Carbon-13 Nuclear Magnetic Resonance Analysis of Intact Oilseeds

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ABSTRACT

High resolution natural abundance ^{13}C nuclear magnetic resonance (NMR) spectra of intact oilseeds have been obtained by Fourier transform techniques. The spectra can be interpreted in terms of the relative concentrations of the major fatty acids in the oilseed. The ^{13}C NMR spectra are well resolved despite the fact that ^{1}H NMR spectra of the same seeds are poorly resolved. The difference in resolution can be attributed to the simplicity of the ^{13}C NMR spectra in which all spin-spin coupling can be removed, in which the separation of lines is increased by a factor of 30, and in which line broadening due to intermolecular dipolar interactions is not important. The ^{13}C NMR Fourier transform technique is sufficiently sensitive that a high quality spectrum can be obtained from a single soybean, for example, in about 10 minutes. Similar spectra of single seeds can be obtained in comparable times for corn, castor bean, peanut, sunflower seed, and rapeseed. Because the NMR technique is nondestructive, it can be used to select individual oilseeds for use in breeding programs designed to improve oil quality. By employing some special experimental NMR line narrowing techniques, it also appears feasible to obtain moderately well resolved, natural abundance ^{13}C NMR spectra of the immobile, rigid protein, and carbohydrate components of an intact oilseed, as well as the more mobile oil components.

INTRODUCTION

A complete compositional analysis of the oil, protein, and carbohydrate content of a single intact oilseed, such as corn or soybean, is technically possible today using Fourier transform ^{13}C nuclear magnetic resonance (NMR) spectroscopy. In this paper, we will introduce the basic ingredients of a Fourier transform ^{13}C NMR experiment, show how standard ^{13}C NMR procedures have been used to obtain compositional analyses of the oil content of individual intact oilseeds, and then discuss the use of some specialized NMR techniques which have the potential of leading to a determination of the protein and carbohydrate concentrations of the same intact seeds.

Fourier Transform Carbon-13 NMR

Fourier transform ^{13}C NMR experiment (1,2) intense radio frequency pulses are repeatedly applied to a sample placed in a strong static magnetic field. The pulses excite the ^{13}C nuclear spins (1% of the carbons of all organic materials have nuclear spins) and so perturb the macroscopic ^{13}C nuclear magnetization of the sample. The return to the steady state of this radio frequency magnetization can be detected by what amounts to a carefully tuned, highly sensitive radio receiver. The ^{13}C radio signal following each pulse is taken from the receiver and stored in a computer, where it is added to the accumulated sum of all the previous signals. This process is called time averaging, and results in an improvement in sensitivity. Because the radio signal from the sample is always the same after each pulse, but the noise is random, by adding together the signals following many pulses, a net improvement is obtained in the signal-to-noise ratio. This improvement is of critical importance in a natural abundance ^{13}C experiment because of its low inherent sensitivity (ca. 5000 times less sensitive than a similar experiment performed on the more abundant ^{1}H spins of the same sample). In principle, the only limitation on the sensitivity enhancement which can be achieved by time averaging is the length of time one is willing to spend accumulating the data.

The reason for using pulses to obtain a ^{13}C NMR signal is primarily because the frequency components of each pulse have the desirable property of exciting all the chemically different kinds of carbons at the same time, regardless of their exact resonance frequencies. The resonance frequencies of various carbons will differ, depending upon their molecular environment. This is the source of chemical information in the experiment. Because all the carbons are excited at the same time, the observed radio signal (which is referred to as a free induction decay) has frequency components which correspond to the various resonance frequencies of the carbons in the sample. Thus, the observed signal is an interferogram. To extract the chemically useful component frequencies and their relative intensities from the interferogram (which can be extremely complicated), a Fourier or spectral analysis is performed, generally by the same small on-line laboratory computer used to accumulate the data. The final result is an NMR spectrum (relative intensity versus relative frequency or chemical shift) which can be immediately interpreted in terms of the various chemical components present in the sample.

