Solid-State ^{13}C NMR Analysis of Forage and Byproduct-Derived Fiber and Lignin Residues. Resolution of Some Discrepancies among Chemical, Infrared, and Pyrolysis–Gas Chromatography–Mass Spectroscopic Analyses

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Solid-state ^{13}C NMR was used to investigate changes in the composition of residues from red clover and peanut hulls obtained by four commonly used gravimetric methods for lignin in forages. The samples had also previously been analyzed by infrared and pyrolytic mass spectrometric procedures. The solid-state ^{13}C NMR spectra enabled the confirmation of a structural interpretation of why the different gravimetric procedures are not chemically equivalent. The three instrumental techniques together enabled a more accurate evaluation of the chemical composition of lignin obtained by each of the four different gravimetric procedures, as well as a comparison of the advantages and limitations of each of the instrumental methods for forage analysis.

Keywords: *Lignin assays, forages, fiber, solid-state ^{13}C NMR*

INTRODUCTION

Lignin is a polymeric substance found in plants and formed by polymerization of phenylpropenyl units. It is widely believed to be responsible for the difficulty ruminants have in completely digesting forages and byproducts and for the structural strength of trees and other plant materials. Although it is still unresolved whether lignin content or composition is more important in determining lignin's role in plant strength and digestibility (Reeves, 1985, 1988; Jung and Himmelsbach, 1989), lignin content is determined by many researchers in studies varying from feed digestibility to the environmental degradation of forest litter (Reeves and Galletti, 1993; Galletti et al., 1993). Methods ranging from spectrometric (Morrison, 1972) to gravimetric (Goering and Van Soest, 1970; Collings et al., 1978; Edwards, 1973) have been used to estimate lignin content. Studies also have demonstrated that the various procedures, generally, do not produce similar results (Reeves, 1993a).

Chemical analysis (Reeves, 1993a), infrared (IR) spectroscopy (Reeves, 1993b), and pyrolysis–GC–mass spectrometry (PY–GC–MS) (Reeves and Galletti, 1993) have previously been used to investigate the nonequivalence among the four gravimetric lignin procedures: 72% sulfuric acid extraction of acid detergent fiber (ADF), known as acid detergent lignin (ADL) (Goering and Van Soest, 1970); permanganate extraction of ADF (Goering and Van Soest, 1970); chlorite extraction of ADF (Collings et al., 1978); and triethylene glycol extraction of ADF (Edwards, 1973).

Differences still unresolved by the chemical, IR, and PY–GC–MS procedures are as follows: 1. Does triethylene glycol remove all of the lignin from samples? 2. Does ADL contain carbohydrates or altered forms of it? 3. Does permanganate lignin also remove or alter carbohydrates? 4. What is the fate of protein in the chlorite lignin procedure? 5. Does the chlorite procedure remove all of the residual lignin from triethylene glycol lignin residues? 6. What is the nature of the residues resulting from 72% sulfuric acid extraction of permanganate lignin residue and from permanganate extraction of 72% sulfuric acid lignin (Reeves, 1993a,b; Reeves and Galletti, 1993)? The primary objective of this study was to determine if solid-state ^{13}C NMR could provide some answers to the above questions. A second objective was to compare the value of solid-state ^{13}C NMR with that of the other nongravimetric methods on identical samples in evaluating fiber assay procedures.

MATERIALS AND METHODS

Batch Fiber and Lignins. Fibers and lignin residues were produced in batch by exact scaling of procedures used for individual assays (Reeves, 1993a). Initial plant materials were prepared by grinding to pass a 1-mm screen using a Christy Norris mill (Christy Norris Ltd., Chelmsford, England). Acid detergent fiber was produced according to the methods of Goering and Van Soest (1970), with the addition of a neutral detergent (Goering and Van Soest, 1970) wash to remove any residual acid or detergent. The ADF fiber served as the starting material for the production of batches of lignin (ADL) (Goering and Van Soest, 1970) or lignin-extracted residues [permanganate (Goering and Van Soest, 1970), chlorite (Collings et al., 1978), or triethylene glycol (Edwards, 1973)]. In addition, some residues of lignin procedures were extracted a second time by other lignin procedures (i.e., extraction of triethylene glycol residue with permanganate or chlorite).

