Non-destructive Analysis of the Oil Composition of Soybean Seeds by Natural Abundance Carbon-13 Nuclear Magnetic Resonance Spectroscopy

Mitsuru YOSHIDA, Hiromi KANO,* Nobuaki ISHIDA**
and Takashi YOSHIDA***

*National Institute of Agro-Environmental Sciences, Kannondai 3-1-1,
Tsukuba, Ibaraki 305, Japan*

** National Institute of Agrobiological Resources, Kannondai 2-1-2,
Tsukuba, Ibaraki 305, Japan*

*** National Food Research Institute, Kannondai 2-1-2,
Tsukuba, Ibaraki 305, Japan*

**** National Institute of Vegetables, Ornamental Plants and Tea,
Kusawa, Ano, Mie 514-23, Japan*

Received December 22, 1988

The signals of fatty acids in the form of triglycerides were observed in the ^{13}C NMR spectrum of an intact soybean seed. The major fatty acid component composition of triglycerides in a soybean seed, which includes linoleic acid, oleic acid and palmitic acid, was estimated by subtracting the spectra of authentic fatty acids from the spectrum of the intact soybean seeds. The fatty acid compositions of seeds of 11 Japanese soybean cultivars and 5 lines bred at the Asian Vegetable Research and Development Center (AVRDC) were estimated by this rapid (within 1 hr for one seed) and non-destructive analytical method.

Soybeans are one of the major crops for oil production. Recently, since the unsaturated fatty acids in vegetable oils have become regarded to be healthy, the fatty acid composition of soybean oil is an important consideration for breeding. And the rapid analysis of the fatty acid composition of the progeny is necessary. Moreover, non-destructive analysis is desirable, because the number of one progeny, which is to be grown in the field to examine its inherited nature, is usually very small.

Nuclear magnetic resonance (NMR) spectroscopy is considered to be a promising technique for such a purpose. NMR, which has been used in various physiological studies on microorganisms, cultured cells, tissues, organs and whole bodies of animals,¹⁾ provides information on the soluble compounds in cells without destruction of the cells by homogenization or solvent extraction. The spectra of plant tissues and seeds have also been pub-

lished, the signals of major soluble compounds being observed.^{2~8)} Schaefer and Stejskal²⁾ reported the detection of signals due to unsaturated fatty acid components in the ^{13}C NMR spectra of intact soybean seeds and showed the possibility of the application of the ^{13}C NMR method to oil composition analysis. In the current investigation, the compositions of fatty acids in various soybean cultivars and breeding lines were analyzed by essentially the same rapid non-destructive analytical technique as that described by Schaefer and Stejskal²⁾ with appropriate modifications; subtracting the spectra of authentic fatty acids, including saturated fatty acid, from the spectra of intact seeds, using ^{13}C NMR.

Materials and Methods

Cultivation of soybeans. Various soybean (*Glycine max*) cultivars were sown on June 20, 1983, in an open field at the National Agriculture Research Center, at the density

of 20 plants/10 m². N (3 kg/10 a), P₂O₅ (10 kg/10 a), K₂O (10 kg/10 a), calcium carbonate (120 kg/10 a) and compost (1100 kg/10 a) were applied as the basal dressing. Cultivation was carried out by the conventional method. Seeds were harvested in October at a suitable stage of seed maturation.

¹³C NMR spectrum measurement. A mature soybean seed was put into a 10 mm NMR sample tube and then the ¹³C NMR spectrum was measured with a JEOL FX-90Q spectrometer. External D₂O was used for the magnetic field lock. A one second repetition time with a 14.7 μsec (60°) pulse width was employed, in the complete proton noise decoupling (COM) mode. A spectrum was obtained by 3000 times accumulation with a FID. A broadening window of 12 Hz was used for the Fourier transformation. The chemical shift was determined, assuming the signal of dioxane to be 67.4 ppm. The spectra of authentic fatty acids, which were used as standard spectra for the subtraction, were measured using a 60.8 sec repetition time with a 45° pulse, in the non-NOE (NNE) mode. An appropriate broadening window was used in order to adjust the line width of the signals to those in the case of soybean seeds when the spectrum subtraction was carried out.

Measurement of fatty acid compositions by gas-chromatographic analysis. Soybean seeds were ground into powders and then extracted with a chloroform-methanol (2:1) solution. After the solvent had been evaporated off completely, the extracted fatty acids were dissolved in chloroform and then passed through a SEP-PAC silica column to remove phospholipids derived from membranes. Then the fatty acid composition was analyzed by gas-chromatography after methylation.