In view of the lack of sensitivity, i.e., poor signal-to-noise ratio, of a ^{13}C NMR experiment, it is reasonable to wonder why we bother with it at all. There are, in fact, 3 compelling reasons. First, the separation of lines within a ^{13}C NMR specturm, i.e., the chemical shift range characterizing the differences between chemically different kinds of carbons, is quite large (3). This translates into a well resolved, highly informative spectrum, far more informative than an ^{1}H NMR spectrum of the same system (4). Second, all of the complicated spin-spin scalar interactions which generally result in confusing multiplets in NMR spectra can be removed easily in a ^{13}C experiment by heteronuclear decoupling (2). This translates into a spectrum in which each observed line can be directly and simply associated with a magnetically unique carbon. Third, the spin-spin dipolar interactions which result in obscuring line broadening (especially for immobile or rigid materials) can also be experimentally removed readily by heteronuclear decoupling (5), by providing enough decoupling power (usually 10-100 times the power necessary for ordinary decoupling). The spectral simplification provided by dipolar decoupling translates into a spectrum in which every single type of carbon in the sample will give rise to an observable, and in most cases, characteristic NMR resonance.

Oil Composition of Intact Oilseeds

The first 2 of the 3 advantages mentioned above are illustrated by the results of an ordinary Fourier transform ^{13}C NMR experiment performed on an intact castor bean (Fig. 1). Under the conditions of this experiment, only the ^{13}C signal arising from the liquid like oil is observed; the signals from the other solid components are too broad to be observed. The resonance of the carboxyl carbon appears at the left hand side of the spectrum. This line has the highest frequency separation from an external reference, the latter

not shown. The resonances from the olefinic carbons appear at somewhat lower frequency separation, while the acyl carbon resonances appear slightly to the right of the center of the spectrum, and the intense group of lines arising from the methylene carbons appear at still lower frequency separation. The methyl carbon resonance is at lowest relative frequency separation of all at the extreme right of the spectrum (6). To a large extent, almost all of the chemically unique carbons of the ricinoleic acid of the castor bean can be individually identified in this ^{13}C NMR spectrum. In short, the spectrum is well resolved, easily interpreted, and highly informative.

A complicating feature of pulsed NMR experiments is also illustrated in Figure 1. The relative intensities of some of the lines (and, hence, determinations of relative concentrations of the various components) are often dependent on the rate at which radio frequency pulses are applied to the sample. We will postpone a discussion of this relaxation phenomena until later, when the results of some relaxation experiments on model systems have been introduced.

The dependence of the ^{13}C resonance frequency of a carbon to subtle changes in molecular structure is illustrated in Figure 2. The substitution of a hydroxyl group for a proton on going from oleic acid to ricinoleic acid not only drastically changes the resonance frequency of the carbon directly involved with the substitution, but also substantially affects carbons several covalent bonds distant. It is just this dependence on small changes in molecular environment which allows a ^{13}C NMR experiment to identify the different kinds of fatty acids present in an intact seed. Naturally, there is a limit to the differences which can be distinguished. Thus, the differences arising, for example, from various distributions of fatty acids among the 3 ester positions of a mixed triacylglycerol cannot be observed in the ^{13}C NMR spectrum of an intact seed.

In addition to identifying the presence of different kinds of fatty acids, a ^{13}C NMR experiment also must be able to establish quantitatively relative concentrations of the oil components to be a useful analytical tool. This capability is illustrated by the comparison of some methylene carbon spectra of radish and soybean seeds shown in Figure 3 (6). Even though radish and soybean have the same kinds of fatty acids, these acids are present in significantly different concentrations, which can be established, in part, by comparison of the relative intensities of lines 3, 5, and 6, for example. The intensity measurements are made best by a computer simulation of the overlapping lines of the spectra, using known line positions and line shapes as inputs, as well as various constraints established by intensity relationships between resonances arising from single carbons of a given fatty acid. Once a reasonable fit of the simulated spectrum to the experimental spectrum has been obtained, using either a visual or some least squares criterion, the intensity measurement can be made by measuring the areas under the various totally resolved lines. Oil composition of radish and soybean determined in this way by ^{13}C NMR are in agreement with chromatographic analyses to within a few percent (6).

