$^{13}$C NMR Studies of Lipids and Starch Digestion

*J. Agric. Food Chem.*, Vol. 27, No. 2, 1979 **435**

LITERATURE CITED

Calvin, M., *Science* 184, 375 (1974).

Calvin, M., *Photochem. Photobiol.* 23, 425 (1976).

Chippendale, G. M., "Eucalypts of the Western Australian Goldfields", Australian Government Publishing Service, Canberra, 1973.

Dolejš, L., Motl, O., Souček, M., Herout, V., Sorm, F., *Collect. Czech. Chem. Commun.* 25, 1483 (1960).

Hall, N., Johnston, R. D., Chippendale, G. M., "Forest Trees of Australia", Australian Government Publishing Service, Canberra, 1975.

Laurent, P. A., da Cunha, J., *Compt. Rend.* 259, 4391 (1964).

Nielsen, P. A., Nishimura, H., Otvos, J. W., Calvin, M., *Science* 198, 942 (1977).

Nishimura, H., Philp, R. P., Calvin, M., *Phytochemistry* 16, 1048 (1977).

Prakash, S., Sinha, G. K., Pathak, R. C., *Indian Oil Soap J.* 37, 230 (1972).

Pryor, L. D., "Biology of Eucalypts", Camelot Press Ltd., Southampton, 1976.

Stenhagen, E., Abrahamsson, S., McLafferty, F. W., "Registry of Mass Spectral Data", Wiley-Interscience, New York, 1974.

Yamashita, T., *Perfume* (Takasago, Japan) **No. 95**, 45 (1970).

Yukawa, Y., Ito, S., "Spectral Atlas of Terpenes and the Related Compounds", Hirokawa Publishing Co., Tokyo, 1973.

# Carbon-13 Nuclear Magnetic Resonance Studies of Lipids and Starch Digestion in Intact Seeds

Shi-chow Chen,* Richard M. Elofson, and John M. MacTaggart

The carbon-13 pulse Fourier transform nuclear magnetic resonance (NMR) technique for measurement of intact plant tissue has been used for the characterization and estimation of fatty acid composition in seeds of *Leucas cephalotes*, *Stocksia brahuica*, and *Avena fatua*. The carbon-13 NMR analysis of monoenoic and dienoic fatty acid concentrations was compared with destructive gas chromatographic analysis. The presence of laballenic esters in *L. cephalotes* and cyanolipids in *S. brahuica* can be detected by carbon-13 NMR spectroscopy. The isoprenoid hydroxynitrile moiety of the cyanolipid was shown to have the trans configuration. Carbohydrate digestion in the excised endosperm of the postgerminated *A. fatua* was observed by the carbon-13 NMR technique.

Isolation of pure substances through a multistep purification procedure has been a standard method for the characterization and recognition of the presence of certain chemical constituents in a biological system. It is not always certain, however, whether a compound so obtained does truly reflect its structure in an intact biological environment. Until recently there has been a lack of a specific technique for the direct observation and determination of chemical constituents in certain living matter, such as viable plant tissue, despite the fact that such a capability could provide a great deal of information. A fast and nondestructive method of chemical analysis of plant tissue has now become feasible as a result of spectacular advances in pulse Fourier transform NMR (FT NMR) in the past decade.

Compared with proton wide-line NMR, the $^{13}$C FT NMR provides both improved sensitivity gain and resolution necessary for the study of heterogeneous material. This relatively new, sophisticated technique has already afforded an efficient analytical method for seed selection in a plant breeding program which requires the nondestructive selection of seeds on the basis of composition (Schaefer and Stejskal, 1974). More recently, the application of $^{13}$C NMR analysis to intact plant tissues has been extended to the study of fruit endocarp (Kainosho, 1976), as well as seed coat (Kainosho and Konishi, 1976).

High-resolution, natural abundance $^{13}$C NMR spectra of many constituents in the plant tissues can be obtained by the Fourier transform technique in a reasonably short time. Sharp NMR spectra are observed from these heterogeneous materials because of local mobility in the cytoplasm and in the intercellular fluid, even though they may be confined in a lignified tissue.

