Carbon-13 Nuclear Magnetic Resonance of Herbaceous Plants and Their Components, Using Cross Polarization and Magic-Angle Spinning

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^{13}C NMR spectra, obtained with cross polarization and magic-angle spinning (CP/MAS), have been obtained on the following five untreated herbage samples: forage sorghum, sorghum × Sudan grass, Jerusalem artichoke, Kochia, and Russian thistle. The diversity of composition of these materials is manifested in the complexity of the NMR spectra. Detailed assignments for forage sorghum and Jerusalem artichoke are made via studies of crude cell wall fractions, holocellulose, α-cellulose, and hemicellulose and with the aid of difference spectra and interrupted decoupling. Spectra of representative nonstructural carbohydrates are also presented for comparison. The lignins in forage sorghum and Jerusalem artichoke were found to be very different. This conclusion is based on the detailed appearance of the aromatic signals in the NMR spectra and on the observation that the lignin of Jerusalem artichoke was substantially retained in the holocellulose fraction, in contrast to the results for forage sorghum. Some features of the ability and limitations of ^{13}C CP/MAS NMR for quantitative analysis in plant samples of this type are discussed.

^{13}C nuclear magnetic resonance (NMR) with cross polarization (CP) and magic-angle spinning (MAS) has made possible a number of detailed studies of compounds and materials in the solid state (Schaefer and Stejskal, 1976; Yannoni, 1982; Wasylishen and Fyfe, 1982). ^{13}C CP/MAS NMR has been applied to the study of wood and wood-derived materials (Kolodziejski et al., 1982; Taylor et al., 1983; Haw et al., 1984), seeds (Rutar et al., 1980; O'Donnell et al., 1981; Haw and Maciel, 1983), and grasses (Himmelsbach et al., 1983). There have also been ^{13}C CP/MAS NMR studies of the principal structural components of plant materials, cellulose (Atalla et al., 1980; Earl and VanderHart, 1980, 1981; Maciel et al., 1982; Horii et al., 1982; Dudley et al., 1983), hemicellulose (Kolodziejski et al., 1982), and lignin (Bartuska et al., 1980; Maciel et al., 1981; Schaefer et al., 1981; Haw et al., 1984).

Much of the previous ^{13}C NMR work on plant materials has been of a qualitative nature. However, in a recent study of wood and wood pulps (Haw et al., 1984), it was possible to quantify lignin on the basis of integrated ^{13}C CP/MAS signal intensities and a reasonable mathematical model. Good agreement was obtained with lignin content determined by permanganate titration. Lignin quantification via NMR was possible in that study because wood and wood pulps have relatively simple compositions, compared to most plant materials. Therefore, it was possible to assign precise spectral regions to specific structural components and to sort out the integrated intensities into separate contributions due to lignin and carbohydrate. Detailed relaxation studies were also conducted in that investigation to ascertain that accurate relative signal intensities could be obtained for extractives-free wood. Herbaceous plant tissues are more chemically diverse than extractives-free wood, and considerable variation among and within species is possible. Therefore, one might expect more complex ^{13}C NMR spectra, with more interferences (overlaps) between resonances of interest and more difficulty in spectral interpretation. There has, nonetheless, been a report recently suggesting that ^{13}C CP/MAS spectra of grasses can yield information on the contents of carbohydrate, lignin, and protein for one sample relative to another (Himmelsbach et al., 1983). The present paper provides a detailed examination of the ^{13}C CP/MAS analysis of herbages from five species and fractions derived from two of them.

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MATERIALS AND METHODS

Herbages. The plant materials selected for study included aerial herbages from two different sorghums [*Sorghum bicolor* (L.) Moench], forage sorghum and a sorghum × Sudan grass hybrid, Jerusalem artichoke (*Helianthus tuberosus* L.), kochia [*Kochia scoparia* (L.) Schrad.], and Russian thistle (*Salsola kali* L.). The primary criterion for selection was the diversity in structural (cell wall) and nonstructural composition of these entries (Smith et al., 1983). All samples were collected from field plots at the Fort Collins Agronomy Research Center during 1982. The sorghums and Jerusalem artichoke were harvested when the upper portions of the plants reached the mature-seed stage of development.

Sample Preparation. Whole-plant herbage, harvested at ground level, from each of the entries was ground immediately after harvest in a Fitz hammermill, frozen in liquid N_2, and stored at −20 °C prior to subsequent processing and analysis. Prior to anlaysis, each sample was dried for 48 h at 60 °C in a forced draft oven and ground to pass a 1.0-mm screen in a Wiley mill. Samples subjected to NMR spectroscopic analysis included untreated samples of each of the five entries and crude cell wall, holocellulose, α-cellulose, and hemicellulose residues from forage sorghum and Jerusalem artichoke.

Crude cell wall preparations were obtained by sequentially digesting untreated plant material in a phosphate-buffered (0.05 M, pH 7.0) solution (5.0 mL of solution containing 5.0 mg of enzyme/g of plant material) of commercial protease (Sigma catalog no. P 5147) and an acetate-buffered (0.05 M, pH 4.5) solution (20.0 mL of solution containing 20.0 mg of enzyme/g of plant material) of amylogucosidase (Sigma catalog no. A7255). Both digestions were conducted for 24 h at 40 °C with occasional agitation, and residues were filtered and washed with distilled water by using 50-mL coarse-porosity fritted glass crucibles after each digest. Washed residue following the amyloglucosidase digest received two successive washings

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with acetone (50 mL/g of wet residue). The crude cell wall extract was then allowed to air-dry for 48 h prior to subsampling for NMR analysis.