As mentioned earlier, the determination of relative concentrations in a pulsed NMR experiment can be complicated by the rate at which the radio frequency pulses are applied to the sample (1,7). Some of the lines of pure linoleic acid, for example, are reduced in intensity by more than a factor of 2 when the pulse rate is changed from 1 per 10 sec to ca. 17 per 10 sec (Figure 4). These differences in intensities reflect differences in the individual rates of relaxation for different carbons, which, in turn, are determined by differences in the microscopic dynamics of molecular rotational reorientations (1). Of course, fast pulsing rates are generally desirable, so that time averaging can be performed quickly, and a large number of samples can be analyzed in as short a time as possible.

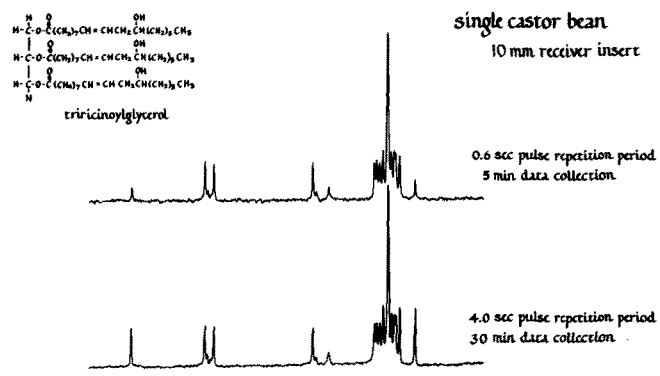

FIG. 1. Carbon-13 NMR spectra of an intact castor bean.

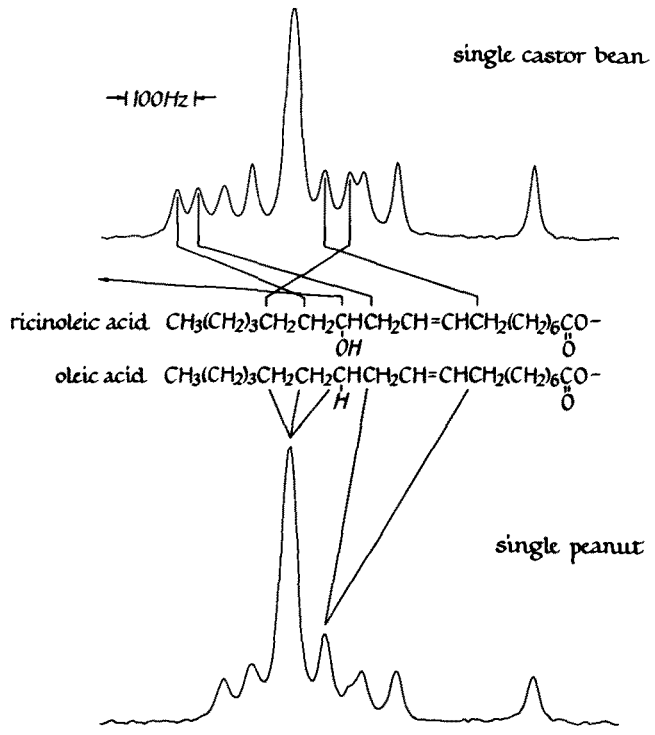

FIG. 2. The methylene and methyl carbon regions of the ^{13}C NMR spectra of 2 kinds of intact oilseeds.

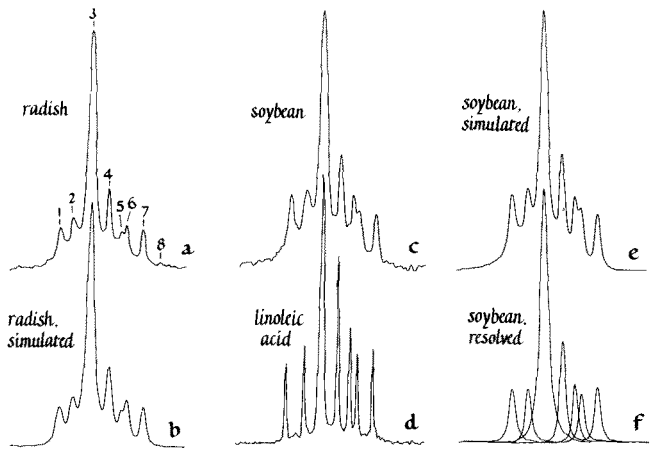

FIG. 3. The methylene carbon region of the ^{13}C NMR spectrum of (a) radish seed, (b) its computer simulation, (c) soybean, (d) linoleic acid, (e) the computer simulation of the soybean spectrum, and (f) the spectral resolution of the simulated spectrum of the soybean.

