Nondestructive Determination of Protein Content of Viable Seeds by Proton Enhanced ^{13}C NMR

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Proton enhanced ^{13}C NMR spectroscopy was used for a non-destructive determination of the protein content in single viable soybean, horse-bean, bean and corn seeds. The intense ^{13}C oil lines were suppressed by a series of saturating pulses and spin temperature reversal so that only the ^{13}C protein and carbohydrate signals of the solid part of the seed were detected. Significant seed to seed variations in the protein content were found which can be used as a basis for rapid seed selection in plant breeding programs.

Introduction

The use of proton wide-line NMR in plant breeding for higher oil content is by now well established [1, 2]. The method allows a fast and nondestructive determination of the total oil content of seeds and offers several advantages in comparison with classical chemical techniques which are usually destructive and slow. When non-destructive NMR techniques are used, only the best seeds are planted so that new plant varieties with a higher oil content can be obtained several times faster than with classical plant-breeding methods [1, 2].

It has been recently discovered [3] that plant breeding for higher oil quantity deteriorates to a certain extent the oil quality and decreases the protein content of seeds. In developing new plant varieties with a higher nutritional value it is therefore advisable to check not only the oil quantity but also the oil composition and protein content of seeds. This and the specific needs of plant breeding for a higher protein content require the development of non-destructive NMR techniques for the determination of the total composition of the individual plant seeds.

Since oil exists in seeds in a liquid-like form, the oil composition and quality can be determined by standard ^{13}C high resolution NMR spectroscopy [4, 5]. This, however, is not the case for the protein content. The ^{13}C lines from the solid part of the seed are severely broadened by dipole-dipole interactions and are therefore too weak to be observed directly. The very existence of the proton-^{13}C dipole-dipole interactions in the solid part of the seed – which prevents the direct observation of the ^{13}C lines – enables one to use a double resonance technique [6] which substantially enhances the sensitivity for the detection of the ^{13}C signals by transferring polarization from the abundant proton reservoir to the dilute ^{13}C spins and simultaneously increases the resolution by removing the heteronuclear dipolar broadening by coherent decoupling [6, 7]. Using this technique Schaefer and Stejskal [8] obtained proton enhanced ^{13}C spectra of intact seeds where carbohydrate and protein resonances could be resolved. The protein ^{13}C lines were, however, masked by the overlapping ^{13}C oil lines from the liquid-like part of the seed and no quantitative determinations of the protein content have been made [8].

In this paper we describe a somewhat modified version of the proton-^{13}C double resonance determination of the protein content of individual intact plant seeds. The undesired ^{13}C signals from the liquid-like part of the seed are completely suppressed and the protein content of the individual seeds can be quantitatively determined. The technique is fast enough to be usefull as routine method for the characterization of the protein content of seeds. Here we review results obtained with individual soybean, horse-bean, bean and corn seeds.

Experimental

The pulse sequence used for the quantitative determination of the protein content of seeds is shown in Fig. 1. The ^{13}C magnetization of the seed is first destroyed by a series of saturating pulses. The

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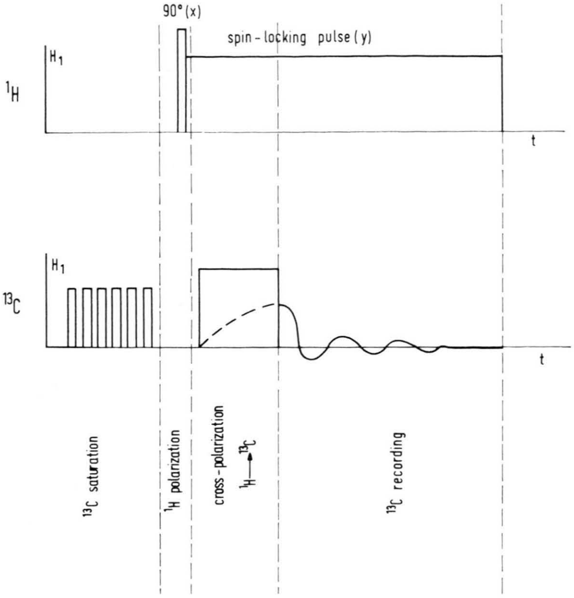

Fig. 1. Schematic representation of the double resonance pulse sequence which is used for the detection of the protein and carbohydrate proton enhanced ^{13}C NMR spectra in oil rich heterogeneous seeds. In spin temperature reversal experiments the phase of the spin-locking pulse is changed periodically by 180 °.