Holocellulose samples were obtained by subjecting the crude cell wall residues to sodium chlorite delignification as described by Collings et al. (1978). The oxidation periods used were 45 and 60 min for the forage sorghum and Jerusalem artichoke samples, respectively. The α-cellulose samples were prepared from air-dried holocellulose preparations. Hemicellulose was extracted from holocellulose by using 17.5% (w/v) sodium hydroxide in a 4% (w/v) aqueous boric acid solution at a ratio of 30 mL of solution/g of holocellulose. Extraction was conducted for 4 h under N_2 gas with a wrist-action shaker. Following extraction, the α-cellulose was filtered by using a coarse-porosity, fritted-glass crucible, and the filtrate, containing the hemicellulose, was collected. Acetic acid acidified ethanol was added to the filtrate to precipitate the hemicellulose. Final hemicellulose residues were then obtained by centrifuging and drying by solvent exchange.

Wet-Chemical Analysis. Herbages from each of the entries were subjected to wet-chemical analysis to obtain quantitative estimates of total cell wall, Klason lignin, α-cellulose, hemicellulose, and crude protein. Total fiber content (AOF, ammonium oxalate fiber) was determined by digestion of untreated samples in 0.5% ammonium oxalate, as described by Collings and Yokoyama (1979). Klason lignin estimates were obtained from samples that had been first extracted in an acid detergent by the Van Soest method (1963). The acid detergent residues were digested in 5.0 mL of 72% (w/v) sulfuric acid for 2 h, at room temperature. The mixture was then diluted with distilled water to 3% (w/v) sulfuric acid and refluxed for 3 h. The resulting Klason lignin residues were filtered by using a coarse-porosity fritted-glass crucible and dried for 24 h at 100 °C. Results are expressed as a percentage of the original sample dry weight used prior to acid detergent extraction.

α-Cellulose (Buchala et al., 1971) was determined quantitatively by sequential treatment of untreated samples in ammonium oxalate, sodium chlorite, and sodium hydroxide–boric acid. The final α-cellulose residue was dried for 24 h at 100 °C, and the results were expressed as a percentage of the original untreated sample dry weight. Hemicellulose content was calculated as the difference between AOF and the sum of Klason lignin and α-cellulose. Crude protein was determined by micro-Kjeldahl analysis for nitrogen and multiplication of the resulting value by a factor of 6.25.

Carbohydrate Samples. The following samples were obtained from Sigma Chemical Co., St. Louis, MO: inulin (from dahlia tubers), inulin (from chicory root), levan (from *Aerobacter levanicum*), amylose (from potato), amylopectin (from potato), and β-D (−) fructose. Sucrose was obtained from a table sugar sample. ^{13}C CP/MAS spectra were obtained on all samples as received.

NMR Spectroscopy. ^{13}C CP/MAS NMR spectra were acquired on a home-built spectrometer at a carbon frequency of 25.27 MHz (field strength 2.3 T). This spectrometer was equipped with a Nicolet 1180 computer and 293 B pulse programmer. Sampes were spun at approximately 3.5 kHz in sleeve-type bullet spinners. The spectra width was 14 kHz (±7 kHz). 1K points were digitized and zero-filled to 4K points prior to Fourier transformation (with the excepton of the fructose spectrum, which was collected at 2K points and zero-filled to 8K). No exponential line broadening or resolution enhancement techniques were used to obtain the spectra presented here

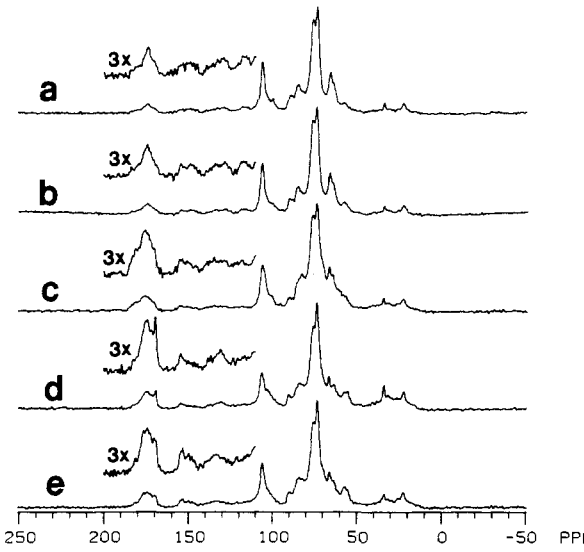

Figure 1. ^{13}C CP/MAS spectra of untreated herbage samples: (a) forage sorghum; (b) sorghum × Sudan grass; (c) Jerusalem artichoke; (d) kochia; (e) Russian thistle.

(except in the interrupted-decoupling spectra, where a 20-Hz exponential line broadening was used). The decoupler field strength was 12 G, and a 1-ms contact time was used for all samples. Delay times were 1.5 s for plant material samples, 1.5 s for polysaccharides, and 60 s for crystalline sugars. The latter value was motivated by the very long proton spin–lattice relaxation times in crystalline sugars, as has been previously observed, e.g., 10 s for fructose (Pfeffer et al., 1983a,b).