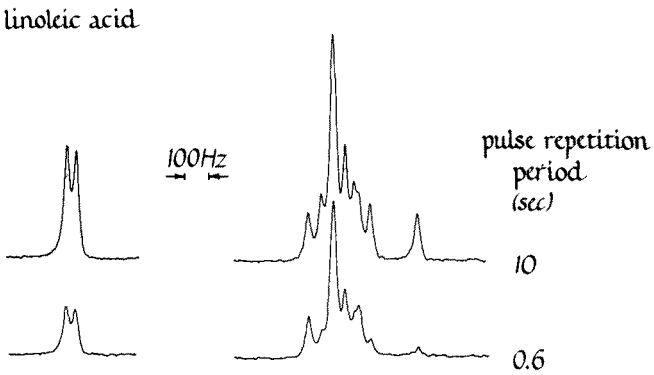

FIG. 4. The olefinic (left), methylene, and methyl carbon regions (right) of the 13C NMR spectra of pure linoleic acid under 2 kinds of pulse repetition conditions. The resolution of the spectra has been matched to that normally observed for oil in intact corn kernels.

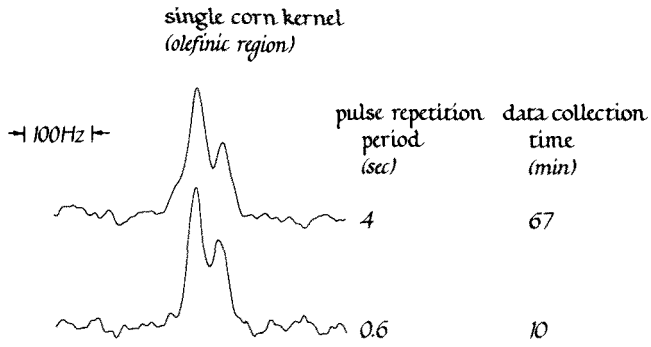

FIG. 5. The olefinic carbon region of the 13C NMR spectra of an intact corn kernel under 2 kinds of pulse repetition conditions. The resolution of these spectra of a corn kernel is poorer than the corresponding spectra of a castor bean. In general, the resolution of spectra of dry, low oil content seeds is poorer than that of either wet or oily seeds.

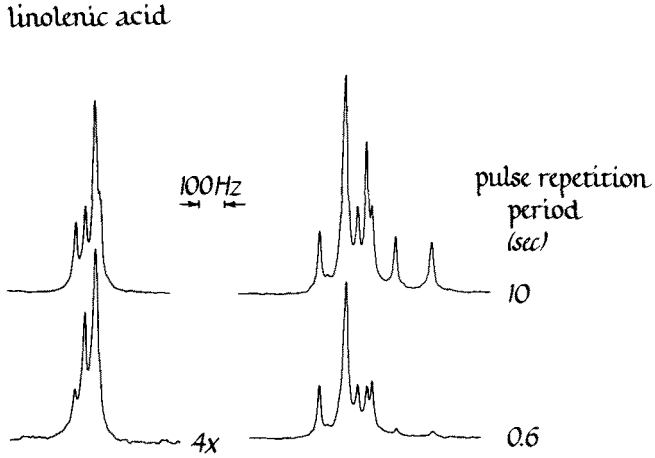

FIG. 6. The olefinic (left), methylene, and methyl carbon regions (right) of the 13C NMR spectra of pure linolenic acid under two kinds of pulse repetition conditions. The resolution of the spectra has been matched to that normally observed for oil in dry, intact soybeans.

