Carbon-13 Nuclear Magnetic Resonance Measurement of Oil Composition in Single Viable Soybeans

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ABSTRACT

High-resolution, natural abundance ^{13}C NMR spectra of intact, viable soybeans have been obtained by Fourier transform techniques. The spectra are interpreted in terms of the relative concentrations of the major fatty acids present in the soybean triglycerides. This nondestructive analysis is sufficiently fast and accurate to permit the selection of individual soybeans for use in a genetic breeding program designed to reduce the undesirably high linolenic acid content of soybean oil.

INTRODUCTION

The poor flavor and flavor stability of soybean oil have been attributed to the presence of substantial amounts of linolenic acid (1), a $C_{18:3}$ fatty acid which is part of the triglyceride reserves of the soybean. Because of the increasing importance of soybeans for human consumption (in terms of both protein and oil content), efforts have been made to develop a hybrid soybean which is low in linolenic acid (2). These efforts have been hampered by the fact that the soybean paternal parent appears to have some influence in determining the amount of linolenic acid present in the resulting hybrid (unlike the situation for oleic and linoleic acids [3]). Therefore, an efficient breeding program requires the nondestructive selection of beans on the basis of composition (4). In the past, this capability was not available.

The use of natural abundance ^{13}C NMR spectroscopy now provides this capability. Carbon-13 NMR permits the nondestructive quantitative analysis of the triglyceride composition of individual soybeans.

Essentially high-resolution ^{13}C NMR spectra of fatty acids or lipids have been observed in heterogeneous or partially immobilized systems in studies of membranes, lipid bilayers, and nerve tissues (5). Because of their local motional flexibility, the lipids have spectra which are reasonably well resolved, even though they may be present in what amount to solid-like materials. Well resolved ^{13}C NMR spectra are observed in seeds, despite the fact that the corresponding ^{1}H NMR spectra are poorly resolved. Thus, while the ^{1}H resolution may be adequate to determine, for example, the total oil content of corn kernels (4), and in some cases the degree of unsaturation of the oil fraction (6), it is inadequate to distinguish between different types of unsaturated fatty acids. The difference in resolution between the two kinds of spectra can be attributed to the simplicity of the ^{13}C NMR spectra in which all scalar spin-spin coupling can be removed, in which the range of chemical shifts, i.e. the separation of lines, is increased by a factor of ca. 30, and in which line broadening due to intermolecular dipolar interactions is not important (7). Furthermore, the ^{13}C measurements generally are made at a substantially lower radio frequency than comparable ^{1}H NMR measurements, and so line broadening, due to magnetic susceptibility variations within the heterogeneous material, is a much less important effect (6). All of these factors combine to permit the ^{13}C NMR spectrum of a single, intact viable soybean to be used for the nondestructive quantitative analysis of its oil composition.

EXPERIMENTAL PROCEDURES

High-resolution, natural abundance ^{13}C NMR spectra were obtained at 22.6 MHz and 25 C by standard pulsed techniques (8) using a Bruker HFX spectrometer, some details of which have been described earlier (7). Repetitive, intense radio frequency pulses excited the ^{13}C spin system; and the resulting NMR transient responses, or free induction decays, were digitized and accumulated in a Nicolet 1074 time averaging computer. After a suitably strong signal had been accumulated, a Fourier transform was performed by a Digital Equipment Corp. PDP-8/I, a small laboratory computer which was interfaced to the Nicolet 1074. This calculation produced an absorption NMR spectrum (8) whose line positions and intensities could be interpreted in the usual way, assuming the spacing of the repetitive pulses was sufficiently long (9). Some experiments were performed using a Nicolet 1085 data system, a laboratory computer which performs the function of both the 1074 and the PDP-8/I, as well as provide greater storage and programing capabilities. In addition, these experiments employed a quadrature detector (10) capable of simultaneously detecting the absorption and dispersion of the ^{13}C magnetization, and an extremely low-noise preamplifier (Avantek, Santa Clara, Calif.) which replaced the standard Bruker preamplifier. The last two changes produced a 2-1/2-fold improvement in the sensitivity of the receiver. Spectra were obtained using both 15 and 10 mm diameter receiver coils. All ^{13}C NMR spectra were obtained under conditions of proton decoupling, so that each line in the ^{13}C spectrum represented a magnetically unique chemical environment (5).