RESULTS AND DISCUSSION

A ^{13}C NMR experiment designed to measure proton spin–lattice relaxation times (Sullivan and Maciel, 1982) was carried out on forage sorghum as a representative sample. The range of T_{1H} values obtained is 0.15 s for the peak at about 173 ppm, 0.18 s for the peak at about 150 ppm, 0.31 s for the peak at 105 ppm, and 0.33 s for the peak at 73 ppm. This dispersion of T_{1H} values in itself indicates that there is substantial domain heterogeneity in this sample; otherwise ^1H–^1H spin diffusion would yield the same T_{1H} value for all structural moieties (e.g., lignin and cellulose) throughout the sample. In order to obtain an estimate of cross polarization relaxation times (T_{CH}) and proton $T_{1\rho}$ values, which govern the efficiency and quantitative reliability of cross polarization experiments, a variable contact time experiment (Sullivan and Maciel, 1982) was carried out on the forage sorghum sample. The cross polarization relaxation times (T_{CH}) thereby obtained were 0.33 ms for the 173-ppm peak, 0.28 ms for the 150-ppm peak, 0.078 ms for the 105-ppm peak, and 0.051 ms for the 73-ppm peak. The corresponding $T_{1\rho H}$ values derived from the experiment are 4.4, 5.0, 5.3, and 4.6, respectively. This combination of relaxation data shows that reasonable confidence can be placed in the quantitative significance of intensities obtained for the forage sorghum sample under the experimental conditions employed in this study (1-ms contact time, 1.5-repetition time) and implies that one can reasonably expect that the NMR intensities obtained for the other plant samples of this study have at least semiquantitative significance. However, more detailed studies on each sample would be necessary if one wished to place precise limits on the degree to which quantitation is achieved in these systems in ^{13}C CP/MAS experiments.

^{13}C CP/MAS spectra of the untreated samples from the five entries are shown in Figure 1. The results of standard

Table I. Chemical Analysis Data for Whole Herbage Samples

plant material	% of dry matter			
	α-cellulose	hemi-cellulose[a]	Klason lignin	crude protein
forage sorghum	28.32	21.07	7.73	5.06
sorghum × Sudan grass	29.13	23.57	8.26	6.34
Jerusalem artichoke	23.73	15.59	9.98	7.03
kochia	25.78	26.41	7.82	13.52
Russian thistle	35.33	23.42	10.16	9.66

[a] Assumed to be the difference between ammonium oxylate fiber (AOF) and the sum of α-cellulose and Klason lignin.

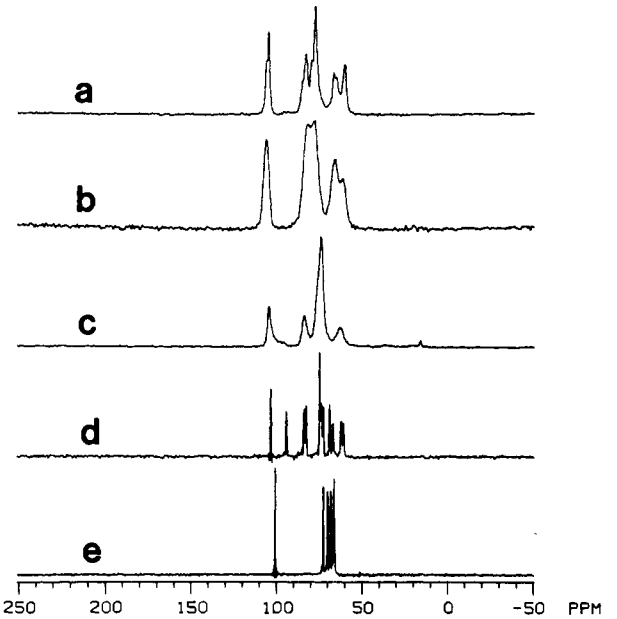

Figure 2. ^{13}C CP/MAS spectra of representative nonstrutural carbohydrates: (a) inulin (from dahlia tubers); (b) levan (from *A. levanicum*); (c) amylose; (d) sucrose; (e) α-D-–(–)-fructose.

(wet) chemical analyses for these samples are reported in Table I. A comparison of the spectra in Figure 1 with those of extractives-free wood (Kolodziejski et al., 1982; Haw et al., 1984) reveals that the spectra from herbaceous plant tissue are somewhat more complex and that some of the spectral features are less well resolved than for extractives-free wood. These observations are consistent with the more complex composition of forages.

In order to make detailed spectral assignments for the herbages, we acquired NMR spectra of various fractions prepared from forage sorghum and Jerusalem artichoke. Since nonstructural carbohydrates are present in significant quantities in herbaceous plants (Smith, 1973), we also acquired spectra of several of the more important members of this class.