Fortunately, it is almost always possible to find lines in the spectrum whose relative ratios do not change with changing pulse rate. These lines are then suitable for the determination of relative concentrations of component fatty acids. Thus, the ratio of the olefinic lines of a single corn kernel are effectively independent of the pulse repetition rate (Figure 5). Actually, even if these 2 lines showed a small dependence on pulse rate, this problem could be handled by the use of suitable scaling factors determined in separate experiments on seeds whose composition was determined later by a separate chromatographic analysis. In other words, the experimental ratio, r, of the 2 olefinic lines in the corn spectrum can be attributed to the concentrations, c_1 and c_2, of oleic and linoleic acids, respectively (6). The olefinic carbons of oleic acid contribute to the low field line and have a relaxation behavior which can be described by a scaling factor, a_1. The olefinic carbons of linoleic acid contribute to both lines. Those contributing to the low field line have a relaxation behavior described by a scaling factor, a_2, and those to the high field line by a_3. When a fast pulsing rate is used, $r = (a_1c_1 + a_2c_2)/a_3c_2$. The only limitation on the pulse rate is that severe spectral intensity distortions must be avoided. The scaling factors are evaluated at the rapid pulse rate, and the ratio of oleic to linoleic acid in corn is then $(r - a_2/a_3)(a_3/a_1)$.

An exception to the applicability of this simple scaling procedure is provided by linolenic acid. The spectrum of pure linolenic acid (with resolution adjusted to match that generally found in dry, intact seeds) is shown in Figure 6. All of the linolenic acid resonances are significantly and characteristically dependent on the pulse rate. In particular, the line on the extreme left of the spectrum is more sharply reduced in intensity under rapid pulsing conditions than the other olefinic carbon lines. Thus, even though this line is unique to linolenic acid, and can be used directly to determine the concentration of linolenic acid in intact soybeans (6), for example, it is usually not observable in any experiment with a pulse repetition period much shorter than about 1 sec. This complicates the efficient, rapid screening of soybeans for linolenic acid content, a procedure which realistically must probably involve some kind of a combination ^{13}C NMR chromatographic analysis, as discussed previously in detail (6).

The total time required to obtain a usable ^{13}C NMR spectrum of a single, intact oilseed is determined by the inherent sensitivity of the spectrometer. This sensitivity is crucially linked to the geometry of the receiver coil. The receiver coil can be thought of as the antenna of the radio detection system of the spectrometer. The most efficient coupling between the sample and the receiver coil occurs when the sample just fills the internal volume of the coil. Under these conditions, a high quality spectrum can be obtained for most oilseeds, even those having diameters as small as 2-3 mm, in times on the order of 10 min. An example of the kind of spectrum obtained from a single soybean in 20 min, using a 10 mm receiver coil, has been shown in a previous paper (6). If a slightly smaller receiver coil diameter of about 7 mm were used, a spectrum of this caliber could be obtained in about 5-10 min. However, when a substantially worse match between the coil geometry and the sample is made by placing a 6mm diameter soybean in a 15mm diameter receiver coil, for example, the time required to obtain a usable spectrum increases from a few minutes to several hours (6). Thus, a proper match is essential. As a practical matter, it is not realistic to suppose that a receiver coil can be changed to meet slight size variations from seed to seed in order to ensure optimum sensitivity. Nevertheless, it is still important to realize that the coil geometry should be chosen so that unnecessarily oversized receiver coils are avoided, thereby avoiding gross losses in sensitivity.

The motivation for performing these ^{13}C NMR experiments on single, intact oilseeds is, of course, to select seeds of desired oil composition for use in breeding programs designed to improve oil quality. Not only is the NMR analysis nondestructive, with no effect on the viability of the seed, it also leaves the seed coat intact. Thus, selected seeds need not be germinated immediately, but can be stored and germinated at some later date at the breeder's convenience, perhaps under optimum field growing conditions.

Total Composition of Intact Oilseeds

So far we have discussed how the high resolution and simplicity of a ^{13}C NMR spectrum can be used to advantage in determining the oil composition of an intact oilseed. These spectra are obtained by what today can be considered completely routine operations. There are, however, some special NMR techniques which can be employed in order to obtain moderately well resolved ^{13}C NMR lines from the rigid protein and carbohydrate components of the oilseeds as well. Spectra obtained under these conditions should ultimately permit the determination of the overall chemical composition of an intact oilseed, and in times on the order of minutes.