protons are brought into the rotating frame by a $\pi/2$ pulse followed by a long spin-locking pulse H_{1I} which is shifted in phase by 90 ° with respect to the first $\pi/2$ pulse. Level matching in the rotating frame is obtained by irradiating the ^{13}C spins by a resonant radiofrequency field H_{1S} which fulfills the Hartmann-Hahn condition [9]

$$\gamma(^{13}\mathrm{C})\, H_{1S} = \gamma(^{1}\mathrm{H})\, H_{1I} \tag{1}$$

and allows for polarization transfer [9] from the proton reservoir to those ^{13}C spins which are dipolarly coupled to the protons [6]. This is the case for the protein and carbohydrate ^{13}C nuclei in the solid part of the seed but not for the "oil" ^{13}C nuclei in the liquid-like part of the seed where C-H dipolar interactions are averaged out by molecular motion. Finally the ^{13}C field H_{1S} is switched off and the proton enhanced ^{13}C free induction decay spectra are recorded. Since the proton H_{1I} field is kept on while the ^{13}C spectra are recorded, the protons are decoupled from the ^{13}C spins during the observation period resulting in a high resolution ^{13}C spectrum from the solid part of the seed. The sequence is repeated and the spectra are accumulated until a satisfactory signal to noise ratio is obtained.

It should be noted that the saturation of the "dilute" ^{13}C spins in the beginning of the sequence is usually omitted in proton enhanced ^{13}C spectroscopy [6]. In heterogeneous systems where both solid-like and liquid-like regions coexist it is however absolutely essential to suppress the signal from the liquid-like region if quantitative measurements of the spectra from the solid-like region are to be made. In principle this can be achieved either by spin temperature reversal [10] or by presaturation (Fig. 1). In practice, however, a combination of these two techniques was necessary to minimize experimental artifacts and make a quantitative analysis possible. Very short "mixing" times ($t_{\mathrm{mix}} \approx 0.5$ msec) had to be used in order to remove cross-polarization effects in the liquid part of the seed due to the presence of scalar coupling [11]. In quantitative measurements of the protein content we used the presaturation technique and in addition periodically reversed the spin temperature in the rotating frame to remove possible artifacts [10].

The experiments were performed with the help of a superconducting magnet at $\nu_L(^{13}\mathrm{C}) = 67.9$ MHz and $\nu_L(^{1}\mathrm{H}) = 270$ MHz. Typical radiofrequency fields used to accomplish the pulse sequence shown in Fig. 1 were $H_{1I} \approx 10$ G and $H_{1S} \approx 40$ G.

Results and Discussion

The effect of the saturating pulses described above was first tested on an artificial heterogeneous sample. A tube containing solid adamantane and another with liquid benzene were simultaneously placed into the radiofrequency probe head and the resulting proton enhanced ^{13}C spectra were recorded without (Fig. 2a) and with (Fig. 2b) the saturating pulses at the beginning of the double resonance pulse sequence. It can be clearly seen that the saturating pulses remove the benzene signal even without spin temperature reversions while the lines of solid adamantane are not affected.

The proton enhanced ^{13}C spectrum of a single soybean seed without the saturating pulses is shown in Fig. 3a while Fig. 3b shows the same spectrum obtained with the help of the pulse sequence described in Fig. 1. A comparison of two spectra shows that all ^{13}C oil lines from the liquid-like part of the seed are suppressed by the saturating pulses and that only ^{13}C lines from the solid part of the seed are displayed. Two groups of lines which represent

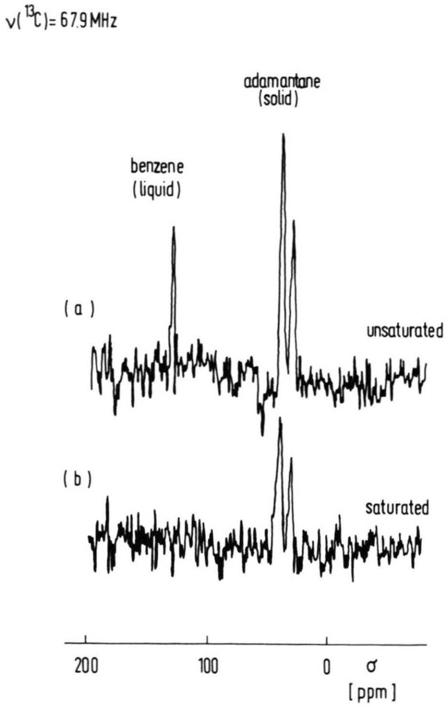

Fig. 2. Proton enhanced ^{13}C spectrum of a heterogeneous sample of liquid benzene and solid adamantane. The saturating pulses selectively suppress the lines of liquid benzene.