Representative Nonstructural Carbohydrates. ^{13}C CP/MAS NMR spectra of five nonstructural carbohydrates that are present in various herbacious plants (Smith, 1973) are presented in Figure 2. The spectrum of an inulin preparation from dahlia tubers is shown in Figure 2a. Inulins are β(2→1)-linked D-fructofuranose polymers. The spectrum of inulin from chicory root (spectrum not shown) was essentially identical with the spectrum shown in Figure 2a. The signal in Figure 2a at 103.9 ppm was assigned to the anomeric carbon (C-2) of the fructofuranose ring and was confirmed with an interrupted-decoupling experiment (spectrum not shown), which distinguishes low-mobility carbons with directly bonded hydrogens from carbons that do not have hydrogen directly attached (Opella and Frey,

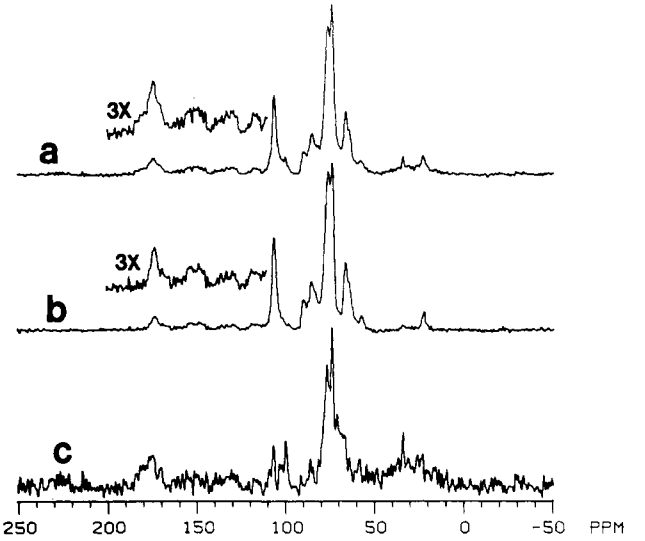

Figure 3. ^{13}C CP/MAS spectra of forage sorghum: (a) untreated material; (b) crude cell wall preparation; (c) difference spectrum obtained by the subtraction of (b) from (a).

1979). By analogy to the ^{13}C NMR spectra of starch (Dorman and Roberts, 1971; O'Donnell et al., 1981) and cellulose (Atalla et al., 1980; Earl and VanderHart, 1980; Maciel et atl., 1982), the signals in Figure 2a betwen 70 and 90 ppm are assigned to C-3, C-4, and C-5 and the signals between 50 and 70 ppm are assigned to C-1 and C-6.

Figure 2b shows the spectrum of a levan preparation. This carbohydrate is a β(2→6)-linked D-fructofuranose polymer (Smith, 1973). The anomeric (C-2) resonance of levan is at a chemical shift of 104.8 ppm; the remaining resonances are assigned by analogy to the spectrum of levan. The spectrum of an amylose preparation is presented in Figure 2c. This spectrum is virtually identical with that of an amylopectin preparation (spectrum not shown). The chemical shifts of starch resonances are very close to those of the analogous carbons in the structural carbohydrates (vide infra).

Parts d and e of Figure 2 are the spectra of sucrose and fructose, respectively. The anomeric carbon of sucrose is at 102.8 ppm, while that of fructose is at 100 ppm. ^{13}C CP/MAS spectra of both glucose anomers have recently been assigned (Pfefer et al., 1983b, 1984).

Plant Materials. Figure 3a presents the ^{13}C CP/MAS NMR spectrum of the forage sorghum sample, which is repeated there for direct comparison with the spectrum of the crude cell wall preparation (Figure 3b). Many of the spectral features in Figure 3a are analogous to those in the spectra of wood and wood-derived materials (Kolodziejski et al., 1982; Taylor et al., 1983; Haw et al., 1984). The broad peak, showing hints of structure, between 162 and 190 ppm is assigned to the carbonyl carbons of proteins, hemicellulose, lignin, and possibly extractives such as tannins. The broad signal between 140 and 162 ppm is due to aromatic carbons adjacent to oxygen, such as those found in lignin, tannins, and, to a much lower extent, tyrosine residues in protein. The remaining intensity on the low-shielding side of 108 ppm are attributed to aromatic carbons not adjacent to oxygen. Solution-state NMR studies of lignin model compounds (Lüdemann and Nimz, 1973) suggest that much of the intensity between 108 and 121 ppm is specifically due to nonprotonated aromatic carbons two bonds removed from oxygen (e.g., C-2 and C-5 of guaiacyl units).

We designate the portion of Figure 3a between 92 and 112 ppm the anomeric carbon region for the purposes of

the following discussion. Much of the intensity in the peak at 105.4 ppm in Figure 3a is due to C-1 of the anhydroglucose repeat unit of cellulose (Atalla et al., 1980; Earl and VanderHart, 1980). There is a poorly resolved shoulder at 103 ppm in Figure 3a that is consistent with starch (Figure 2c) and hemicellulose (vide infra). The peak at 99.2 ppm in Figure 3a has not been observed in wood spectra (Kolodziejski et al., 1982) and, as will be shown, probably corresponds to C-2 of fructose.

The peak at 89 ppm in the spectrum of the forage sorghum sample (Figure 3a) is due to C-4 of cellulose. The peak at 84 ppm in this spectrum can be assigned primarily to hemicellulose (Kolodziejski et al., 1982), although starch could also contribute to this signal. Amorphous and/or fibril-surface cellulose could also contribute to the 84-ppm signal (Atalla et al., 1980; Earl and VanderHart, 1981). The intense signal at 73 ppm in Figure 3a is due to C-2, C-3, and C-5 of cellulose and the analogous carbons of hemicellulose and nonstructural carbohydrates. The peak at 65 ppm in Figure 3a is attributed to C-6 of cellulose and the analogous carbons of other carbohydrates.