In the light of the rapid developments in this fascinating technique for the study of chemistry *in the gross*, $^{13}$C NMR measurements of some seeds which are related to a number of research interests in this laboratory were undertaken. It has been meticulously demonstrated that solid polymers give well-resolved $^{13}$C spectra by using cross-polarization experiments with magic angle spinning (Schaefer and Stejskal, 1976). However, there are limitations in routine experiments in that NMR signals of minor constituents or rigidly bound components in a complex biological matrix cannot be elicited without an extensive acquisition of pulsed transients or without some means of modification of the raw materials (Kainosho and Ajisaka, 1978). Nevertheless, highly abundant cellular components could be easily identified by repetitive technique with overnight acquisitions (60K). Thus $^{13}$C NMR may be used as a simple and effective method for the direct characterization of fatty acids and sugars in seeds. Studies on seeds of *Leucas cephalotes* (Labiatae), *Stocksia brahuica* (Sapindaceae), and *Avena fatua* (wild oats) are reported herein.

EXPERIMENTAL SECTION
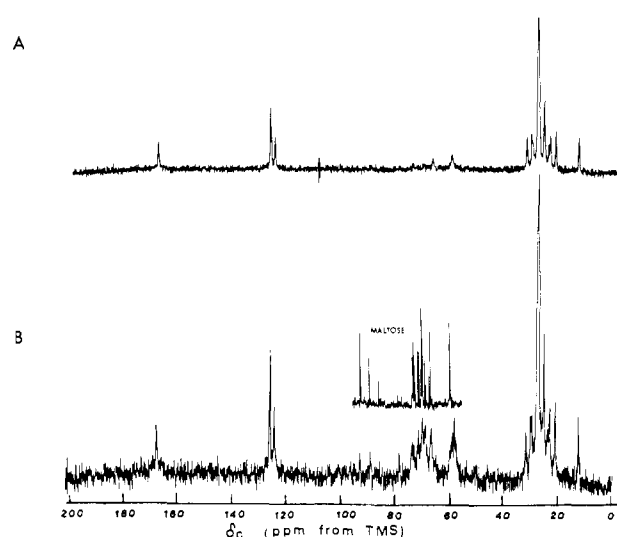
Detailed experimental procedure for the standard pulse

Special Projects Division, Alberta Research Council, Edmonton, Alberta, Canada T6G 2C2.
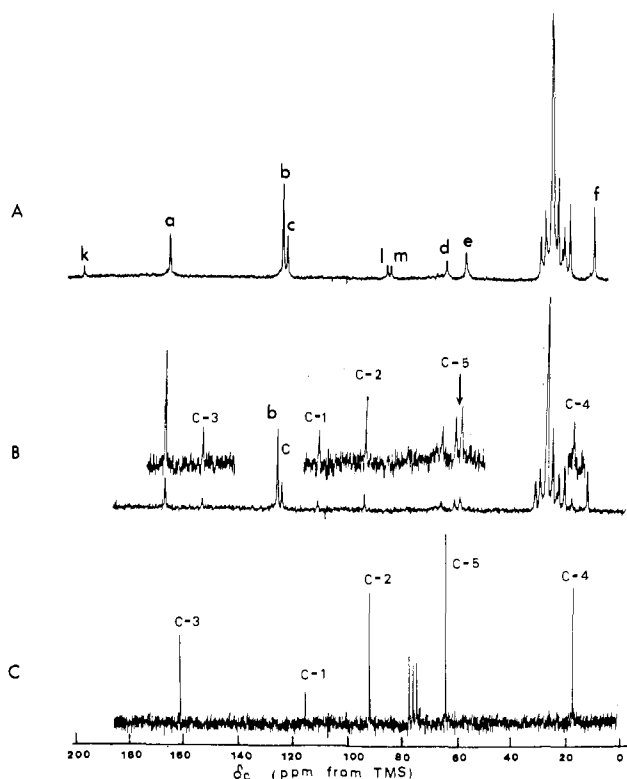
**Figure 1.** Carbon-13 nuclear magnetic spectra (22.6 MHz) of (A) seeds of *Leucas cephalotes*; (B) seeds of *Stocksia brahuica*; (C) (*E*)-4-hydroxy-3-methylcrotononitrile (in CDCl₃).



**Figure 2.** Carbon-13 nuclear magnetic resonance spectra (22.6 MHz) of (A) wild oat seeds; (B) excised seed reserve of 2-day-old wild oat plantlets (inset maltose).

