and thereby release hesperidin. An observation by C. P. Verdon at the Fruit and Vegetable Chemistry Laboratory is of interest in this respect. A navel orange purchased at a local market showed white spots on the segment walls, and the fruit had a strongly bitter taste characteristic of the triterpenoid constituent limonin. Navel oranges normally contain high concentrations of the tasteless precursor of limonin, limonoate A-ring lactone. When fruit cell membranes are broken, this compound comes in contact with an enzyme, limonin D-ring lactone hydrolase, which converts it to the bitter limonin (Maier et al., 1977). This enzymic process is slow, so normally the fruit is eaten before it becomes bitter. In the case of the fruit referred to above, its bitterness indicated that cell membranes had been broken previously. The presence of the white spots showed that the fruit had been exposed to low temperatures. These observations provide strong evidence that freezing conditions cause damage to cell membranes, which would explain the formation of white spots composed of crystalline hesperidin.

It remained to be explained why crystallization of hesperidin occurs in only certain small areas rather than uniformly throughout the tissue. This was clarified by an experiment in which a Valencia orange was immersed in ethanol for 3 days. Treatment of hesperidin-containing tissue with ethanol causes the flavonoid to crystallize within the cells in which it is present in high concentration. The segment walls from the ethanol-treated fruit showed crystallization only in local clusters of cells. The distribution of such clusters was similar to that of freeze-induced white spots. Apparently, therefore, hesperidin is unevenly distributed in the tissue, and groups of cells with high concentrations of the flavanone form white spots in response to freezing conditions.

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Comparative Study of Whole Seed Protein and Starch Content via Cross Polarization–Magic Angle Spinning Carbon-13 Nuclear Magnetic Resonance Spectroscopy

Daniel J. O'Donnell, Joseph J. H. Ackerman, and Gary E. Maciel*

A series of nuclear magnetic resonance (NMR) spectra of whole seeds of various types was obtained by using cross polarization–magic angle spinning (CP/MAS) techniques. Select signals in the spectra provided a means of comparing the protein content relative to the starch content within a group of seed varieties. Seeds obtained from legumes were found to be high in protein content, with different legumes showing a range of protein NMR signal intensities. A series of sorghum varieties and a series of grain types were also analyzed, and the protein content of these series was compared to that of the legumes and to that of one another. The potential of ^{13}C CP/MAS NMR for the study of seeds and other intact plant materials is strongly indicated.

The need for rapid, nondestructive methods of chemical analysis of seeds has long been recognized by agronomists. The traditional method of Kjeldahl protein determination destroys the seed, as does the newer estimation using near-infrared light reflectance (Hymowitz et al., 1974). More recently, proton activation of whole and ground seeds has permitted the measurement of total nitrogen content (Dohan and Standing, 1976). However, this and other similar methods require the use of high-energy (~16 MeV) proton beams.

In the past, wide-line ^{1}H nuclear magnetic resonance (NMR) and ^{13}C NMR spectroscopy have been used to estimate the oil content of whole seeds (Brown and Craddock, 1972; Schaefer and Stejskal, 1975; Alexander et al., 1967; Schaefer et al., 1979) in a nondestructive manner. The oil and water components of the seeds

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constitute a highly mobile, almost liquid phase, surrounded by a solid matrix of protein, starch, and other more rigid constituents, and can be detected by liquid-state NMR approaches. By contrast, the more rigid components of the seed would not be expected to contribute to the spectrum obtained by liquid-state techniques, because of long ^{13}C spin–lattice relaxation times and the broadening influences of ^{1}H–^{13}C dipole–dipole interactions and ^{13}C chemical shift anisotropy. Hence, with a spectrometer designed to give sharp ^{1}H or ^{13}C resonance signals for liquids and essentially no (or extremely broad) ^{1}H or ^{13}C signals for solids, the oil (and water) content can be measured with only minute interference from the solid components.