Results

¹³C NMR spectra of intact soybean seeds

The ¹³C NMR spectra of seed of a Japanese soybean cultivar and two lines bred at the Asian Vegetable Research and Development Center (AVRDC) are shown in Fig. 1. The sharp signals of fatty acids, which were considered to be present as triglycerides in oil droplets in the lipid cells,^{9,10)} were detected. The assignment of these signals to individual groups is described in the figure. The spectral patterns reflected the fatty acid compositions of the cultivar and lines. There were prominent differences among the soybean seeds, especially in the *sp*² carbon region, which represented the unsaturated fatty acids present. The signal at 128.5 ppm was assigned to C₁₀ and

Fig. 1. ¹³C NMR Spectra of Seeds of a Japanese Soybean Cultivar and Lines Bred at the Asian Vegetable Research and Development Center (AVRDC).

A, Enrei; B, G 219; C, AGS 62.

Fig. 2. ¹³C NMR Spectra of Authentic Fatty Acids.

A, linoleic acid (neat); B, oleic acid (neat); C, palmitic acid (in CH₃Cl).

C₁₂ of linoleic acid (Fig. 2A). The signal at 130.2 ppm was assigned to linoleic acid C₉ and C₁₃ and oleic acid C₉ and C₁₀ (Fig. 2A, B).²⁾ On the other hand, the signal at 131.9 ppm of linolenic acid C₁₆, which was reported to be present in soybean seeds^{11~13)}, was not observed in the soybean spectra (Fig. 3). This suggested the low content of linolenic acid in the lipid body, though it was reported to be abundant in membranes.¹⁴⁾ Saturated fatty

Fig. 3. sp^2 Regions of the ^{13}C NMR Spectra of Authentic Fatty Acids and a Soybean Seed.

A, oleic acid; B, linoleic acid; C, linolenic acid; D, soybean seed.

acids were also expected to contribute to the soybean spectra in the sp^3 region from 14.6 to 34.4 ppm. But their signals were considered to overlap with those of the unsaturated fatty acids (Fig. 2C).

Estimation of fatty acid composition of soybean oil

Usually the non-NOE (NNE) mode with a long repetition time (approximately 5 times T_1 in order to avoid saturation) is employed for quantitative analysis by NMR.¹⁵⁾ However, this requires too much time to be adopted as an analytical method in the agricultural field. In this experiment, the complete proton noise decoupling (COM) mode with a short repetition time (1 sec) was used for soybean seeds to reduce the time required for the analysis, because of the high signal-noise ratio.

First the ratio of linoleic and oleic acids was determined from the sp^2 region of the spectrum. The intensities of the sp^2 carbon signals in the spectrum obtained with the COM mode with a short repetition time hardly differed from those with the NNE mode with a long (30 sec) repetition time (data not shown). Subtraction of the authentic linoleic acid spectrum from that of a soybean seed was carried out, until the signal at 128.5 ppm, which is characteristic of linoleic acid, decreased to the baseline (Fig. 4A, B). This was

Fig. 4. Spectral Subtraction of the sp^2 Region. A, soybean seed; B, A – linoleic acid; C, B – oleic acid.

Fig. 5. Spectral Subtraction of the sp^2 Region. A, soybean seed; B, A – linoleic acid; C, B – oleic acid; D, C – palmitic acid.

followed by subtraction of the authentic oleic acid spectrum from the subsequent spectrum, in which the signal at 130.2 ppm ascribed to oleic acid remained (Fig. 4B, C). The ratio of linoleic and oleic acids in seeds was calculated from the magnitude of the spectra of the authentic fatty acids used for subtraction.

As the next step, the saturated fatty acid content was determined by subtraction, using the methyl signal at 14.6 ppm. In the case of the signals of methyl and methylene groups, the short repetition time (1 sec) for rapid measurement decreased the signal intensity. The intensity of the methyl signal obtained with the COM mode decreased to 60% of that with a 30 sec repetition time in the NNE mode. Therefore, the spectral subtraction at the methyl group was carried out with the standard spectra at 60% magnitude (Fig. 5). The

spectrum of palmitic acid was used as the standard one for saturated fatty acids assuming all the saturated fatty acid in soybeans to be palmitic acid, because the amount of stearic acid in soybean oil is small compared with those of other fatty acids.^{11,12)} And the percentage of the saturated fatty acid was calculated from the magnitude of the palmitic acid spectrum used.

The fatty acid compositions of seeds of two soybean lines (G 219 and AGS 66), bred at the Asian Vegetable Research and Development Center (AVRDC), were determined by NMR in the complete proton noise decoupling (COM) mode and in the non-NOE (NNE) mode, and by the gas-chromatographic technique (Table I). The percentages of the saturated fatty acid which was assumed to be palmitic acid, and oleic acid determined by the three methods agreed well. Linolenic acid was detected on gas-chromatography, which was estimated to account for approximately 6% of the total fatty acids in G 219 and 11% in AGS 66. On the other hand, its characteristic signal was not observed in the sp^2 region of the ^{13}C NMR spectrum, probably because of the low resolution of the spectrometer used. Taking into account the signal shapes of unsaturated

fatty acids (Fig. 3), undetectable linolenic acid was anticipated to result primarily in overestimation of the amount of linoleic acid, with slight underestimation of oleic acid, *i.e.*, the amounts of linoleic and linolenic acid together accounted for that of linoleic acid estimated by the ^{13}C NMR method. Consequently, fatty acid compositions determined by ^{13}C NMR well agreed with the results obtained with the gas-chromatographic technique, though linoleic and linolenic acids could not be distinguished.