FT NMR measurement of intact seeds has been well documented (Schaefer and Stejskal, 1974). All spectra studied in this report were obtained at room temperature with a Bruker HFX 90-Nicolet 1085 spectrometer operating at 22.6 MHz ($D_2O$ lock) in a 10-mm sample tube, in which an appropriate amount of plant material was placed for the measurement. Each seed sample was studied under two different conditions for data acquisition to ensure no intensity variation caused by differences in $T_1$; settings for repetitive pulsing were pulse width 5 $\mu$s (32 °C) with data acquisition of 0.8 s and those conditions for pulse and delay technique were pulse width 17 $\mu$s (90 °C) with 4.8 s delay between pulses. All spectra reproduced in Figures 1 and 2 were taken under rapid pulsing conditions. Chemical shifts (ppm) were determined from an offset taken immediately after recording of the spectrum. An external capillary containing 50% v/v $Me_4Si$ in carbon tetrachloride placed in $D_2O$ was used as standard. Each spectrum, except that of a pure compound, was the result of overnight acquisitions (about $6 \times 10^4$ transients). A sample 0.6 g each of *L. cephalotes* or dehulled *A. fatua* (wild oats) and five seeds (ca. 0.7 g) of *S. brahuica* were used directly. For measurement of germinating seeds, excised endosperms of wild oats plantlets, which were collected about 2 days after extrusion of coleoptile, were used.

Extraction of seed oils of *L. cephalotes* and wild oats were carried out in two steps. The seeds were ground and extracted simultaneously with petroleum ether (bp 30–60 °C) in a Waring Blendor. The pulverized seed residue was separated and subsequently extracted continuously with a Soxhlet extractor. Total oil contents of *L. cephatoles* and hulled wild oat seeds were 28 and 9.5%, respectively. Fatty acid methyl esters were prepared by transesterification of seed oils with methanol and 1% sulfuric acid. Gas chromatography of the esters was performed using a column of 5% FFAP on Chromosorb P. Peak assignments and composition analysis of the methyl esters were made

by comparison with chromatograms of authentic samples obtained from Applied Science Laboratories, Inc.

## RESULTS AND DISCUSSION

*Leucas cephalotes*, Spreng (Labiatae). The seed of *L. cephalotes*, which contains about 28% seed oil having a high proportion of laballenic acid (octadeca-5,6-dienoic acid) (Sinha et al., 1978), gave a well-resolved $^{13}$C FT NMR spectrum taken under the pulse and delay conditions after about 2 h of accumulation time. The spectrum so obtained showed three weak peaks in addition to the relatively strong absorptions due to the presence of common unsaturated fatty acids. These peaks appeared distinctly in a spectrum obtained with prolonged accumulation time under both repetitive pulsing (Figure 1A, peaks k, l, and m) and pulse and delay conditions. Spectra obtained under the two different pulse rates showed no significant differences in olefinic carbon absorptions when data were accumulated over an extended period of time. However, pulse and delay was decidedly beneficial for the detection of peak k (205 ppm). Peaks k, l, and m are characteristic for allenic carbons; the signal due to the sp-hybridized central allenic carbon is found in the sparsely populated low field region at around 200 ppm from $Me_4Si$ whereas signals due to the two allenic sp² carbon atoms appear close to 90 ppm (Crandall and Sojka, 1972). Although laballenic esters were reported to contribute to nearly one-third of the total seed oil in *L. cephalotes*, the signals which are ascribed to allenic carbons are relatively weak because of a diminished nuclear Overhauser enhancement in these carbons.

The fact that the pattern of the olefinic carbon peaks in the vicinity of 130 ppm did not significantly change with varying pulse interval and flip angle clearly indicates the absence of any significant concentration of linolenic acid in the fatty acid mixture (Shoolery, 1973; Shaefer and Stejskal, 1975). The $^{13}$C NMR spectra of oleic and linoleic acids are well known (Shoolery, 1973). Resonances appropriate to the carboxy carbon, olefin carbons, and terminal methyl carbon appear at 171 ppm (a), 130 ppm (b, c), and 14 ppm (f) downfield from $Me_4Si$, respectively. The methine and methylene carbons of the glycerol moiety in the lipid appeared as two weak peaks (d, e) around 70 ppm and the methylene carbons of fatty acid chains exhibited multiple peaks centered at about 30 ppm. It has been shown that in the absence of linolenic acid, the in-

tegrated intensities of olefinic peaks b (130.6 ppm) and c (129 ppm) can be used for an estimation of molar concentration ratio of oleic and linoleic acid (Shaefer and Stejskal, 1974; Rutar et al., 1977). Since oleic acid contributes two carbons to peak b and linoleic acid two carbons to both b and c, the molar concentration ratio of the monoenoic to dienoic acid may be formulated as $C_{18:1}:C_{18:2} = (b - c):c$.