Recently, ^{13}C NMR spectra of rigid, solid bioorganic materials have been obtained (Miknis et al., 1979; Maciel et al., 1979; Rutar et al., 1980) by using cross polarization–magic angle spinning (CP/MAS) (Schaefer and Stejskal, 1979). Interestingly, the spectra obtained using these techniques contain only signals from carbons in structures of *low* mobility. It is the object of this paper to qualitatively assess the usefulness of CP/MAS ^{13}C NMR spectroscopy in the analysis of seeds. Because only carbons in nonfluid environments give rise to signals in the CP/MAS experiment, spectra of seed proteins and carbohydrates should be obtained presumably without interference from the fluid fractions. Experiments using whole, mature soybean seeds have graphically illustrated this point (Schaefer and Stejskal, 1975; Schaefer et al., 1979). Nonspinning, as well as spinning, CP NMR spectra of structural and storage protein and starch as well as scalar-decoupled Fourier transformed (FT) NMR spectra of fats and lipids were obtained. Rutar and Blinc (1979) and Rutar et al. (1980) have reported some results obtained on seeds by these techniques.

In the present study, a series of ^{13}C NMR spectra were acquired of different seed types and varieties, and individual ^{13}C signals were assigned based upon model compound studies. The results of standard methods of protein analysis were used to correlate the differences observed in these spectra and to demonstrate the potential usefulness of the CP/MAS method.

EXPERIMENTAL METHODS

Discussions of solid-sample NMR techniques appear elsewhere (Miknis et al., 1979; Schaefer and Stejskal, 1979). A brief discussion follows to aid the reader.

In the cross polarization experiment, the carbon-13 magnetization is obtained by a transfer of magnetization from the proton spin reservoir under a specific double-resonance condition called the Hartmann–Hahn match (Hartmann and Hahn, 1962; Pines et al., 1973). This provides two types of advantages: (1) the contact between the carbon and proton spin systems allows one to use the relaxation of protons to circumvent the ^{13}C relaxation time bottleneck, permitting much more rapid repetitions; (2) an almost 4-fold increase in the carbon-13 magnetization is obtained over the magnetization that would be obtained in a normal ^{3}C 90° pulse NMR experiment. Since this transfer of magnetization is most effective when there is little or no molecular motion, the fluid portions of the seed are essentially nonobservable. A high radio frequency (rf) field at the ^{1}H resonance frequency is also applied during the carbon sampling period to eliminate line broadening in the carbon spectrum due to dipolar coupling between the carbons and protons.

Dramatic line broadening is observed in nonspinning ^{13}C CP/NMR experiments due to ^{13}C chemical shift anisotropy. This arises from the fact that the chemical shift of a carbon depends not only on its chemical structural environment but also on the orientation of that environment relative to the applied magnetic field. In a fluid, rapid isotropic molecular motion averages the effects of molecular orientation, resulting in narrow lines. A similar averaging effect can be obtained in the solid by rapid mechanical spinning of the sample about an axis that makes an angle of 54.7° (the magic angle) relative to the applied magnetic field. This angle is unique in that all of the line broadening interactions due to chemical shift anisotropy disappear, resulting in relatively small line widths.

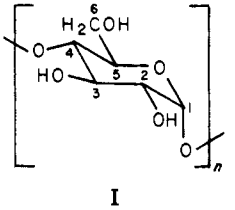
A home-built CP/MAS spectrometer, based on a Varian HR-60 magnet, operating at 15.08 MHz for ^{13}C was used for this work. Most samples were packed in diatomaceous earth (which contributed no ^{13}C signal) to prevent rotor instabilities under high-speed spinning conditions. Pinto beans were examined as single seeds; for other samples a large enough number of seeds was employed to fill the spinner volume (~0.6 cm^3). Spinning rates ranged from 2 to 2.5 kHz. Contact times for the CP experiments were 0.5–1.0 ms, with delays of 2–4 s between sampling periods. Data were collected over 2047 points at a frequency of data acquisition of 8 kHz. Spin temperature inversion (Schaefer et al., 1979) was employed to minimize experimental artifacts and unwanted ^{13}C signals from more mobile (liquid-like) components (e.g., lipids). Typically 1500–33 000 acquisitions were necessary to obtain a sufficiently high signal-to-noise ratio. All spectra are referenced to an external standard of tetramethylsilane (Me_4Si; TMS in the figures). Protein analyses were obtained (when possible) via the dye absorption method.