In the forage sorghum spectrum (Figure 3a) the poorly resolved signal at 56 ppm is due to methoxy groups. Most of the intensity in the region between 15 and 52 ppm is due to nonfibrous material (vide infra). The signal at 22 ppm is consistent with the methyl carbons of acetyl groups, which may be associated with hemicellulose (Kolodziejski et al., 1982). Figure 3a resembles previously reported ^{13}C CP/MAS spectra of wood, although many of the familiar peaks have intensity contributions from components not present in wood. This diversity of structure also accounts for the slight loss of detail in the 50–90-ppm region of Figure 3a relative to typical wood spectra obtained under identical conditions. For those reasons a better understanding of the various contributions to herbage spectra such as Figure 3a requires that NMR spectra of suitable herbage-derived fractions be obtained.

Figure 3b is the ^{13}C CP/MAS spectrum of the crude cell wall preparation from forage sorghum. This sample was expected to contain mostly structural polysaccharides and lignin. The features attributable to hemicellulose in Figure 3b (e.g., the peak at 83.5 ppm) are more intense than those in wood spectra. This result is consistent with the high ratio of hemicellulose to cellulose for this forage sorghum sample (Table I). The carbonyl signals in the region betwen 25 and 52 ppm in Figure 3b are also reduced relative to the spectrum in Figure 3a, a pattern that is consistent with the removal of protein and other extractives from forage sorghum. The peak at 99.2 ppm is essentially absent in Figure 3b, establishing that this component is due to nonstructural carbohydrates.

A commonly used method for emphasizing differences between two spectra is to subtract digitally one from the other. Figure 3c is the difference spectrum created by subtracting the spectrum of the forage sorghum crude cell wall preparation (Figure 3b) from that of untreated forage sorghum (Figure 3a). The weighting factor used in the subtraction was the maximum that could be applied without introducing negative peaks. According to the strategy of this comparison, one should be able to view Figure 3c as a spectrum of the nonstructural components of forage sorghum. The region in Figure 3c between 60 and 105 ppm is assigned to nonstructural carbohydrates. The signal at 99.2 ppm is greatly enhanced in this difference spectrum. A careful comparison of Figure 3c with the spectra of representative nonstructural carbohydrates in Figure 2 suggests that much of the intensity in Figure 3c can be attributed to sugars and possibly some starch

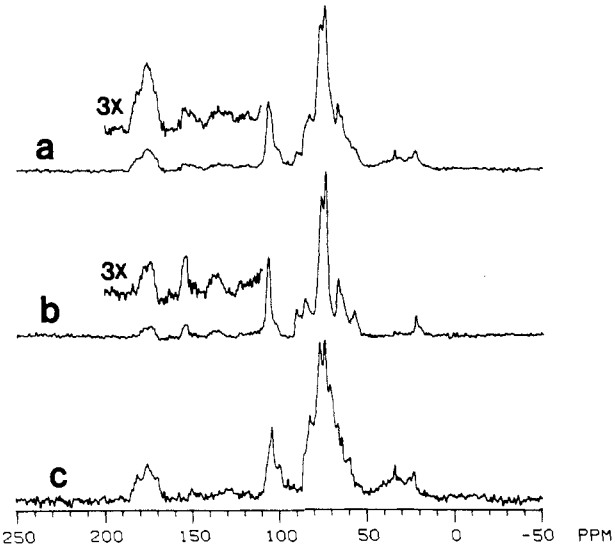

Figure 4. ^{13}C CP/MAS spectra of Jerusalem artichoke: (a) untreated material; (b) crude cell wall preparation; (c) difference spectrum obtained by the subtraction of (b) from (a).

but not to the fructosans, inulin or levan.

The broad and weak carbonyl and aromatic carbon signals in Figure 3c are at least in part due to protein. The possibility of other extractives such as phenolics contributing to the weak signals in the aromatic carbon region cannot at this point be excluded. The intensity between 15 and 50 ppm in Figure 3c can be attributed to the aliphatic carbons of protein and other extractives.

The ^{13}C CP/MAS spectrum of the Jerusalem artichoke samples is repeated in Figure 4a for direct comparison with the spectrum of the crude cell wall preparation (Figure 4b). The spectrum of Figure 4a is qualitatively very similar to that of the forage sorghum sample (Figure 3a). The spectrum of the crude cell wall preparation of Jerusalem artichoke (Figure 4b) is also similar to that of the corresponding preparation of forage sorghum (Figure 3b) but does show some obvious differences from that spectrum. In particular, the aromatic carbon pattern between about 146 and 157 ppm in Figure 4b shows major differences from that in Figure 3b, indicating that there are significant differences between the structure of lignin in Jerusalem artichoke and lignin in forage sorghum.