The observed integrations of peaks b and c in the spectrum of *L. cephalotes* led to a value $C_{18:1}:C_{18:2} = 2.7:1$.

The ^{13}C NMR analysis of fatty acid composition in intact tissue may be compared with data obtained by destructive gas chromatographic analysis. Our gas chromatographic study of fatty acid methyl esters derived from seed lipids showed that the oil consists of $C_{16:0}$ (15%), $C_{18:0}$ (5%), $C_{18:1}$ (46%), $C_{18:2}$ (15%), and methyl laballenate (19%). Data from both the present and the earlier (Sinha et al., 1978) studies showed similar amounts of oleic and linoleic acids and the ratio of the two fatty acids analyzed chromatographically is rather close to that estimated by the ^{13}C NMR technique. The proportion of methyl laballenate was considerably lower than that reported earlier. Unfortunately, the concentration of laballenic esters cannot be estimated from a simple analysis of the *L. cephalotes* spectrum. The discrepancy in laballenic acid concentration between the two chromatographic studies may be due to seed crop differences. It has been shown that differences in fatty acid composition were found between varieties of several cereal grains as well as in crops differing only in sowing date (Welch, 1975).

***Stocksia brahuica* Benth (Sapindaceae).** The seed oil of *S. brahuica* shifts reported to contain 35% of 4-hydroxy-3-methylcrotononitrile esters of fatty acids in which a $C_{20}$ monoenoic acid predominates (Mikolajczak et al., 1970). The isoprenoid cyanoalcohol 1, the structure of which was partially elucidated by chemical transformation, has not been isolated from the hydrolysate of the lipids since cyanolipids are somewhat unstable, especially under hydrolysis conditions. Although a reported proton NMR spectrum of the cyanolipid, in which an olefinic proton appeared as a quartet, may be suggestive of a trans configuration of the cyanoalcohol (Mikolajczak, 1977), no definite stereochemistry concerning the geometrical structure of the compound has been postulated. Having compound 1 readily available as a synthetic intermediate in our laboratory, we were interested in a direct comparison of the ^{13}C NMR spectrum of 1 with that of *S. brahuica*. It would, therefore, unambiguously establish the stereochemistry of the cyanoalcohol in the naturally occurring lipids.

The ^{13}C NMR spectrum of the seed (Figure 1B) clearly revealed five carbon peaks in addition to the resonances of ubiquitous oleic and linoleic acids. On the basis of their chemical shifts and relative peak intensities, these five peaks may be assigned to the isoprenoid carbons of the structure 1 or its cis isomer. However, no information is



**1**
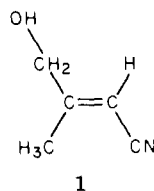
readily available as to the question of the geometrical isomerism. Figure 1C shows a spectrum of compound 1. The spectrum correlates very well with non-fatty acid peaks (insert Figure 1B) of the intact seed spectrum, despite the profound differences between two samples.

This spectral comparison further supports the identity of the alcohol moiety of the cyanolipid to compound 1. The assignments of the spectrum of 1 were confirmed by an off-resonance spectrum.

The absence of any discernible signal at about 2 ppm downfield from peak b in the spectra of *S. brahuica* taken under various conditions agreed with the result obtained from an earlier chromatographic analysis which showed a very low concentration of linolenic acid (1%) in the seed oil. Furthermore, a value of 2.9 obtained for the ratio of (b − c) to c, i.e., the concentration ratio of monoenoic to dienoic fatty acids, can be compared favorably with the chromatographic analysis of the methyl esters which showed 68% of monoenoic vs. 22% of the dienoic acids.