Solid-State ^{13}C NMR. Analysis by ^{13}C NMR was carried out on a Bruker MSL-400 at a field strength of 9.4 T. Samples were spun at 4–5 kHz in a ceramic Al_2O_3 rotor inside a Bruker MAS probe head with magic angle spinning. The ^{13}C 90° pulse width was 8 μs. Spectra were obtained with a 1-ms contact time, a 1.5-s recycle time, and a 125-W decoupling power. The ^{13}C spectra were recorded at 100.63 MHz and a spectral width of 26 000 Hz. Adamantine was the external intermediate reference standard used to set the relative chemical shift scale. Chemical shifts were relative to tetramethysilane (TMS). The typical number

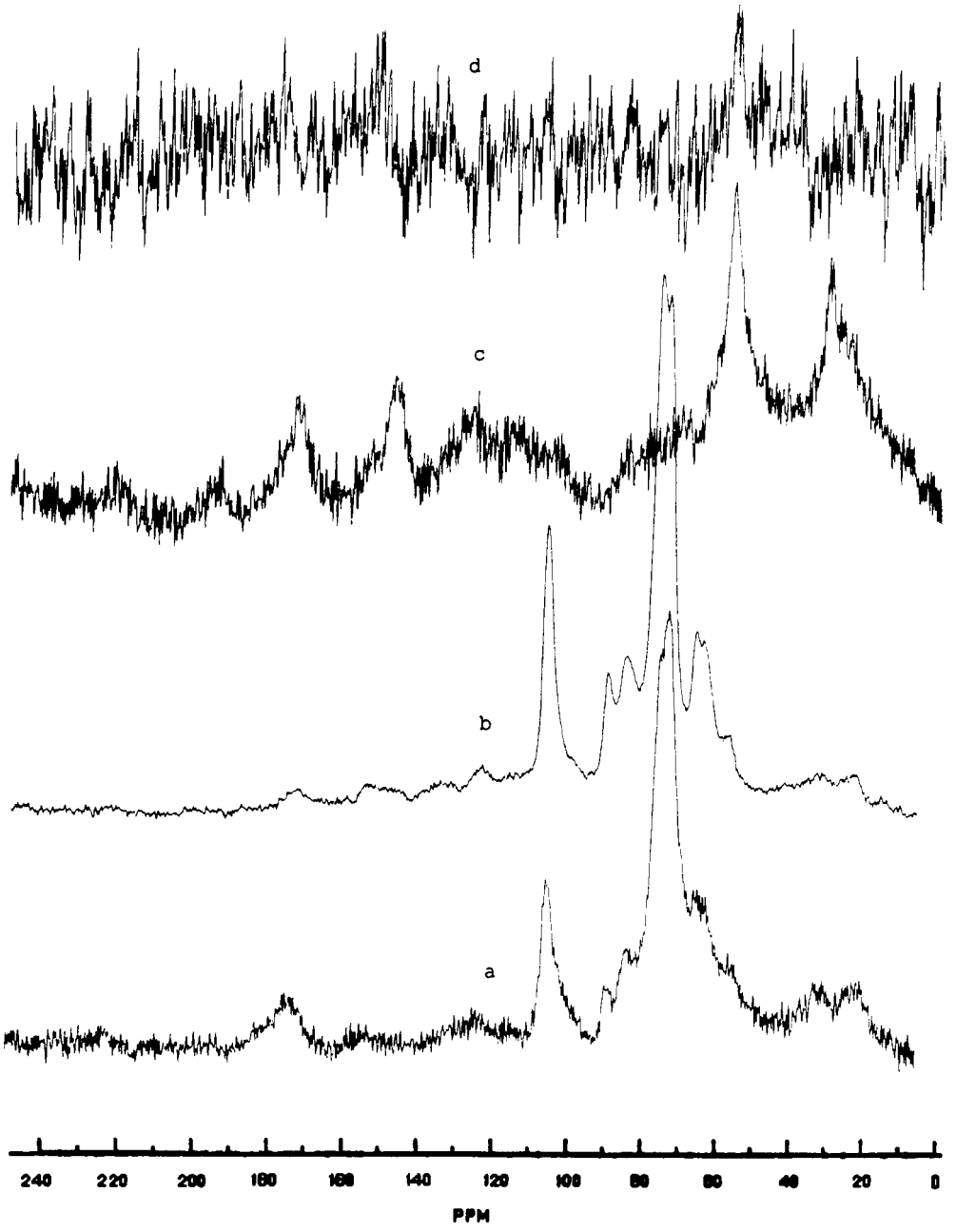

Figure 1. ^{13}C NMR spectra of (a) red clover hay (RC), (b) RC acid detergent fiber (ADF), (c) 72% sulfuric acid lignin (ADL) performed on RC ADF, and (d) residue from permanganate extraction of RC ADL.

of scans was 1024 with 16-Hz line broadening. The rotors (0.7 cm o.d. × 1.8 cm), volume about 0.5 mL, were packed with sample. Because of low sample density, both before and after treatment, about 100 mg of sample was required.