Some questions remain on the quantitative significance of relative intensities in CP/MAS ^{13}C spectra; definitive answers will require additional work on each type of sample. However, comparisons of the resonances of a given carbon type among similar samples are not expected to be problematical.

RESULTS AND DISCUSSION

The seeds used in this study can be considered in the following three categories: legumes, cereal grains, and sorghum varieties. This choice of categories permits the evaluation of the NMR technique for high protein seeds (legumes), low protein seeds (cereal grain), and varieties within a single plant type (sorghum).

The CP/MAS NMR spectra of a selection of legumes are shown in Figure 1. The sharp signal at ~75 ppm, from an external standard of liquid tetramethylsilane, compares favorably to the chemical shifts of carbons 2–5 in amylose,



I

I (Dorman and Roberts, 1971) and can therefore be assigned to starch in the seeds. Likewise, the signals observed at ~105 ppm in each spectrum of Figure 1 can be assigned to carbons in seed starch similar to carbon 1 in amylose. It was also noted that in spectra D and E in Figure 1, a signal was observed at ~62 ppm, which corresponds to carbons similar to carbon 6 in amylose. However, this signal is not observable in spectra A, B, and C of the legumes due to interference from other signals, as discussed below. A spectrum of corn starch is shown in Figure 2, with signals at 103, 74, and 64 ppm. This

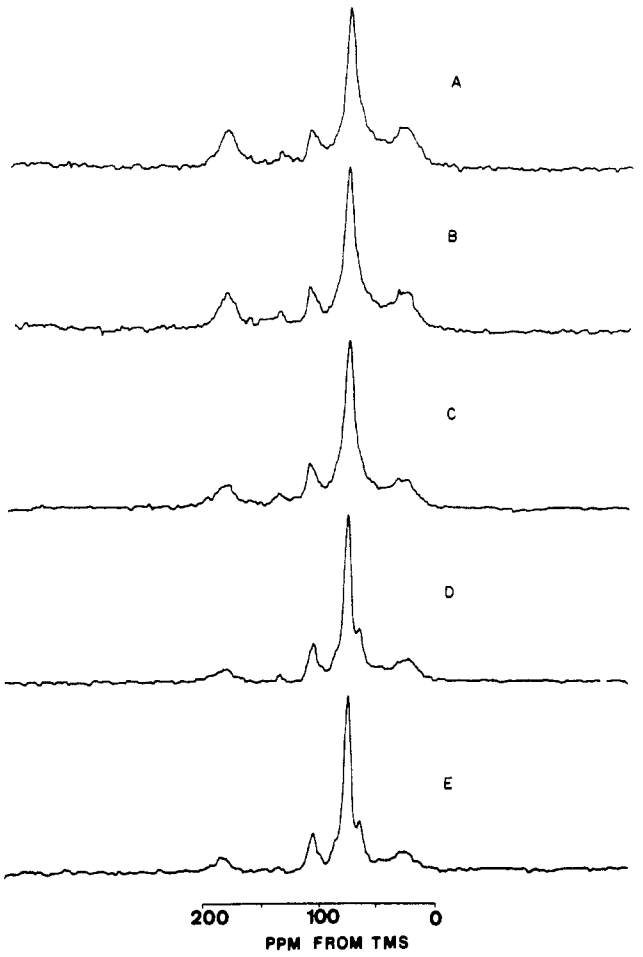


Figure 1. CP/MAS ^{13}C NMR spectra of a selection of legumes. (A) Ranger alfalfa; (B) red clover; (C) white clover; (D) pinto bean; (E) lentil.