Pulsed Fourier transform measurements were performed on intact soybeans (*Glycine max* L. cv. Dare and cv. Wayne), radish seeds (*Raphanus sativus* L.), and on various neat fatty acids (Sigma Chemical Co., St. Louis, Mo.). Pulse repetition rates of 0.5-2.0 sec were used in measurements on soybeans and radish seeds and 10.0 sec on the more slowly relaxing neat fatty acids. Data accumulation times varied from 10 hr for a single soybean experiment using a 15 mm diameter receiver coil and a standard detector to 20 min for a single soybean experiment using a 10 mm diameter receiver coil and a high-sensitivity quadrature detector to ca. 2 min for the neat fatty acids and the standard receiver system. Experiments performed on intact seeds were made with the seed suspended in a dry, inert powder of poly(vinyl chloride). This technique ensured high resolution NMR measurements by minimizing magnetic susceptibility variations within the sample coil, while at the same time not affecting the viability of the intact seed.

The partially overlapping peaks of the ^{13}C NMR spectra of the soybean and radish seeds were resolved totally using a DuPont 310 curve resolver, an analogue computer which generates a series of component peaks and synthesizes a sum curve matching the original data. The vinyl-carbon region of the NMR spectra of the seeds was assumed to consist of three Lorentzian lines (with slightly broadened bases) and the methylene-carbon region of eight. The quality of the fit was estimated visually; and the sum curve plotted, as well as each of the individual components. Integrated intensity measurements were made on the individual component lines.

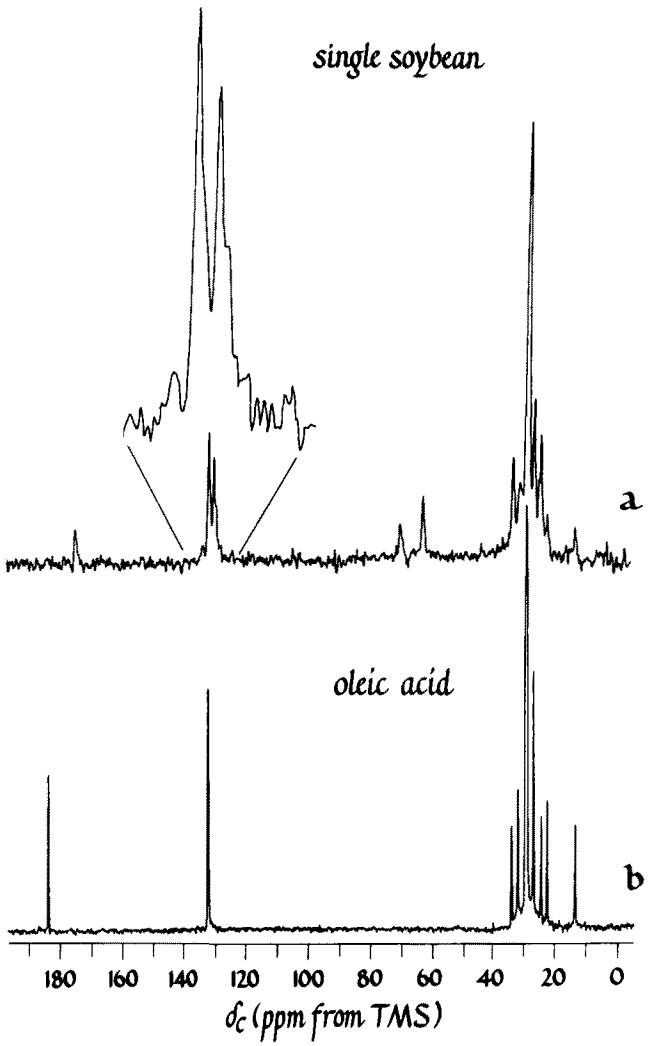

FIG. 1. Natural abundance ^{13}C NMR spectra of (a) one intact soybean (cv. Dare) and (b) neat oleic acid. The high-resolution spectrum of the solid soybean is due exclusively to the triglycerides in its oil fraction. Tetramethylsilane (TMS) is the external reference.

RESULTS

The ^{13}C NMR spectrum of a single, viable soybean is shown in Figure 1. The carboxyl-carbon line appears at ca. 170 ppm from tetramethylsilane, those from the vinyl, i.e. olefinic, carbons at 130 ppm, the lines due to the glycerol methine- and methylene-carbons appear at ca. 70 ppm, the methylene carbons of the fatty acid chains of the glycerides are centered ca. 30 ppm, and the line due to the terminal methyl carbon of the fatty acids appears at ca. 17 ppm from tetramethylsilane. For comparison, a fully relaxed spectrum of neat oleic acid ($C_{18:1}$) under comparable conditions of resolution also is shown in Figure 1.