Infrared Analysis. FTIR analysis of samples was carried out by diffuse reflectance on samples diluted to 5% with KBr, using a DigiLab FTS 65 FTIR. Samples were scanned 64 times, at a resolution of 4 cm^{-1} between 4000 and 400 cm^{-1} with data presented for 1800 to 400 cm^{-1} (Reeves, 1993b).

RESULTS AND DISCUSSION

NMR Frequency Assignments. Spectra a–d of Figure 1 show the ^{13}C NMR results for red clover hay (RC), its ADF, ADL, and the residue from permanganate extraction of its ADL, respectively. The main features seen for RC and its ADF belong to the carbohydrate and protein constituents. Although lignin comprises perhaps 10–20% of RC [depending on the assay used (Reeves, 1993a)], its concentration is not represented very prominently. In addition, protein and lignin frequencies overlap in some places, making distinctions using a single ^{13}C NMR plot difficult. Thus, in the 52–56 ppm region, chemical shifts are due to both protein and methoxy groups in lignins (Scalbert et al., 1986; Baianu, 1989; Jung and Himmelsbach, 1989). The same ambiguity occurs between 120 and 130 ppm, where both proteins and guaiacyl chemical shifts can be found.

Since the ADF procedure removes most of the protein (Goering and Van Soest, 1970; Reeves, 1993a) and hemicelluloses from forages, we can delineate the true nature of the frequencies for ADF by comparing the RC and RC ADF results. This is important since ADF is the fiber upon which all subsequent assays were based. Examination of the changes in intensities for the chemical shifts at 174, 125, and 33 ppm before (Figure 1a) and after the ADF procedure (Figure 1b) shows a large decrease for all three compared with other peaks, such as that at 152 ppm which is due to guaiacyl and syringyl groups in lignin (Horil, 1989; Lapierre et al., 1984; Scalbert et al., 1986; Haw, 1989).