Fatty acid compositions of the oils of various cultivars and breeding lines of soybeans

The results of fatty acid composition analysis of eleven Japanese cultivars and three lines bred at the Asian Vegetable Research and Development Center (AVRDC), other than G 219 and AGS 66, by this ^{13}C NMR method are presented in Table II. The percentage of palmitic acid varied a little among the cultivars and lines. Unsaturated fatty acids accounted for from 80% to 90% of the oils, in which the oleic acid content varied widely from 2% to 34%. Accordingly, the linoleic acid content varied inversely as to the oleic acid content.

Table I. FATTY ACID COMPOSITIONS OF SOYBEAN SEEDS DETERMINED IN THE COM AND NNE MODES BY NMR SPECTROSCOPY AND GAS-CHROMATOGRAPHY

Lines	COM mode (%)	NNE mode (%)	Gas-chromatography (%)
G 219			
Linoleic	52.8 ± 2.7	48.5	46.8 (41.0)
(Linolenic)			(5.8)
Oleic	33.7 ± 2.8	34.0	40.0
Palmitic	13.5 ± 1.6	16.8	13.2 (11.0)
(Stearic)			(2.2)
AGS 66			
Linoleic	64.7 ± 3.7	66.7 ± 1.7	65.7 (55.0)
(Linolenic)			(10.7)
Oleic	21.3 ± 2.5	20.0 ± 1.4	20.8
Palmitic	13.7 ± 3.4	13.3 ± 1.3	13.5 (10.5)
(Stearic)			(3.0)

The figures in parentheses are the contents of individual fatty acids included in the figures to the left.

Table II. FATTY ACID COMPONENTS OF TRIGLYCERIDES IN DIFFERENT SOYBEAN CULTIVARS

Cultivar	Content (%)		
	Linoleic acid	Oleic acid	Palmitic acid
Enrei	79	10	11
Raiden	65	13	22
Bon-minori	66	12	22
Tamahikari	67	19	14
Fujimijiro	80	4	16
Tokachi-nagaha	57	22	21
Shirome-nagaha	72	17	11
Kakushin No. 1	73	5	22
Norin No. 1	61	29	10
Tsukui-zairai	76	12	12
Natto-shoryu	73	7	20
G 488 ^a	70	17	13
AGA 62 ^a	81	2	17
China ^a	70	8	22

^a Provided by the Asian Vegetable Research and Development Center.

The deviation of the linoleic acid–oleic acid ratio among six samples of the same cultivar, with this method, was within 7%, which was smaller than that among the cultivars and the lines.

Discussion

In the natural abundance ^{13}C NMR spectrum of an intact soybean seed, signals of fatty acids in the form of triglycerides were exclusively observed,^{2,16)} which are considered to be contained in the cells as oil droplets,^{9,10)} judging from their narrow line widths. The intensity of each signal provides quantitative information on the fatty acid component of the triglyceride. On the other hand, signals of other cell components, such as proteins, saccharides and nucleic acids, were not detected, due to the extreme broadening of the line width resulting from the low molecular mobility in dry seeds. This enabled us to estimate the fatty acid composition easily using the soybean spectrum.^{2,16)}

The major fatty acids in soybean triglycerides are linoleic, oleic and palmitic acids. The ratio of these fatty acids in the oil of soybean seeds was estimated by sequential subtraction of the spectra of the authentic fatty acids from the soybean spectrum. This method does not require destruction of samples and thus is valuable for analysis in connection with breeding. To shorten the measurement time to a practical level, we adopted complete proton noise decoupling and a short repetition time for the pulse (1 sec). The changes in the signal intensity due to NOE in the proton decoupling and to incomplete relaxation after the short repetition time were overcome by the introduction of appropriate factors in the subtraction procedure for the spectra. Because of the differences in the NOE and relaxation time of the carbon nuclei in individual groups, the signals of methylene clusters remained after completion of the subtraction procedure (Fig. 5D). This allowed great shortening of the time required for analysis and enabled us to finish a

measurement within 1 hr.