Computer simulation and spectral resolution of the three line vinyl-carbon region of a typical, soybean spectrum are shown in Figure 2. There are only three major unsaturated fatty acids in soybean triglycerides: oleic, linoleic, and linolenic acids (11). Oleic acid ($C_{18:1}$) has 2 vinyl carbons, both of which contribute to the center line, or line 2 (Fig. 2). Linoleic acid ($C_{18:2}$) has 4 vinyl carbons, 2 of which contribute to line 2 and 2 of which contribute to line 3. Finally, linolenic acid ($C_{18:3}$) has 6 vinyl carbons which contribute to lines 1, 2, and 3 in the ratios of 1:1:4, respectively. These assignments are made easily by comparisons of the spectra of the isolated acids. From Figure 2, the observed integrated intensities of the 3 soybean lines are 0.028, 0.528, and 0.444, respectively. These values lead directly to the determination of the relative concentrations

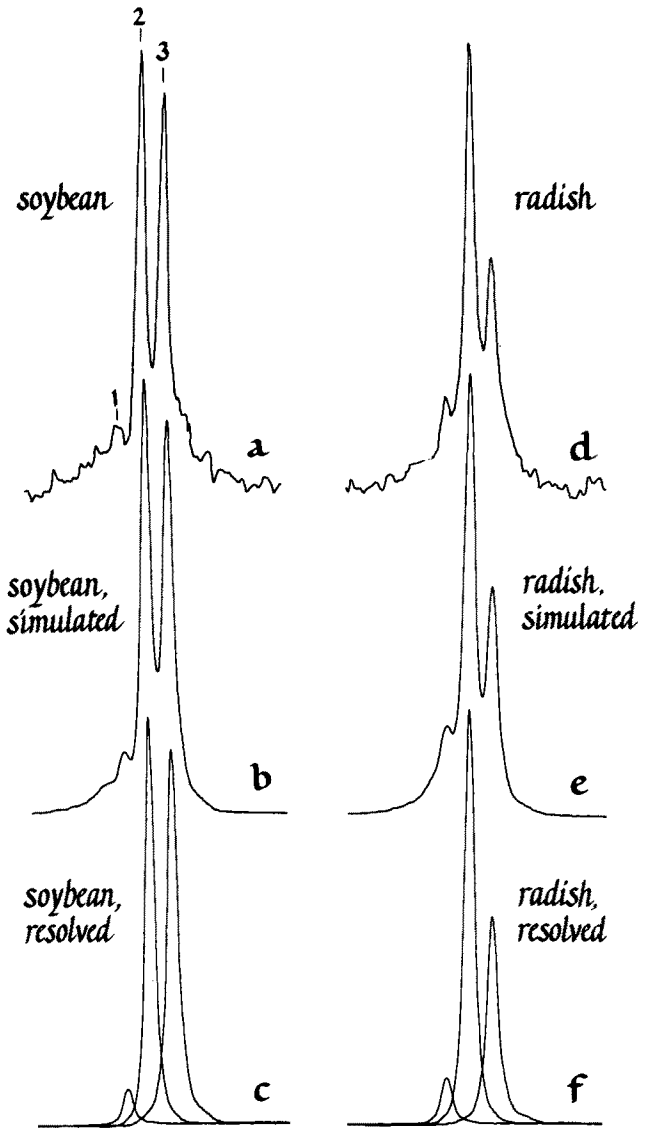

FIG. 2. The vinyl-carbon region of the ^{13}C NMR spectrum of (a) intact soybean along with (b) its computer simulation and (c) the spectral resolution of the simulation. The corresponding spectrum, computer simulation, and spectral resolution for intact radish seed are shown in d, e, and f, respectively.

of oleic, linoleic, and linolenic acids of 0.30, 0.60, and 0.10, respectively. These values compare favorably with the chromatographically determined (11) values of 0.33, 0.57, and 0.10, respectively. Of course, the chromatographic analysis is based upon extracted oil and, hence, is a destructive analysis. This agreement is representative of the kind of agreement obtained between chromatographic results and NMR analysis in over 50 experiments.