Under standard spectrometer conditions, the resonances of the rigid protein and carbohydrate components of the oilseeds are not observed. This is due to the dipolar broadening of the ^{13}C lines of the rigid components by directly bonded or nearby protons. The resonances of the rigid components in the solid state are simply too broad to be observed in a standard experiment. This static dipolar broadening can be removed by strong ^{1}H decoupling (5,8), which we refer to as dipolar decoupling. Dipolar decoupling involves the intense radio frequency irradiation of the ^{1}H spins in the sample, thereby removing the effects of the dipolar coupling between carbon and proton spins. The irradiation of the protons, in effect, stirs the spins and has a result not dissimilar from the line narrowing obtained from increased molecular motion (5). Dipolar decoupling requires about 100 times as much power as ordinary scalar decoupling (and so can lead to some overloading and noise problems in the ^{13}C receiver), but removes static dipolar interactions in the same way weaker decoupling removes weaker scalar interactions. The experiment is not very subtle; it is simply a matter of brute radio frequency force.

Examples of dipolar decoupled spectra of some common seeds, along with some other materials of biological interest have been shown previously (9). As discussed there, the resonances due to the proteins and carbohydrates in the seeds can be identified by comparison with the spectra of various isolated materials. Because the major resonances of protein and carbohydrate components occur at different frequencies, it is clear, in principle at least, that the relative concentrations of each can be determined from such spectra.

Actually, a major limitation of the usefulness of this dipolar decoupled spectra (9) is that the oil lines tend to dominate or obscure the weaker, broader lines in the spectra, thereby complicating the determination of the concentrations of the rigid components in the seeds. This limitation can be overcome, at least in part, by performing what is known as as a cross polarization ^{13}C NMR experiment. This experiment also involves dipolar decoupling, but now relies for the polarization of the carbons, i.e., the establishment of a net carbon magnetization which can be detected by the receiver, not upon ordinary means of relaxation, but instead upon direct interactions with immobilized protons in the sample. The net result is that the observed spectrum discriminates against the mobile components of a mixed phase system such as an oilseed, and gives, instead, a much clearer picture of the rigid components. Some examples of some cross polarization ^{13}C NMR spectra obtained in our laboratory are shown in Figure 7.

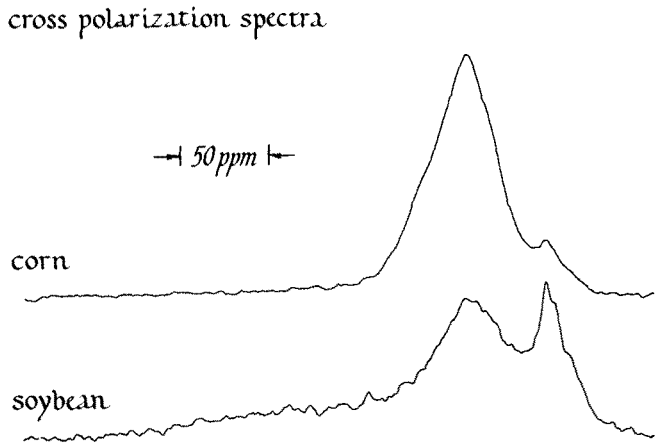

FIG. 7. Cross polarization ^{13}C NMR spectra of intact corn and soybean. (1 ppm = 22.6 Hz.)

The high protein concentration of the soybean is most clearly shown in the broad resonance at the left hand side of the spectrum, while the high starch concentration of the corn kernel is evident in the intense resonance just to the right of the center of the spectrum. Interferences from intense oil lines are not present in these spectra. The way in which cross polarization spectra are calibrated, and the extent to which immobilized, rigid lipids can contribute to such spectra are beyond the scope of this review (8).

A major source of residual line broadening in both dipolar decoupled and cross polarization spectra of seeds is due to what is called chemical shift anisotropy (8). The broadening is especially pronounced for asymmetrically substituted carbons, but has been shown both theoretically (10) and in practice (11) to be subject to removal by high speed, mechanical sample spinning This spinning can produce ^{13}C NMR spectra in which the resonances arising from the rigid components are far better resolved and richer in detail than those shown in Figure 7. Such spectra, therefore, ultimately will be well suited for the analysis of the total composition of an intact oilseed.

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