The results of fatty acid analysis by this method (Table I) coincided with those obtained with the usual method involving solvent extraction.^{11,12)} The soybean seeds were rich in linoleic acid, which accounted for 50% to 80% of the total oil, and oleic acid, 2% to 35%. The remaining 10% to 20% of the total oil was saturated fatty acid (Table II). Linolenic acid, an unsaturated fatty acid, was present in soybean oil,^{11~13)} however, its characteristic signal was not observed in the sp^2 region (Fig. 3). The amount of linolenic acid was included in that of linoleic acid, resulting in an apparent increase in the amount of linoleic acid. Also, the amount of stearic acid is small, though it is present in soybean oil together with the fatty acids mentioned above.^{11,12)} Therefore, the minor fatty acid, stearic acid, was neglected in this analysis. But the ratio of linoleic including linolenic, oleic and palmitic acids is still a useful index for the breeding to crops with high contents of unsaturated fatty acids.

Enrei, a cultivar widely used in our country, contains a high percentage of unsaturated fatty acids, approximately 90%. Raiden, a nematoda-tolerant cultivar which was obtained with the radiation technique, is not suitable for oil production with regard to unsaturated fatty acids. Of two cultivars with long leaves, Tokachi-nagaha and Shirome-nagaha, selected for high photosynthetic activity, Shirome-nagaha is preferable as to oil quality. Tsukui-zairai which exhibits high tolerance to wet soil, like in lowland fields after rice cultivation, contains a high percentage of unsaturated fatty acids, and therefore is valuable as a parent material in breeding as to oil quality. Bonminori, Tamahikari, Fujimijiro, Kakushin No. 1 and Norin No. 1 have gone out of favor, due to various defects as to seed quality or cultivation management, however, Norin No. 1 will be a suitable parent material for the selection of cultivars with high contents of unsaturated fatty acids. Breeding lines of the Asian Vegetable Research and Development Center (AVRDC), G 219, G 488 and AGS 66, are approved as to the oil compo-

sition quality and only AGS 62 showed a little lower unsaturated fatty acid content than the other three lines.

Although the precision is inferior to that of the conventional method, ^{13}C NMR constitutes a rapid and non-destructive analytical method which is useful for the evaluation of oil quality in breeding. Besides, considering that its characteristics are suitable for automatic routine analysis, the method developed in the current investigation is also useful for the quality control of soybean seeds in the food industry.

Acknowledgments. The authors wish to thank Dr. H. Taira and Mr. A. Nagao of the National Food Research Institute for their valuable advice.

References

- 1) D. P. Hollis, "Biological Magnetic Resonance," Vol. 2, ed. by L. J. Berliner and J. Reuben, Plenum Press, New York and London, 1980, pp. 1 ~ 44.
- 2) J. Schaefer and E. O. Stejskal, *J. Am. Oil Chem. Soc.*, **51**, 210 (1974).
- 3) M. Kainosho, *Tetrahedron Lett.*, **1976**, 4279.
- 4) M. Kainosho and H. Konishi, *Tetrahedron Lett.*, **1976**, 4757.
- 5) M. Kainosho and K. Ajisaka, *Tetrahedron Lett.*, **1978**, 1563.
- 6) J. K. M. Roberts, "The Biochemistry of Plants," Vol. 13, ed. by P. K. Stumpf and E. E. Conn, Academic Press, San Diego, New York, Berkeley, Boston, London, Sydney, Tokyo and Toronto, 1987, pp. 181 ~ 227.
- 7) J. K. M. Roberts, "Modern Methods of Plant Analysis," Vol. 2, ed. by H. F. Linskens and J. F. Jackson, Springer-Verlag, Heidelberg, 1986, pp. 43 ~ 49.
- 8) N. Ishida, H. Kano, T. Kobayashi, H. Hamaguchi and T. Yoshida, *Agric. Biol. Chem.*, **51**, 301 (1987).
- 9) R. F. Bils and R. W. Howell, *Crop Sci.*, **3**, 304 (1963).
- 10) K. Saio, K. Kondo and T. Sugimoto, *Food Microstructure*, **4**, 191 (1985).
- 11) H. Taira, H. Taira and M. Saito, *Proc. Crop Sci. Soc. Japan*, **43**, 482 (1974).
- 12) H. Taira and H. Taira, *Nippon Shokuhin Kogyo Gakkaishi*, **22**, 42 (1975).
- 13) O. Hirayama and K. Hujii, *Agric. Biol. Chem.*, **29**, 1 (1965).
- 14) Y. Nakayama, K. Saio and M. Kito, *Cereal Chem.*, **58**, 260 (1981).
- 15) E. Breitmaier and W. Voelter, " ^{13}C NMR Spectroscopy," 2nd Ed., Verlag Chemie, Weinheim and New York, 1978, p. 43.
- 16) M. Yoshida, H. Kano, N. Ishida and T. Yoshida, Abstracts of Papers, the Annual Meeting of the Agricultural Society of Japan, Tokyo, April, 1984, p. 647.