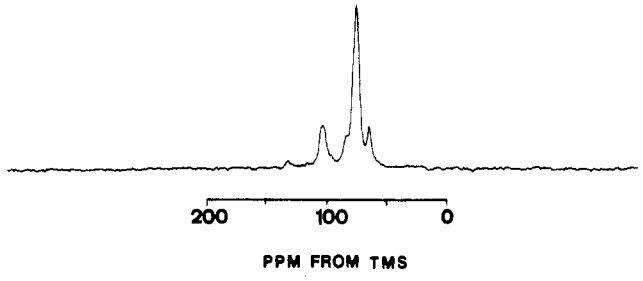
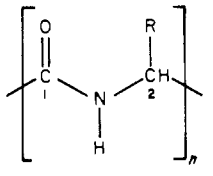


Figure 2. CP/MAS ^{13}C NMR spectrum of corn starch.

CP/MAS NMR spectrum supports the above assignments.

As can be seen in Figure 1, there are two broad, prominent signals in the spectra that cannot be attributed to starches in the sample. Since it was initially assumed that the only compound types that would be observable were proteins and starches (vide infra), it was reasonable to assume that the signal centered at ~175 ppm arose from the carbonyl carbon (1) in a protein peptide bond, II. The



II

broad signal centered at ~22 ppm in each spectrum was assigned to the variety of methyl, methylene, and methine carbons that occur in the various aliphatic side chains of the amino acids in the proteins.

Table I. ^{13}C CP/MAS NMR Data for Select Amino Acids and Dipeptides

compd	chemical shift, ppm from Me_4Si		
	>C=O	C-α	R group signals
glycine	176.1	49.7	
L-serine	174.1	55.5[a]	62.2
L-cysteine	173.6	57.2[a]	28.5
L-tyrosine	176.1	58.0	155.9, 131.6, 123.8, 118.6
L-valine	175.3	55.0	31.3, 21.5, 18.9
L-asparagine	174.6	51.0	190.6
L-proline	174.1	55.3	33.7
L-tryptophan	176.7	55.7	148.7, 129.5, 121.7, 111.9, 30.0
L-phenylalanine	175.6	49.7	135.7, 129.0
L-tryptophyl-L-alanine	177.7 170.2[a,b]	55.3[a]	119.2, 110.5, 22.1, 18.1
glycyl-L-tryptophan	179.2 169.3[a,b]	50.8[a]	118.6, 108.8, 24.9
L-tyrosylglycine	178.2[c]	53.2	158.0, 132.1, 121.2, 35.2
glycyl-L-tyrosine	179.8[c]	~50[d]	153.3, 128.4, 116.5, 36.2
L-phenylalanyl-L-leucine	180.2 170.2[a,b]	~50[d]	135.7, 129.5, 39.4, 25.4, 23.2
glycyl-L-phenylalanine	177.2 170.2[a,b]	64.1[a]	139.9, 128.0, 39.9

[a] Center of broad doublet. [b] Shift of >C=O in the peptide bond. [c] Peptide >C=O not observable. [d] Very broad signal.

For confirmation of these assignments, as well as for investigation of the possible resonances that might interfere with the signal at 62 ppm, a series of amino acids and dipeptides was used in a model compound study. The results are summarized in Table I. It is immediately obvious from the table that indeed the carbonyl carbons in both the free acids and the peptide bonds give rise to a signal at 170–180 ppm. The carbonyl carbons in the peptide bonds yield very broad signals, usually distinguishable as a doublet, due to interaction with the ^{14}N quadrupolar nucleus. This same interaction produces a similar effect in the signals for the α-carbon in the amino acid residues. Thus, even though the center of the signal for the α-carbon was found to be ~50 ppm, the signal extends to both lower shielding and higher shielding, which causes interference with the starch signal at 62 ppm in the seed spectra.