Figure 4c is the difference spectrum obtained by subtracting the spectrum of the crude cell wall preparation of Jerusalem artichoke (Figure 4b) from that of the untreated Jerusalem artichoke sample (Figure 4a). The spectral features of Figure 4c in the regions 10–50 and 110–190 ppm are qualitatively roughly similar to those in the difference spectrum of forage sorghum (Figure 3c). However, the signals between 50 and 110 ppm in Figure 4c indicate that the nonstructural carbohydrates of Jerusalem artichoke are significantly different from those of forage sorghum. A close comparison of Figure 4c with Figure 2a indicates that inulin may be a major nonstructural carbohydrate in this Jerusalem artichoke sample. In Figure 4c the peak at about 104 ppm and the well-resolved maxima at about 82, 75, 65, and 60 ppm are all within about 1 ppm of the peak maxima in the inulin spectrum (Figure 2a). The clearly resolved peak at 99.8 ppm that is more prominent in the corresponding difference spectrum of forage sorghum (Figure 3c) is due to fructose. Further evidence for this assignment and for the assignment of the 103.7 peak to inulin is provided by interrupted-decoupling experiments (vide infra). The presence of inulin in Jerusalem artichoke stems has been previously reported (Bacon and Edelman, 1951). The generally broad

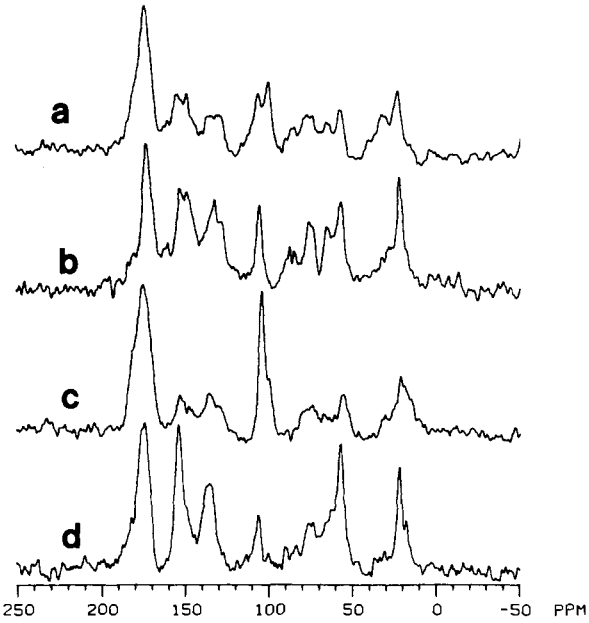

Figure 5. ^{13}C CP/MAS spectra obtained with 40-μs interrupted decoupling: (a) untreated forage sorghum; (b) forage sorghum crude cell wall preparation; (c) untreated Jerusalem artichoke; (d) Jerusalem artichoke crude cell wall preparation.

features of the region between 60 and 90 ppm in the spectrum of Figure 4c suggest that there are other nonstructural carbohydrates contributing intensity in this region. Significant quantities of sucrose and starch are also found in aerial portions of Jerusalem artichoke (Bacon and Edelman, 1951; Edelman and Jefford, 1968).

It should be apparent from the above discussion that it is difficult to distinguish individual nonstructural carbohydrates in herbage samples by means of the standard ^{13C} CP/MAS experiment, even with the aid of difference spectra such as those shown in Figures 3c and 4c. The root of this problem is in the close similarity of the local environments around each carbon type (e.g., anomeric carbons), independent of the longer range structure of the carbohydrate. This results in a close similarity in chemical shifts for analogous carbons. Add to this the resonance frequency shifts and line broadening that can result from differences in hydrogen bonding, which can account for several ppm in solids (Imashiro et al., 1982; Terao et al., 1983), and the difficulty in distinguishing individual carbohydrates in the standard ^{13}C CP/MAS experiments is apparent.

The interrupted-decoupling experiment (Opella and Frey, 1979) is a simple modification of the CP/MAS experiment that is valuable for assigning some of the signals due to nonstructural carbohydrates, as well as those due to other components. This experiment suppresses signals from immobile, protonated carbons, while only slightly attenuating signals from nonprotonated carbons and mobile carbon moieties with directly attached protons (e.g., methyl groups). Figure 5a is the ^{13}C CP/MAS spectrum of the untreated forage sorghum sample obtained with a 40-μs interrupted-decoupling period prior to data acquisition. The low-shielding region of this spectrum (110–190 ppm) contains signals due to carbonyl carbons and nonprotonated aromatic carbons. Signals due to methyl and methoxy groups are seen between 15 and 65 ppm in Figure 5a. Of significance to nonstructural carbohydrates are the signals at 103 and 99 ppm; these correspond to the signals observed in the difference spectrum (Figure 3c). The only nonprotonated-type carbon in the carbohydrates found in herbage, whether structural or nonstructural, is the

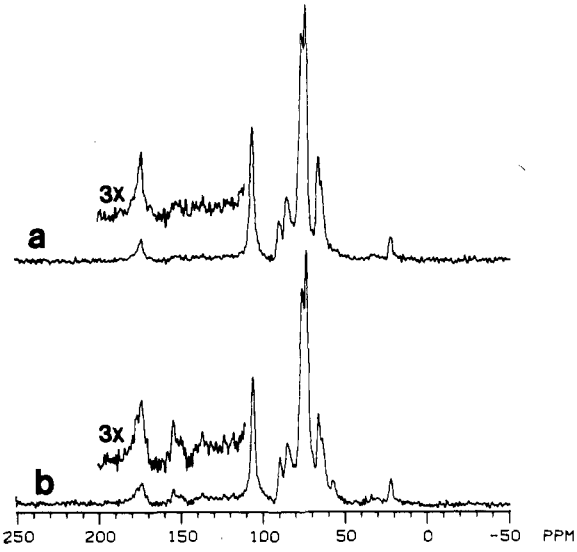

Figure 6. ^{13}C CP/MAS spectra of holocellulose fractions: (a) forage sorghum holocellulose; (b) Jerusalem artichoke holocellulose.

anomeric (C-2) carbon of fructose, fructose-derived oligosaccharides (principally sucrose), and fructose-derived polymers (e.g., inulin). On the basis of chemical shift, it is possible to assign the 99-ppm signal in Figure 5a to fructose. The shoulder at 103 ppm could be due to sucrose or possibly a fructosan polymer.