For comparison, a similar ^{13}C NMR spectrum and computer simulation of intact radish seeds are also shown in Figure 2. Radish seeds are much richer in linolenic and singly unsaturated acids than soybean but contain far less linoleic acid (12). Differences in line intensities are pronounced, as expected. The only effect upon the spectra of having performed the NMR experiment on solid seeds is an occasional slight broadening of the bases of the lines (Fig. 2a and d), which is readily taken into account in the computer simulations of the spectra.

Computer simulation and spectral resolution of the methylene-carbon region of a typical soybean spectrum are shown in Figure 3 (along with the spectra of radish seeds and neat linoleic acid, for comparison). There is a total of eight lines in this region, seven of which are at least

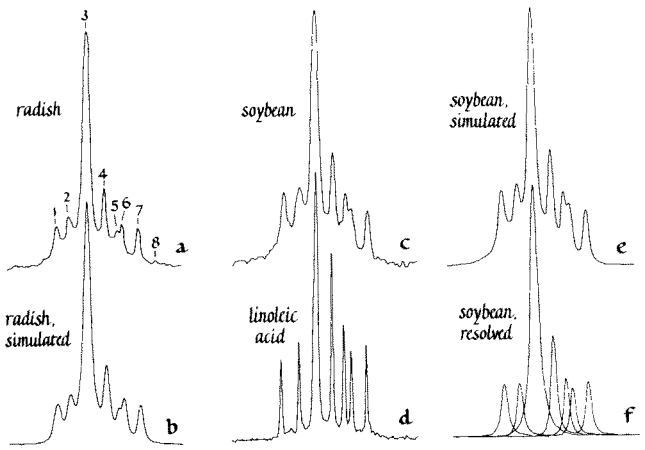

FIG. 3. The methylene-carbon region of the ^{13}C NMR spectrum of (a) radish seed, (b) its computer simulation, (c) soybean, (d) linoleic acid, (e) the computer simulation of the soybean spectrum, and (f) the spectral resolution of the simulated spectrum of the soybean.

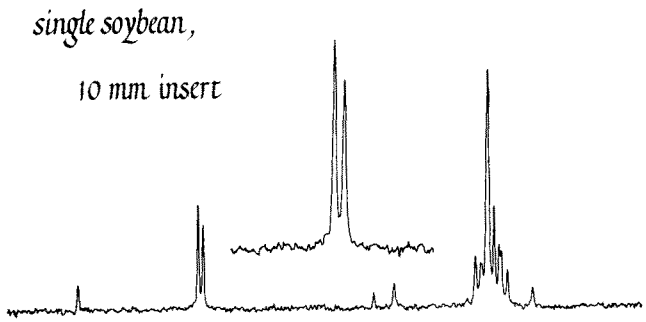

FIG. 4. Natural abundance ^{13}C NMR spectrum of an intact soybean (cv. Wayne) obtained in 20 min using a 10 mm diameter receiver coil and a high-sensitivity quadrature detection system.

partially resolved in most soybean spectra. One way in which the relative concentration of saturated fatty acid in soybean triglycerides can be determined is by the ratio of intensities of lines 3 and 4 (Fig. 3), together with a knowledge of the unsaturated acid composition, as determined by analysis of the vinyl-carbon region of the same ^{13}C NMR spectrum. The only major saturated fatty acid in soybean is palmitic ($C_{16:0}$). Palmitic, oleic, linoleic, and linolenic acids have 10, 8, 5, and 4 carbons, respectively, which contribute to line 3; and they have 0, 2, 2, and 1 carbons, respectively, which contribute to line 4. Again, this can be determined in a straightforward way by comparisons of the spectra of the separate components. From Figure 3, the observed ratio of integrated intensity of line 3 to that of line 4 is 3.80, which leads to a determination of the concentration of palmitic acid, relative to the sum of the unsaturated acids, of 0.12, compared to the chromatographically determined value of 0.15. A somewhat more elaborate analysis of the methylene-carbon region would take into account the presence of the small amounts of stearic acid usually found in soybeans (11). In any event, the major fatty acids of intact, viable soybeans clearly can be determined to within a few percent by the separate use of the relative intensities of the vinyl- and methylene-carbon natural abundance NMR spectra.

An example of the kind of sensitivity that can be achieved in a single-soybean ^{13}C NMR experiment is shown in Figure 4. This spectrum was obtained using a 10 mm diameter receiver coil and a high-sensitivity quadrature detector and required only 20 min of data accumulation time.