***Avena fatua* L. (Wild Oats).** Our primary interest in the analysis of wild oats was to study the digestion of the reserve carbohydrate during seed germination. Both dormant seeds, which were not presoaked in water, and germinating seeds, collected 12 h after protrusion of coleoptile, however, gave similar ^{13}C NMR spectra. This indicated that the massive amount of reserve carbohydrate in the endosperm of the seed at an early stage of germination had undergone little change. Further, change in the starch reserve in the seed by inhibition of water, if any, was not observed by NMR.

The spectrum of wild oats (Figure 2A) can be unambiguously ascribed to the resonance of a mixture of oleic and linoleic acids.

The pattern of the olefinic peaks indicates that the presence of linolenic acid, if any, is negligible. The ratio of monoenoic to dienoic acid was found to be 1.2, showing a preponderance of oleic acid to linoleic acid. A gas chromatographic analysis of methyl esters derived from the extracted oil showed that palmitic ($C_{16:0}$), oleic, and linoleic acids are the predominant acids accounting for about 16, 51, and 33%, respectively, of the total esters. Whereas the chromatographic analysis confirmed the absence of linolenic acid in wild oat seeds, it gave a significantly higher percentage of oleic acid than that estimated by ^{13}C NMR technique. Nevertheless, both methods consistently showed that wild oat seeds contain more oleic acid than linoleic acid in the reserve lipids. This result is in contrast to the reported composition of a number of cultivated oat genotypes in which a higher concentration of linoleic acid (about 50%) than oleic acid (about 30%) was found (Welch, 1975); the presence of palmitic acid in the cultivated oats was reported to contribute 15–10% of the total fatty acid.

The ^{13}C NMR spectrum of excised endosperm of wild oat plantlets, which was determined about 2 days after the emergence of the coleoptile, is shown in Figure 2B. The spectrum still showed a high concentration of oleic and linoleic acids, suggesting that digestion and transportation of fat to the embryo was slow. On the other hand, the hydrolysis of the reserve carbohydrate to maltose was then observed in the excised endosperm. The signal of maltose was partially obscured by two groups of complex absorptions which may be due to the presence of α-starch (Kainosho and Ajisaka, 1978). Thus, at the postgermination stage, cleavage of the polymeric carbohydrate chain in the starch reserve has greatly affected its mobility in the massive endosperm matrix.

This study has demonstrated that under optimum conditions, ^{13}C FT NMR permits a direct characterization and analytical discrimination of the reserve fatty acid and sugar in plant tissues of various species which may contain uncommon constituents. This technique for the characterization of seed oil and sugar is profoundly simple as

**438** J. Agric. Food Chem., Vol. 27, No. 2, 1979

Klepper

compared to the conventional methodology. On the other hand, determination of monoenoic and dienoic fatty acid composition in seeds may possibly involve a 10–15% error. The major problem in this analysis arises from the weakness of olefinic carbon signals as well as some undesirable quantitative aspects of $^{13}C$ NMR spectroscopy (Shoolery and Jankowsky, 1973). An accurate quantitative compositional analysis and determination of total oil content in the seeds will, however, need a somewhat more elaborate procedure. It has been demonstrated that during the germination process catabolic breakdown of the storage carbohydrate into major polysaccharide components in seeds can be detected by $^{13}C$ NMR. The same technique can also be employed for the determination of the quantity of starch converted during malting or hydrolysis of barley (Diner and Elofson, 1977) or other grain seeds. Many aspects of the economically important areas of agriculture and food production that affect crop quality, such as starch aging, storage methods and processing conditions, would seem amenable to $^{13}C$ NMR technique.