Figure 5b is the interrupted-decoupling spectrum of the crude cell wall preparation of forage sorghum. The anomeric carbon signals are not present in this spectrum, again confirming the conclusion that these signals are due to nonstructural components. It should be noted that the apparent intensity increase of the aromatic signals in Figure 5b relative to Figure 5a is, in part, an artifact of the normalization process used in plotting the spectra. Some of the increase in the aromatic signal intensity is due to the concentration of lignin that accompanies the removal of nonstructural components.

Figure 5c is the interrupted-decoupling spectrum of the Jerusalem artichoke sample. The signal at 103 ppm is clearly due to the anomeric carbon of a fructose residue, such as that of inulin. The shoulder at 99 ppm is assigned to fructose.

Figure 5d is the interrupted-decoupling spectrum of the crude cell wall preparation of Jerusalem artichoke. Comparison of the aromatic signals in this spectrum with those in Figure 5b (forage sorghum crude cell wall preparation) reinforces the earlier observation that there are significant structural differences between the lignins of these two forages.

^{13}C NMR spectra of holocellulose fractions derived from forage sorghum and Jerusalem artichoke are presented in parts a and b Figure 6, respectively. By comparing Figure 6a with the spectrum of the crude cell wall preparation of forage sorghum (Figure 3b), one can see clearly that substantial delignification of forage sorghum is achieved by the holocellulose procedure. The aromatic and methoxy signals are greatly reduced in Figure 6a, relative to Figure 3b, but the relative intensity of the carbonyl signal in Figure 6a (relative to the anomeric carbon signal) is essentially identical with that in Figure 3b. This latter observation indicates that in herbage spectra the carbonyl groups in lignin do not contribute significantly to the intensity of the carbonyl signal, relative to the contribution from carbonyl groups in structural carbohydrates.

A comparison of the spectrum of Jerusalem artichoke holocellulose (Figure 6b) with that of the corresponding

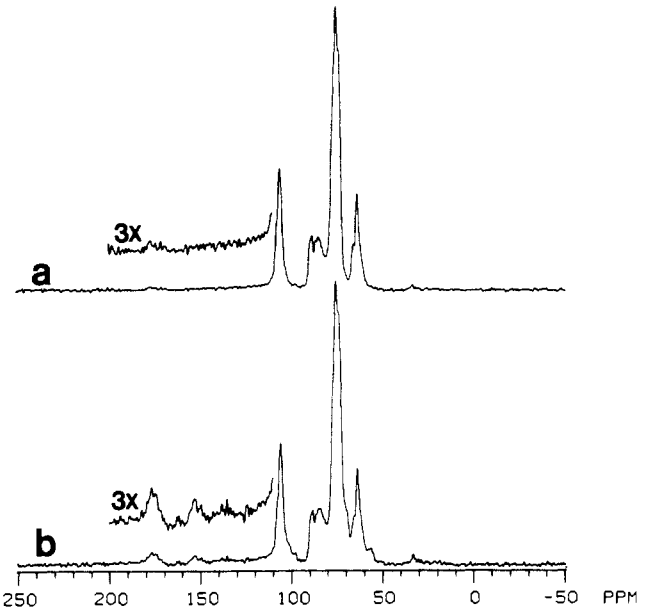

Figure 7. ^{13}C CP/MAS spectra of (a) forage sorghum α-cellulose; (b) Jerusalem artichoke α-cellulose.

crude cell wall preparation (Figure 4b) reveals that the holocellulose procedure affects only modest delignification for this species, in contrast to the results for forage sorghum. This is in spite of the more severe conditions used for the preparation of Jerusalem artichoke holocellulose (see Materials and Methods) and reinforces our view that the lignins of these two species are significantly different.

One could, in principle, obtain difference spectra representative of the lignin components by subtracting the holocellulose spectra from the corresponding crude cell wall spectra. This was attempted for forage sorghum and Jerusalem artichoke, but the signal-to-noise ratios in the resulting difference spectra (not shown) were too low for confident interpretation.

α-Cellulose. Figure 7a is the ^{13}C NMR spectrum of the α-cellulose fraction obtained from forage sorghum. This spectrum has no intensity in the aromatic region and, therefore, shows no evidence of lignin. The anomeric carbon signal is at 105.9 ppm, and the C-4 signal is at 84.5 ppm. The overlapping signals due to C-2, C-3, and C-5 provide a single peak in Figure 7a at 75.4 ppm. The signal due to C-6 is at 65.2 ppm. These chemical shifts are very similar to those of α-cellulose derived from lodgepole pinewood (Kolodziejski et al., 1982). Figure 7a appears to have no features that are clearly consistent with hemicellulose (vide infra).

Figure 7b is the ^{13}C NMR spectrum of the α-cellulose derived from Jerusalem artichoke. In contrast to the result for forage sorghum α-cellulose, this spectrum contains weak aromatic signals indicative of the presence of small amounts of lignin. It also has signals in the carbonyl region that may be due to hemicellulose (vide infra), although the carbonyl intensity in Figure 7b is concentrated at lower shielding than is the case in Figure 6b.