Having established that signals for starch and protein are discernible from the spectra, one can note that the intensities of the signals for the carbonyl carbons (~175 ppm) and the methyl–methylene carbons (20–30 ppm) vary depending upon the type of seed used. From Figure 1, it is clear that the alfalfa and the red clover seeds (spectra A and B in Figure 1) contain substantially more protein than either the pinto beans or the lentils (spectra D and E in Figure 1). It is also apparent that the red clover seeds have a higher protein content than the white clover seeds (spectrum C in Figure 1), although the difference is not great. In addition, it can be noted that in the cases where a high level of protein is indicated, the starch signal at ~62 ppm is obscured. In the samples of lower protein content (spectra D and E, Figure 1), this signal is clearly discernible. A marked difference in the line width of the starch signal at ~75 ppm is also observed, with the narrowest signal being associated with the lower protein content. This is again attributed to the interference of the α-carbon signals of the protein present in the spectra, resulting in larger line widths (spectra A and B, Figure 1).

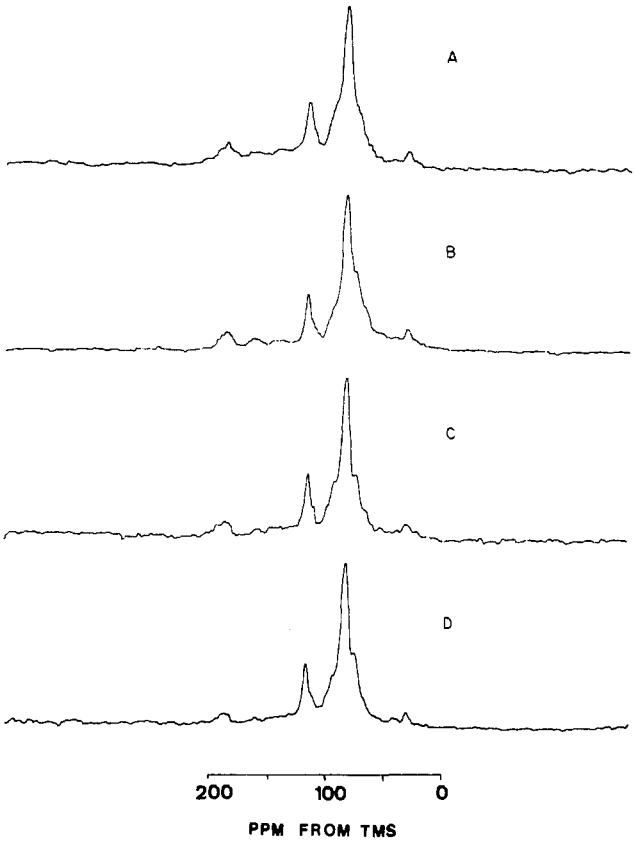


Figure 3. CP/MAS ^{13}C NMR spectra of sorghum varieties. (A) N 3-1; (B) N 2-1; (C) BRM 6-1; (D) N 12-1.

Several low-intensity signals are present between 120 and 140 ppm in each spectrum with a particularly sharp signal occurring at ~128 ppm. When this value is compared with shifts in Table I, it seems very likely that this signal, as well as others observed in this area of the spectra, is due to aromatic signals arising from select R groups in the protein amino acid residues.

A series of different sorghum varieties was also investigated via ^{13}C CP/MAS NMR spectroscopy and the resulting spectra are displayed in Figure 3. The same basic features noted above for the spectra of the legume seeds were found in these sorghum spectra. However, it is obvious that protein content of any one of these sorghum varieties is less than that found in most of the leguminous seeds types. The protein content of the lentil seeds in this study was found to be 15.9% (by weight). On the other hand, an investigation of different sorghum varieties showed a range of protein content over five varieties from 9.8 to only 14.3%. Thus, it was not surprising to find the level of the carbonyl signals at ~175 ppm in the sorghum spectra to be generally lower than that found in the legume seed types. Although the differences among the spectra of the sorghums are not as great as those observed among the legume seed types, it is nevertheless apparent that some differences still exist. Furthermore, it is observed that the line widths of the starch signals at 105 and 75 ppm are on the whole narrower than those in the spectra of Figure 1. In addition, the protein signals at 175 and ~30 ppm, although less intense, seem sharper in the sorghum spectra than in the spectra of the lugumes. Although no diffinitive conclusions can be made from this observation, it may be that this line shape difference results from a difference in the amino acid makeup between the sorghums and legumes.