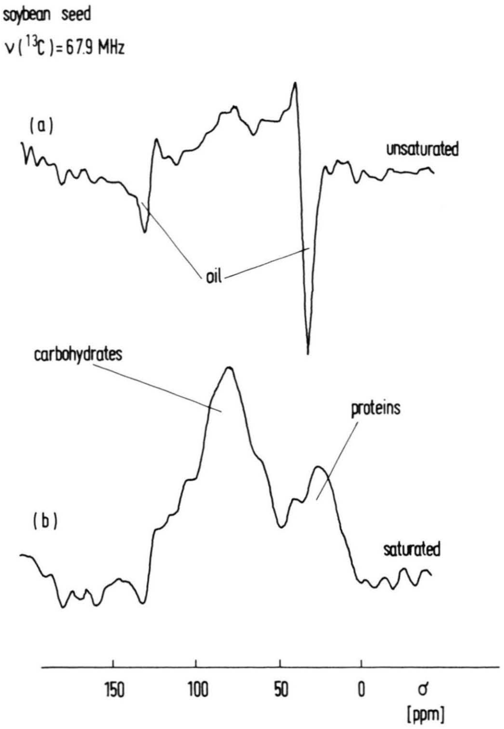

Fig. 3. Proton enhanced ^{13}C spectrum of a soybean seed without (a) and with (b) the saturating pulses. The "oil-free" spectrum (b) clearly shows the resonance of proteins (I_p) at 25 ppm and carbohydrates (I_c) at 80 ppm downfield from the external TMS standard.

protein ^{13}C resonances at 25 ppm and carbohydrate ^{13}C resonances at 80 ppm with respect to TMS are clearly seen.

To check whether this method can be used for a quantitative determination of the protein content of seeds flour samples with a known composition – determined by standard chemical techniques – were prepared and their ^{13}C spectra measured under the same experimental conditions as used for the individual seeds. The integrals of the "protein" (I_p) and "carbohydrate" (I_c) ^{13}C peaks were compared and a linear relation was found between the relative intensity of the protein peak $I_p/(I_p + I_c)$ and the protein content of the flour samples (Fig. 4).

The calibration curve obtained with the flour samples was used to determine the protein content of the various investigated seeds. The protein content in soybean seeds was found to vary between 27% and 31% of the total weight including oil. Seed to seed variations were found to be highest in horse-beans where the protein content varied between 19 and 27% and in beans where it varied between 19

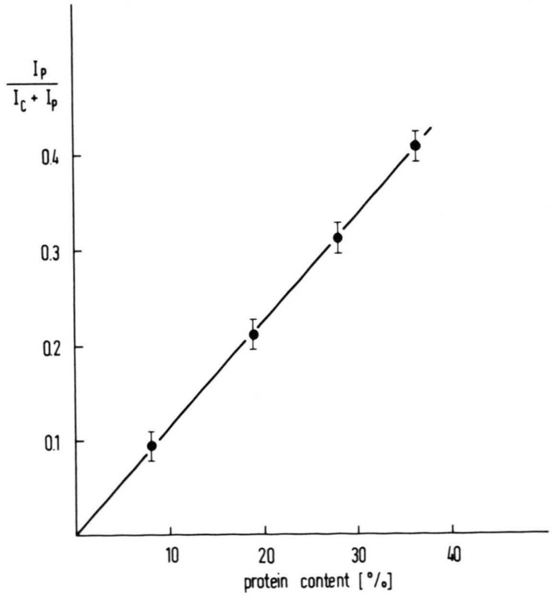

Fig. 4. Calibration curve showing the integral intensity of the ^{13}C protein NMR signal of oil-free flour samples as a function of their protein content as determined by standard chemical techniques.

and 26%. In corn seeds on the other hand, the protein content was found to be as low as 10%. Direct chemical analysis of a few selected seeds gave essentially the same protein content as obtained by ^{13}C NMR and the "flour" calibration curve.

The relative accuracy of the protein determination was better than 10% of the measured value. The seed to seed variations thus clearly represent differences in individual seed compositions and can be used as a basis for seed selection in plant breeding programs.

Conclusions

Proton enhanced ^{13}C spectroscopy enables one to perform quantitative measurements of the ^{13}C protein spectra even in oil rich individual plant seeds if the intense overlapping oil lines are removed by a series of saturating pulses combined with spin temperature reversal. Since the measuring time can be reduced down to a few minutes, the method can be used for a rapid and non-destructive determination of the protein content in intact viable seeds in plant breeding programs.

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