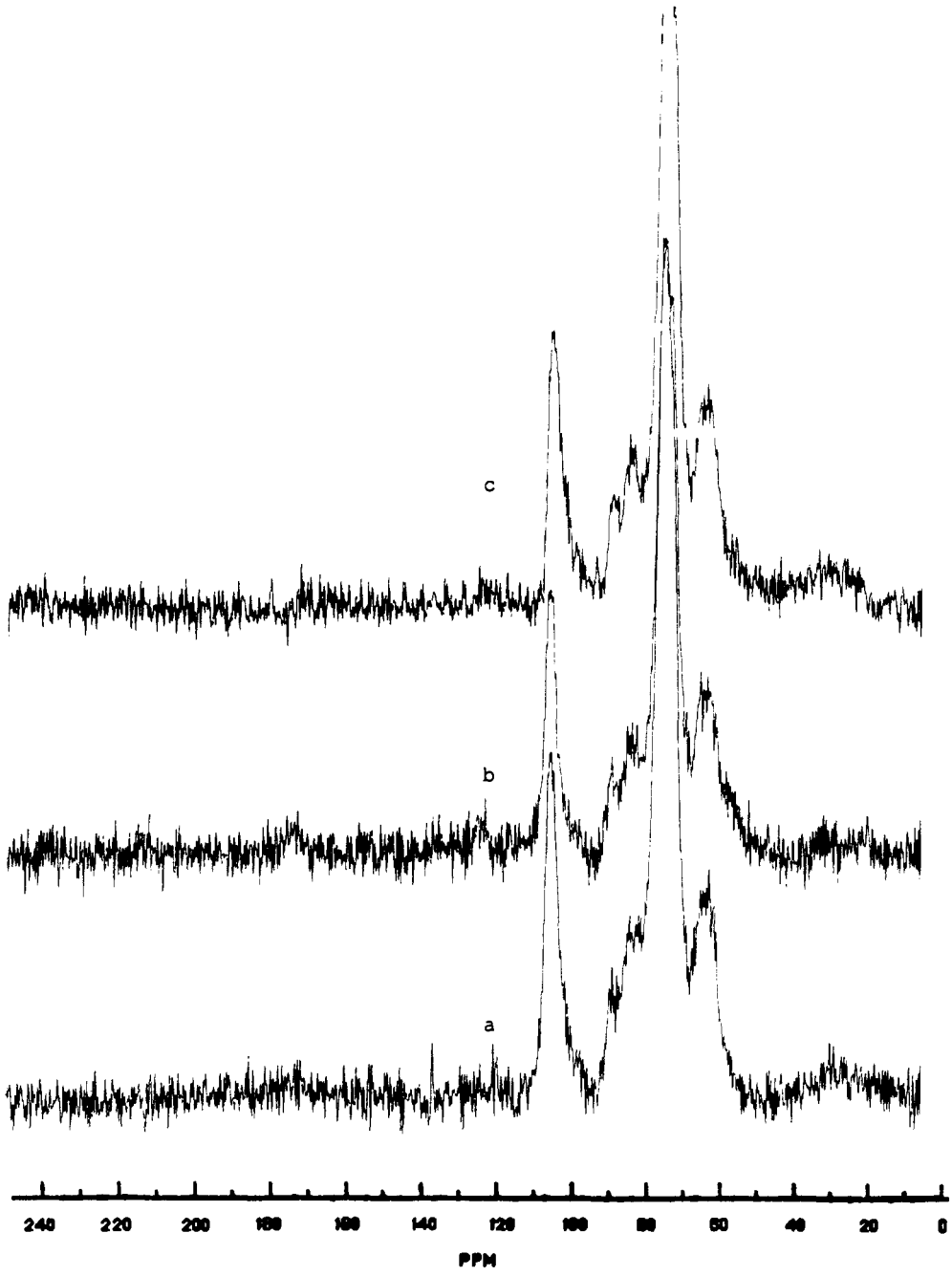

Figure 2. ^{13}C NMR spectra of (a) residue from permanganate extraction of red clover hay (RC) acid detergent fiber (ADF), (b) residue from chlorite extraction of RC ADF, and (c) residue from triethylene glycol extraction of RC ADF.

The remaining part of the peak at 52–56 ppm (ADF spectra) must be due to methoxy groups in lignin (Scalbert et al., 1986; Horil, 1989; Haw, 1989; Jung and Himmelsbach, 1989), or else, like the other protein peaks, there would have been a greater reduction in relative intensity.

There is also a chemical shift due to guaiacyl residues at 115 ppm that was not resolved for the RC or ADF but can be easily seen for ADL lignin (Figure 1c). Aromatic and carbonyl frequencies at 145 and 175 ppm are also present. Nearly all of the lignin disappears on permanganate treatment (Figure 1d). In Table 1, are presented the shift assignments upon which the discussion that follows is based.

Red Clover Lignin Results. Considerable non-lignin material remained in the ADL (Figure 1c). The chemical shifts at 30, 55, and 175 ppm are consistent with protein, which is also supported by both FTIR results (Reeves, 1993b) and chemical analyses (Reeves, 1993a). There is, however, only weak evidence for the presence of carbohydrate residues, whose possibility was indicated by FTIR (Reeves, 1993b). It is also possible that due to the dehydration effects of the 72% sulfuric acid the carbohydrates have been severely altered. Insufficient resolution from other carbohydrate peaks prevents adequate identification of potential structural alterations. Unfortunately, the same kind of situation results with the use of FTIR (Reeves, 1993b). A significant decrease in the largest carbohydrate peaks near 78 ppm supports changes in the chemical structure of any carbohydrate present. Chemical analysis (gas chromatography) showed no known sugars (Reeves, 1993a), but this would be expected if the residues were modified by dehydration.

Spectra a–c of Figure 2 contain the results for permanganate (Goering and Van Soest, 1970), chlorite (Collings et al., 1978), and triethylene glycol (Edwards, 1973) extractions of RC ADF, respectively. In all three cases,