Hemicellulose. Spectra of the hemicellulose fractions are presented in Figure 8. The spectrum of forage sorghum hemicellulose (Figure 8a) has no discernible intensity in the aromatic region. The anomeric carbon signal in Figure 8a is at 103.3 ppm. The signal due to carbons analogous to C-2, C-3, and C-5 of cellulose are at 75.2 ppm; that due to carbons analogous to C-6 of cellulose is at 65.1 ppm. A broad, weak carbonyl signal is also evident in Figure 8a, which is consistent with the presence of glucuronic acid in the hemicellulose sample. With the use of

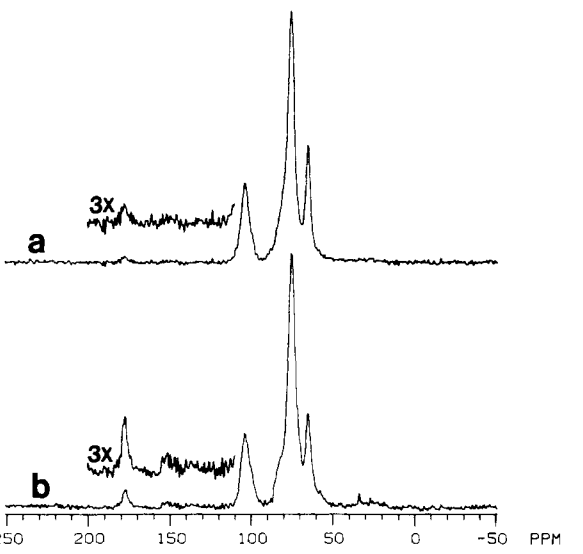

Figure 8. ^{13}C CP/MAS spectra of hemicellulose fractions: (a) forage sorghum hemicellulose; (b) Jerusalem artichoke hemicellulose.

computational resolution-enhancement techniques, it is possible to discern one or more broad signals on the low-shielding side of the 75.2-ppm peak in the 79–85-ppm region, a chemical shift range consistent with carbons analogous to C-4 of cellulose (resolution-enhanced spectrum not shown).

Figure 8b is the spectrum of Jerusalem artichoke hemicellulose. In contrast to the spectrum of forage sorghum hemicellulose (Figure 8a), Figure 8b shows convincing low-intensity signals in the aromatic region. This further supports our view that lignin is more closely associated with carbohydrate (or is of significantly different composition) in Jerusalem artichoke than in forage sorghum. Figure 8b also shows a small peak at about 35 ppm (and possibly one at about 27 ppm), which may be due to small amounts of protein, lignin, or extractables in this sample. The remaining features in Figure 8b are similar to those in Figure 8a.

Having made a detailed assignment of the ^{13}C NMR spectra of herbages based on studies of representative nonstructural carbohydrates and various herbage-derived components, we now turn our attention to the issue of the possible quantification of forage components by ^{13}C CP/MAS NMR. The overall pattern described above is one of a complex array of resonances with substantial overlaps among peaks that one would like to be able to use for quantification. Because of these overlaps in the spectra of herbages, it is very difficult to achieve quantification for this class of plant samples by the standard ^{13}C CP/MAS approach as it is now commonly practiced. It can be expected that emerging solid-state ^{13}C NMR techniques will markedly improve that situation.

CONCLUSIONS

Detailed assignments of the ^{13}C CP/MAS NMR spectra of herbage samples can be made on the basis of spectra of untreated herbage samples, herbage-derived fractions, and representative nonstructural carbohydrates. We have demonstrated the value of crude cell wall preparations for sorting out the contributions of nonstructural components to the ^{13}C NMR spectra of herbage samples; the contributions of nonstructural carbohydrates are particularly significant. Difference spectra obtained by the subtraction of a spectrum of a crude cell wall preparation from that of the untreated material can provide some information about the composition of nonstructural carbohydrates but

not enough for a detailed analysis. The interrupted decoupling experiment is valuable in studies of untreated herbage samples in that it permits the identification of signals due to the anomeric carbon of fructose and fructose residues.

Spectra of holocellulose fractions reveal that structural carbohydrates make significant contributions to the carbonyl signal in spectra of untreated forage samples. Therefore, it is not straightforward to use the carbonyl signal in a standard ^{13}C CP/MAS spectrum to quantify protein in herbages with any degree of confidence.

The value of ^{13}C CP/MAS NMR in the study of herbages is demonstrated by the ease with which differences between the lignins of forage sorghum and Jerusalem artichoke are recognized. These differences are evident in the detailed appearance of the aromatic signals in the spectra of crude cell wall preparations and by the presence of aromatic and methoxy carbon signals in the spectra of holocellulose, α-cellulose, and hemicellulose fractions from Jerusalem artichoke; by contrast, aromatic and methoxy carbon signals are essentially absent in the spectra of the corresponding forage sorghum fractions. The spectrum of Jerusalem artichoke α-cellulose also shows evidence of a considerable amount of hemicellulose, whereas none is observed in forage sorghum α-cellulose.

Registry No. Holocellulose, 8064-26-4; α-cellulose, 9004-34-6; hemicellulose, 9034-32-6.

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