In Figure 4, the spectra of several different grains are shown. These spectra show that the differences among the

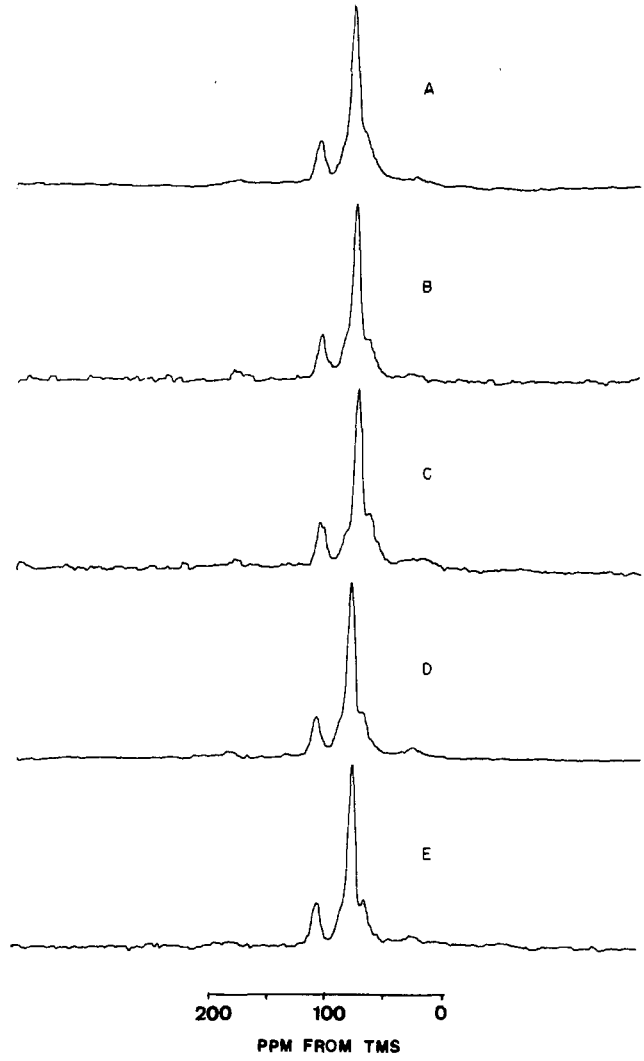


Figure 4. CP/MAS ^{13}C NMR spectra of whole grains. (A) Millet; (B) wheat, siefe cevros; (C) oats, Colorado 37; (D) corn; (E) barley, Highland.

grain types are small. It appears that the amount of protein throughout this series is much lower than that of any of the leguminous types. A close inspection of Figure 4 reveals that the starch signal at 62 ppm, easily discernible in the spectrum of the barley (Figure 4E), is partially obscured in the spectra of wheat, oats, and corn (spectra B, C, and D, respectively of Figure 4) and is totally obscured in the spectrum of millet (Figure 4A). In addition, the spectra of wheat, oats, and corn (spectra B, C, and D of Figure 4) are remarkably similar. A protein analysis of these seeds revealed the following proteins contents: wheat, 13.3%; oats, 12.8%; corn, 12.0%; barley, 10.6% (analysis standards for millet were not available). Thus the seeds which have similar NMR spectra also have very similar protein content, while a difference of less than 2% in protein content was discernible in the NMR spectrum of barley when compared to the other spectra.