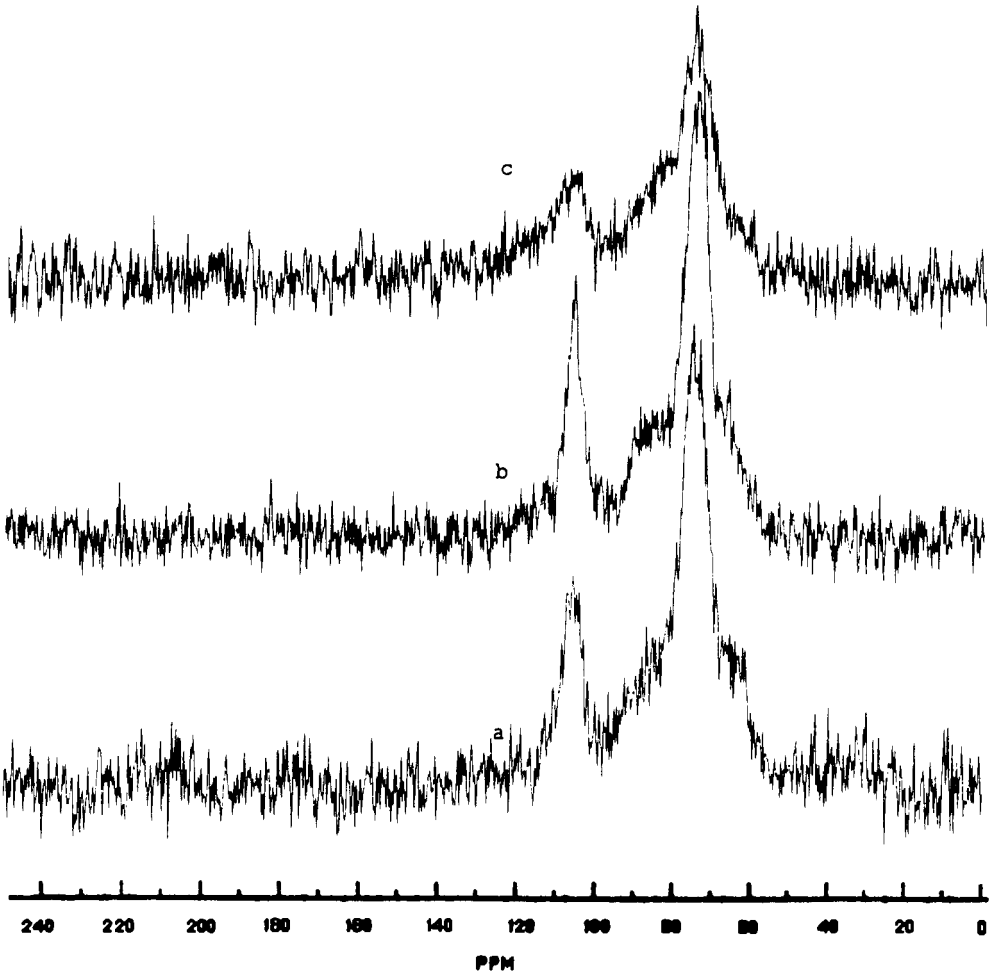

Figure 3. ^{13}C NMR spectra of (a) residue from chlorite extraction of residue from triethylene glycol extraction (TRIG) of red clover hay (RC) acid detergent fiber (ADF) and (b, c) residue from permanganate extraction of residue from (b) RC TRIG and (c) chlorite extraction of RC ADF.

Table 1. ^{13}C NMR Spectral Assignments for Red Clover and Fiber Derived from Red Clover[a]

chemical shift (ppm)	assignment	source[b]
221–243	possibly spinning side bands	?
195	ketones	L
174	ester carbonyls	H, P
153–149	guaiacyl and syringyl groups	L
130–123	aliphatic and aromatic CH	P
115	guaiacyl groups	L
105	C1	C, H
88	C4	C, H[c]
84	C4	C, H[d]
73	C2, C3, C5	C, H
65	C6	C, H
63	C6	C, H[d]
56	methoxy groups	H, L
30–33	aliphatic C—H	P
22	CH_3—C=O	H
16	aliphatic C—H	P

[a] References used: Attala et al. (1980), Baianu (1989), Horil (1989), Lapierre et al. (1984), Nimz et al. (1981), and Scalbert et al. (1986). [b] C, cellulose; H, hemicellulose; L, lignin; P, protein. [c] Crystalline carbohydrates. [d] Amorphous carbohydrates.

there were some shifts in the relative intensities of the carbohydrate bands, representing crystalline and amorphous forms, of cellulose and hemicellulose (Atalla et al., 1980). Examination of the spectra for evidence of lignin showed permanganate to be lignin free, while both of the others contained greatly reduced, but still present, residual lignin, as indicated by the presence of ester carbonyl groups at 175 ppm, of guaiacyl and syringyl units at a frequency of 154 ppm, and of methoxy units at a frequency of 56 ppm. These results agree precisely with FTIR results. However, the triethylene glycol results, while supported by nitrobenzene oxidation results (Reeves, 1993a), disagree with PY–GC–MS results that showed no aromatic fragments (Reeves and Galletti, 1993). It would thus appear that the triethylene glycol residue contains either highly condensed aromatic moieties that do not give rise to volatile aromatic fragments upon pyrolysis or a highly modified lignin for which the aromatic rings are all cracked during pyrolysis.