## ACKNOWLEDGMENT

## LITERATURE CITED

Crandall, J. K., Sojka, S. A., J. Am. Chem. Soc. 94, 5084 (1972).
Diner, U., Elofson, R. M., U.S. Patent 4 044 156 (1977).
Gunstone, F. G., McLaughlan, J., Scrimgeour, C. M., Watson, A. P., J. Sci. Food Agric. 27, 675 (1976).
Hilditch, T. P., Williams, P. N., "The Chemical Constitution of Natural Fats", 4th ed, Chapman and Hall, London, 1964, p 314.
Johnson, L. F., Jankowski, W. C., "Carbon-13 NMR Spectra", Wiley-Interscience, New York, 1972, Spectrum 443.
Kainosho, M., Tetrahedron Lett., 4279 (1976).
Kainosho, M., Konishi, H., Tetrahedron Lett., 4757 (1976).
Kainosho, M., Ajisaka, K., Tetrahedron Lett., 1563 (1978).
Mikolajczak, K. L., Smith, C. R., Jr., Tjarks, L. W., Biochem. Biophys. Acta. 210, 306 (1970).
Mikolajczak, K. L., Prog. Chem. Fats Lipids 15, 97–130 (1977).
Rutar, V., Barger, M., Blinc, R., J. Magn. Res. 27, 83 (1977).
Schaefer, J., Stejskal, E. O., J. Am. Oil Chem. Soc. 51, 210 (1974).
Schaefer, J., Stejskal, E. O., J. Am. Oil Chem. Soc. 52, 366 (1975).
Schaefer, J., Stejskal, E. O., J. Am. Chem. Soc. 98, 1031 (1976).
Sinha, S., Ansai, A. A., Osman, S. M., Chem. Ind., 67 (1978).
Shoolery, J. N., Varian Application Notes, NMR-73-3, 1973.
Shoolery, J. N., Jankowski, W. C., Varian Application Notes, NMR-73-4, 1973.
Welch, R. W., J. Sci. Food Agric. 26, 429 (1975).

# An Improved Method for Nitrite Extraction from Plants

Lowell A. Klepper

An improved technique for aqueous extraction of nitrite from plant tissues is described. Additions of methylene chloride (MeCl$_2$) and insoluble polyvinylpyrollidone (PVP) produced clear, aqueous nitrite-containing extracts. The MeCl$_2$/PVP addition increased recoveries of nitrite from all 15 plant species tested.

Nitrite is a naturally occurring metabolite in the process of nitrate assimilation by green plants. It is considered an "enzyme-bound", intermediate and is not easily detectable in healthy plants. In the past, nitrite analyses have been largely confined to soils tests and nitrate reductase assays. Recently, nitrite has been shown to accumulate in plant tissues as a result of herbicidal action (Klepper, 1974, 1975, 1976) and there has been considerable concern regarding nitrite and its role as a precursor in the formation of nitrosamines. Nitrite has been reported to accumulate in certain processed or raw foods when high nitrate content was coupled with improper handling or storage (Bassir and Maduagwu, 1978; Keating et al., 1973; Keybets et al., 1969). Also, recent studies of the effects of air pollution on plants have required tests for nitrite in vegetation (Zeewart, 1976). These new research approaches would be aided by a reliable nitrite test.

Losses of known quantities of nitrite have been reported upon extraction of plant tissues (Finke et al., 1977) or when free nitrite was in contact with green leaf tissue (Vanecko and Varner, 1965). Nitrite additions to tannic acid solutions, tea, or apple juice resulted in the disappearance of the added nitrite (Bogovski et al., 1972). Up to 100 ppm nitrite added to apple juice could not be detected but a

Department of Agronomy, University of Nebraska, Lincoln, Nebraska 68583.

darkening of the juice was associated with an oxidative change in the tannin content. They concluded that the tannins present in the juice reacted with the added nitrite.

The sulfanilamide/$\alpha$-naphthylethylenediamine/HCl reagent is extremely sensitive to nitrite and can easily be used following extraction. The problem lies in recovery during extraction of nitrite from plant tissues. Nitrite is known to react with many compounds present in the green plant (Klepper, 1974). Upon extraction, both nitrite and reactive metabolites are released into an aqueous medium, permitting the destruction of nitrite during the extraction process. The result is a loss of nitrite before an analysis can be conducted.

I have developed an extraction method that achieves higher recovery of nitrite in plant tissues than ordinary aqueous extraction.

## METHODS AND MATERIALS

**Preparation of Insoluble Polyvinylpyrollidone (PVP).** PVP was obtained from Sigma Chemical Co. Analysis indicated it contained the equivalent of more than 900 nmol of nitrite/g. Thus, the material had to be thoroughly washed before use (Klepper and Hageman, 1969). One hundred grams of PVP was placed in 3 L of 0.1 N HCl and stirred for 4 h. Nitrite quickly disappeared from the PVP. The PVP was strained through four layers of cheesecloth and resuspended in distilled, deionized H$_2$O. This water-washing procedure was repeated four times.