As noted in the discussion of the spectra, some overlapping exists between the starch and protein signals in each spectrum. However, a recently developed technique (Opella and Frey, 1979) utilizing an interrupted decoupled pulse sequence can be applied to seed samples. This method discriminates against protonated carbons. Amylose (I) has only protonated carbons and hence would *not* give rise to any strong signals in the ^{13}C CP/MAS NMR spectrum obtained by using this decoupling technique. The proteins, on the other hand, have carbons which are not protonated and should give rise to strong signals. The

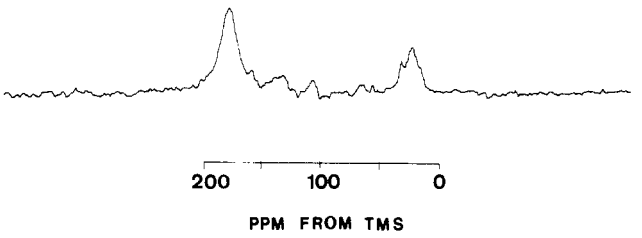


Figure 5. CP/MAS ^{13}C NMR spectrum of red clover seeds by using a 50-μs interruption of the ^{1}H decoupling pulse prior to data acquisition of the ^{13}C signal.

only exception to this rule is the case of methyl groups, which, because of rapid rotational motion, do show strong resonances even under the interrupted decoupling conditions. As can be seen in Figure 5, signals derived from the starch present in red clover seeds were effectively eliminated by interrupted decoupling, while the signal from the carbonyl carbons in the peptide bonds (C-1 in II) is still visible. Methyl signals, presumably from methyl groups in the side-chain R groups of amino acids, also appear in the high-shielding region of Figure 5. It should be noted that the signal-to-noise ratio in Figure 5 was attained only after using 4 times the number of acquisitions used for the normal ^{13}C CP/MAS NMR experiment (Figure 1B). The apparent reduction of overall intensity results largely from the fact that the carbonyl and methyl signals are lower in intensity than the starch signal in the normal spectrum. Also, some small amount of carbon magnetization (roughly one-fourth) is lost from the carbonyl carbons during the interruption of the decoupling, due to neighboring protons, and the methyl carbons are certainly effected by the interruption, even though rapid rotation about the carbon–carbon bond attenuates the effect. Some small signals in the center of the spectrum (110–160 ppm) are also observed in Figure 5 and can be tentatively assigned to substitued aromatic carbons in aromatic amino acids (Opella, et al., 1979).

Although the qualitative importance of the ^{13}C CP/MAS technique is obvious, the quantitative aspects remain to be investigated. Among the factors that require additional study is the extent to which lipid components of varying degrees of mobility can contribute to the ^{13}C spectrum and the analysis. This question has been addressed by Rutar and Blinc (1979). The spin-temperature inversion technique should minimize any contribution due to mobile lipid components. Another factor for additional study is the relative cross polarization efficiencies of the pertinent carbon types in seed spectra. These efficiencies will determine the optimum experimental conditions and the nature of any corrections or calibrations that may be needed for quantitation.

CONCLUSIONS

The use of CP/MAS ^{13}C NMR spectroscopy in the analysis of whole seeds certainly shows great promise. The main limitation would seem to be the length of analysis time. At present, a minimum of 30 min is required per analysis. However, this disadvantage may be offset by the safe, nondestructive nature of the analysis and should be reduced substantially as the equipment and techniques improve. One can expect significant developments in this area during the next few years.

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Isolation and Characterization of the Major Protein Fraction of Sunflower Seeds

E. H. Rahma and M. S. Narasinga Rao*

A method is described for isolating the major protein fraction of sunflower seeds in a homogeneous form. It has an $s_{20,w}$ value of 11.6 S (at 1% protein concentration) and a molecular weight of 300 000. It contains 0.31% carbohydrate and no phosphorus. It consists of at least 10 polypeptide chains. Its structure is predominantly random coil and β structure. Some of the SH groups are buried and are exposed after denaturation of the protein with urea.

The proteins of sunflower seed (*Helianthus annuus*) consist of three protein fractions having sedimentation coefficients of 2, 7, and 11 S (Joubert, 1955; Sabir et al., 1973; Schwenke et al., 1974; Rahma and Narasinga Rao, 1979). The major protein is a high molecular weight protein with a molecular weight of 300 000–350 000 (Sabir et al., 1973; Joubert, 1955). Although there are reports on

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