Re-extraction of Assay Residues. Infrared analysis of residues from the treatment of the triethylene glycol residue with sodium chlorite indicated that while most of the residual lignin present was removed, some might have remained (Reeves, 1993a). From ^{13}C NMR analysis (Figure 3a) no lignin survives the double treatment, indicating that the FTIR results were probably due to residual protein. Permanganate treatment of triethylene glycol (Figure 3b) or chlorite residue (Figure 3c) also appears to remove all remaining lignin, which agrees with previous FTIR analysis (Reeves, 1993b). The disappearance of the small peaks, identified as due to lignin in these two residues, upon treatment with permanganate supports the original conclusions that neither triethylene glycol nor chlorite removes all of the lignin from forages. The same results were found for chlorite-extracted triethylene glycol residue.

In Figure 1d, the results are presented for permanganate treatment of RC ADL. The absence of the peak at 146 ppm indicates that nearly all lignin components are gone.

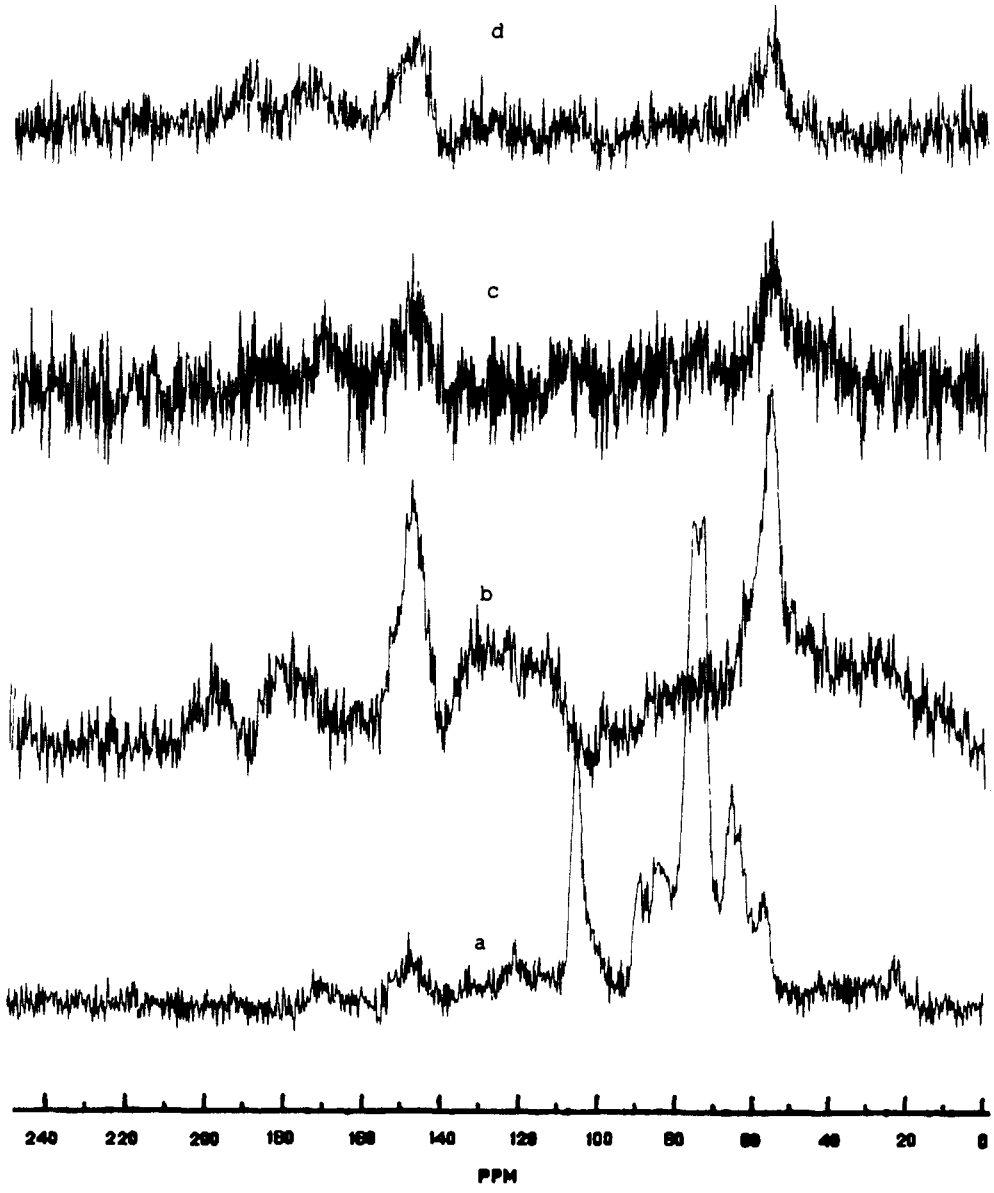

Figure 4. ^{13}C NMR spectra of (a) peanut hull (PH) acid detergent fiber (ADF), (b) 72% sulfuric acid lignin (ADL) performed on PH ADF, (c) residue from permanganate extraction of PH ADL, and (d) residue from 72% sulfuric acid treatment of residue from permanganate extraction of PH ADF.

