemistry and its associated errors.

One problem in using high-field solid-state NMR to determine actual percent composition is the presence of spinning side bands for carbon nuclei that have large chemical shift anisotropies. This is particularly a concern with carbonyl and aromatic carbons. The side bands, which can be detected in lignin spectrum (Figure 1C) around 240 ppm for the aromatic carbons and in the protein spectrum (Figure 1D) for carbonyl carbons around 280 ppm, represent about 10% of the total signal intensity for these carbons. Similar side bands cannot be detected in the spectrum of the whole plant material because the amounts of lignin and protein present are relatively low and the side bands are therefore not distinguishable above the noise. In addition to this, the high-field portions of these side bands appear above 90 ppm and do not interfere with the regions that were used to analyze for carbohydrate, lignin, and protein in this study. The presence of side bands is one reason it is necessary to normalize integrated data over the entire spectrum (to include the side bands). Data presented in the form of ratios of components is not appreciably affected by this decrease in signal intensity in the primary peaks due to side bands, but some systematic error may be introduced. This situation is not exactly ideal even at these field strengths and one would like to go to higher field strengths to improve S/N or to decrease acquisition time. At higher field strengths, however, the side bands become a greater problem and could interfere directly with the peaks of interest. Samples will have to be spun faster to give greater dispersion to the side bands or quantitativeness will have to be established with techniques such as phase alteration of spinning side bands as proposed by Dixon (1981).

Still, it appears that either instrumental technique would be better than the wet chemical technique for fiber constituent analysis. The fact that the solid-state NMR technique is structurally more definitive and NMR results agree so well with NIR results gives more credence to the NIR method vs. the wet chemical method.

CONCLUSIONS

It is doubtful that the CP/MAS ^{13}C NMR method will replace the NIR method for constituent analysis because of the lack of rapidity (3 h vs. 20 s). However, because of the structural definity and ease of interpretation of the NMR spectrum, it does show a great promise as a complementary technique to NIR and potential as a means by which to standardize NIR results. It can be used to check the results of the somewhat more empirical NIR method that is receiving rapid acceptance as *the* forage analysis technique. In the immediate future, one can expect the CP/MAS ^{13}C NMR technique to be expanded to study many other plant materials.

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