DISCUSSION

A single soybean weighs ca. 200 mg, of which some 20% is oil, and has a diameter of ca. 6 mm. The most desirable geometry for the sensitive detection of the natural abundance ^{13}C NMR signal of a soybean involves a reeceiver coil with a 6 or 7 mm diameter. The coil used to obtain the single soybean spectrum of Figure 1 was 15 mm in diameter, far from the optimum. As a result, 10 hr of data accumulation were required to obtain a satisfactory signal to noise ratio. The less noisy soybean spectra of Figures 2 and 3 were obtained from 4 soybeans after 1 hr of data accumulation. Since the soybeans filled only ca. half the space available in the 15 mm coil and since a standard detector and receiver were used, we estimate that the sensitivity of this experiment is comparable to that of a single soybean in a 6 mm coil using a high-sensitivity quadrature detector. In addition, a pulse repetition rate of 2 sec was used to obtain the soybean spectra of Figures 2 and 3 to ensure that all the vinyl- and methylene-carbon lines had close to full intensity (9). However, waiting 2 sec between pulses was not really necessary. Just as for the single soybean spectrum of Figure 1, a fourfold faster pulse repetition rate of 1/2 sec can be used which will still allow most vinyl and methylene carbons to display representative relative intensitites when compared to other vinyl and methylene carbons, respectively (9). (Exceptions include lines 2 and 5 of the methylene region.) This is especially true if, as was done in these experiments, the ^{1}H decoupling field is not allowed to heat the soybean sample and so adversely affect relaxation times and observed relative intensities. (Of course, since the relaxation times are approximately equal for all fatty-acid carbons with the same resonance frequency, intensity scaling factors could be determined for each line in a soybean spectrum. This procedure would reduce intensity variations resulting from a rapid pulse repetition rate.) All of these results together suggest that an optimum sensitivity experiment performed on a single soybean should require only a modest expenditure of time for data accumulation.

An experiment which we consider close to the optimum is illustrated by the single soybean spectrum of Figure 4. A pulse repetition time of 0.6 sec was employed and resulted in a total data accumulation time of 20 min. The much improved sensitivity of this experiment, compared to that illustrated in Figure 1, confirms the fact that as the coupling between the receiver coil and the soybean sample is improved, the length of time required to obtain a usable ^{13}C NMR spectrum is reduced dramatically. In fact, we feel the optimum sensitivity experiment, using quadrature detection, on a single soybean can produce a high-quality ^{13}C NMR spectrum in ca. 10 min. Since we have shown that the major fatty acids of intact, viable soybeans can be determined by the analysis of their ^{13}C NMR spectra, one spectrometer, fitted with a 6 or 7 mm coil, should be able to screen ca. 100 soybeans a day for desirable oil composition. This is a sufficiently large number to make feasible a genetic breeding program for the improvement of soybean oil quality by the reduction of the linolenic acid content.

The estimate that 100 soybeans/day can be screened by a ^{13}C NMR spectrometer assumes that the spectrometer will be operated over a 2 shift 16 hr day and that the raw data produced by the spectrometer system can be reduced by an off-line computing facility, so as not to delay the data collection itself. Neither of these two conditions seems difficult to meet. However, as a practical matter, to screen as many as 100 soybeans a day by ^{13}C NMR for linolenic acid content, use probably should be made of a chromatographic determination of the oleic and linoleic acid contents of a few beans, carefully chosen to be representative

of a given plant. Since these two unsaturated acids are dominated genetically by the maternal parent (3), the chromatographic analysis need not be repeated for other selected beans of that particular plant. Knowing (to a good approximation) the oleic and linoleic acid contents of a single bean, one can determine the linolenic acid content, which, in general, depends upon both parents (3) and is not known from the chromatographic analysis, from the ratio of lines 2 and 3 in the ^{13}C NMR vinyl-carbon region. Since these are both strong lines of comparable intensity and relaxation behavior and since as much as one-fourth of the intensity of line 3 arises from linolenic acid, the determination of the linolenic acid content (to within ± 10%) is practical using spectra of the quality of that of Figure 4. If ^{13}C data alone were to be used in the determination of the unsaturated acid composition of a single bean, all three lines of the vinyl-carbon region must be used (see "Results"). Then, since line 1 of the vinyl-carbon region is weak and has an integrated intensity which is relatively difficult to determine, either more time must be spent in obtaining a spectrum with a better signal to noise ratio than that of Figure 4; or a two- or threefold less accurate determination of the linolenic acid content must be tolerated.

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