The large noise/signal ratio indicates most of the sample has decomposed due to the chemical treatment. The peaks that remain are at frequencies different from those prior to the treatment or previously unresolved due to the more abundant components that were lost on treatment. The remaining peak at 156 ppm is consistent with (C=O)—NH—(C=O) structures, such as in biuret, and that at 56 ppm, with secondary amines (Levy et al., 1980), but both are almost lost in the background spectra. The frequency at 156 ppm is resolved from the lignin peaks removed at 146 ppm. Common metal ions, such as Zn^{2+} and Fe^{3+}, are known to complex with (C=O)—NH—(C=O) groups (Houghton, 1979). Metal–ligand complexes could catalyze protein and/or carbohydrate rearrangement on chemical treatment. Organic ligands in strong metal–ligand complexes may not be detected in the ^{13}C spectra due to line broadening from the metal, effectively leaving only the non-metal-complexed ligands as detectable. This could explain the lower sensitivity, compared to FTIR, observed in this spectrum.

Even though permanganate removes lignin, the above results for permanganate treatment of ADL are at complete odds with FTIR data (Reeves, 1993b), where spectral

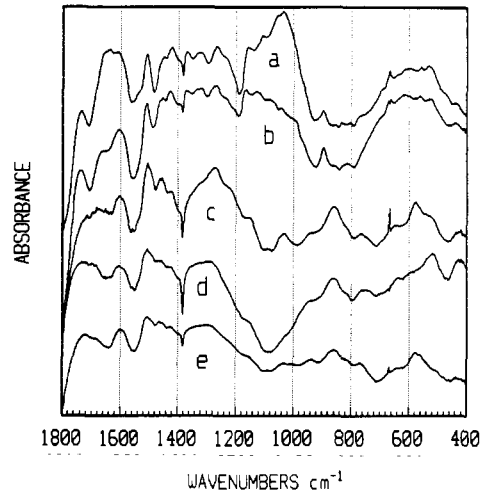

Figure 5. Mid-infrared diffuse reflectance spectra from 1800 to 400 cm^{-1} of (a) peanut hulls (PH), (b) PH acid detergent fiber (ADF), (c) 72% sulfuric acid lignin (ADL) performed on PH ADF, (d) residue from permanganate extraction of PH ADL, and (e) residue from 72% sulfuric acid extraction of residue from permanganate extraction of PH ADF.

changes were minor, and with PY–GC–MS (Reeves and Galletti, 1993), where lignin was abundantly evident, although altered. In the PY–GC–MS results it appeared that the permanganate treatment resulted in a greater number of distinct products, as though the condensed ADL might have been partially cracked, but the abundance of aromatic residues was clear. Considerable material survives the treatment of ADL with permanganate even after two treatments (Reeves, 1993a), too much to be simply inorganic ash.

A possible explanation, consistent with the results of these different procedures, is that permanganate may only remove the mobile lignin. The immobile lignin, e.g., metal complexed, would be observable by FTIR but may not be by NMR under the same analytical conditions due to metal-induced line broadening. If the chemical structures were simply more strongly cross-linked polymers, NMR should have easily detected their presence. A chemical (e.g., mineral) environment that made the NMR signal too broad or the decay too rapid would clarify why in Figure 1d no lignin was detected. Pyrolysis products could also rearrange in the presence of metal catalysts. Further research will be required to resolve these discrepancies.

Peanut Hull Lignin Results. Spectra a–d of Figure 4 show ^{13}C NMR results obtained using peanut hulls (PH) as the starting material. Peanut hulls have a higher lignin and much lower protein content than the RC, although RC is more representative of materials used as feeds for ruminants (Reeves, 1993a). As is easily seen, the lignin frequencies for PH (Figure 4a,b) are much more prominent and the proteins reduced, compared to the RC ADF or ADL (Figure 1a,b). While for RC little residue remained after treatment of permanganate residue with 72% sulfuric acid, this was not true for PH, which is believed to have a high cutin content. Examination of the spectra (Figure 4c) would indicate that virtually all of the residue was lignin, and not cutin, due to incomplete removal of lignin by permanganate in the first treatment stage (Goering and Van Soest, 1970). Infrared results for RC, where much less residue was produced (Reeves, 1993a), were very similar to those for PH, indicating that many cutin values may be largely due to lignin.

Figure 4c contains the results for permanganate extraction of PH ADL. As shown, the results were similar to those for 72% sulfuric acid extraction of permanganate residue (Figure 4d), confirming that neither procedure removes all of the lignin from PH. Chemical shift differences above 160 ppm between the two, however, suggest the chemical compositions and/or chemical environments of the carbonyl regions are not identical.

Comparison among the Instrumental Techniques. In Figure 5 are the infrared spectra of PH (a), PH ADF (b), PH ADL (c), and PH ADL extracted with permanganate (d), and PH permanganate extraction residue treated with 72% sulfuric acid (e). The main points of interest, from a spectral vs compositional standpoint, are as follows: 1750 cm^{-1} represents esterified carbohydrates, the large peak between 1650 and 1600 cm^{-1}(a) is due to nonresolved overlapping bands produced by protein and lignin, the peak at about 1510 cm^{-1} is also due to lignin, and finally the small peak in Figure 5a,b at about 900 cm^{-1} is due to cellulose (Faix, 1989; Reeves, 1993b). Other information is obviously present but can be difficult to definitively assign in a complex material such as this. When most of the protein is removed by the ADF process, a large decrease is seen in the 1650-cm^{-1} area. Note, however, that even for PH, a low protein material, the peak does not disappear, indicating contributions from other substances. The ADL process removes carbohydrates and one sees a large decrease in the area between 1200 and 1000 cm^{-1} and a disappearance entirely of the cellulose peak at 900 cm^{-1} (Reeves, 1993b). Finally, the treatment of ADL with permanganate (d) or the reverse (e) results in very similar spectra, although the yields of residue were very different (Reeves, 1993a). The spectral similarity is in marked contrast to that found with the NMR spectra. Effects of carbonyl complexation with metal ions on NMR spectra could explain this difference.

As can be seen, overall, the information provided by FTIR was comparable in nature and detail to that provided by ^{13}C NMR analysis. In both, the detailed spectral information provided by spectra of pure, single-compound, materials, is largely lost due to the multitude of overlapping peaks [i.e., the large peak between 1650 and 1600 cm^{-1}, due to protein and lignin, (Reeves, 1993b)]. Information is provided by both on materials more as a class, i.e., carbohydrates, lignin, proteins, etc., as opposed to detailed information on a specific molecular bond (e.g., amides in proteins), although, of the two (^{13}C NMR and FTIR), ^{13}C NMR appears to provide the more detailed information. The increase in detail provided by ^{13}C NMR is, however, offset by the decrease in sensitivity for some materials (proteins and particularly lignin). While with FTIR strong absorption bands can be easily found in forage materials representing carbohydrates, proteins, and lignins, with ^{13}C NMR the carbohydrates dominate the spectra. PY–GC–MS is similar to ^{13}C NMR in that lignins dominate, with carbohydrates a strong second and proteins a very distant third. Overall, the three techniques complement each other and are most useful when used together (along with chemical analysis) to analyze problems in forage chemistry.

Conclusions. The ^{13}C NMR results confirm the following conclusions about gravimetric analysis of plant fiber. Triethylene glycol does not remove all lignin from forages and appears to leave a condensed lignin residue. The chlorite procedure can remove residual lignin found in triethylene glycol extracted materials. The sulfuric acid extraction of the residue from the permanganate procedure yields lignin not removed by the latter method. The chemical composition of the residue from the reverse procedure (permanganate treatment of the residue from the sulfuric acid procedure) was dependent on the starting material and can have residual lignin.

However, because of sensitivity and resolution limitations, the fate of carbohydrate and protein in the gravimetric procedures was not fully resolved by the ^{13}C NMR procedure. The permanganate residues showed little evidence for carbohydrate removal or alteration. Residues from the chlorite extraction did not clarify whether protein rearrangement due to the treatment had occurred.

Analysis by multiple instrumental techniques enables a more thorough evaluation of the composition and content of forage obtained from different agricultural products. Primarily because of the lack of its ready availability and the relatively high cost of the instrumentation and maintenance, solid-state ^{13}C NMR procedures are not practical for routine forage analysis but may prove valuable in evaluating which lower cost analytical procedures are most valid.

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