Nondestructive Study of Liquids in Single Fir Seeds Using Nuclear Magnetic Resonance and Magic Angle Sample Spinning

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Magic angle sample spinning reduces line broadening which arises from differences in magnetic susceptibility. NMR spectra of liquid-like components in single fir seeds are recorded with superior resolution and sensitivity, thus facilitating nondestructive determination of oil composition. The results also show that *cis*-5, *cis*-9-octadecadienoic, and *cis*-5, *cis*-9, *cis*-12-octadecatrienoic fatty acids are selectively esterified to the CH_2-O- group of the glycerol backbone.

Determination of chemical constituents in living matter usually involves extraction and purification of substances which are subsequently studied by various analytical techniques. Nuclear magnetic resonance (NMR) certainly plays an important role among them, because it frequently facilitates unambiguous assignment of unknown compounds.

It must be stressed that "successful NMR application" does not imply simple detection of signals, but it rather requires high-resolution separation of resonances for measurements of chemical shifts and J coupling constants. The most obvious and efficient step towards this goal is made by dissolving the sample in a suitable deuterated solvent. Since rapid molecular motions eliminate line-broadening arising from chemical shift anisotropy and magnetic dipolar interaction, the obtained resolution is typically not limited by the natural linewidth. Homogeneity of the external magnetic field remains the dominant obstacle which must be overcome by shimming and better design of superconducting magnets. Such efforts have been very successful and the quality of NMR results is continuously improving.

We do not want to dispute the above experimental strategy which has a significant potential value in studies of metabolic processes (1), however, we feel that more attention should be focused on alternative possibilities which leave the sample intact (2). It is clear that resolution and sensitivity will be inferior, but at least two good reasons favor nondestructive applications of NMR. Firstly, the processes can be followed on the individual object, and secondly, valuable genetic material is not destroyed if the experiment should select seeds for plant breeding purposes.

The complex composition of biological objects makes nondestructive NMR studies especially difficult because overlap of resonances is inevitable. Moreover, many substances exist in a solid state and despite significant technical developments (magic angle sample spinning, polarization transfer, etc.) the obtainable resolution does not allow clear identification of all responses. It seems unlikely that further improvements will solve all remaining problems.

On the other hand, NMR has a great potential value in specialized studies designed to detect signals of liquid-like components. For example, some plant seeds contain oil (3,4) or dissolved carbohydrates (5) which give rise to well resolved signals. From the NMR point of view, such substances behave as liquids, because spin-spin relaxation times are relatively long and chemical shift anisotropy is averaged out by fast microscopic motions. If the spectra are recorded by the traditional "liquid-state" version of NMR, they still show relatively poor resolution which is limited neither by natural linewidths nor by magnetic field inhomogeneity. The broadening arises mostly from the heterogeneous structure of the sample. Liquid components are found in small droplets embedded in the solid matrix and differences in susceptibility create local perturbations of the magnetic field, thus increasing linewidth of resonances.

It has been shown that magic angle sample spinning (MASS) eliminates inhomogeneous broadening (6,7) and it is the method of choice in studies of plant seeds and other biological objects (8). Since any reduction of the linewidth makes signals higher, the signal-to-noise ratio is also improved. Therefore, MASS gives rise to superior resolution and sensitivity in NMR spectra of liquids in inhomogeneous systems.

This approach has some limitations because the object must be confined in a spinner with a typical volume about 1 cm^3. Fast rotation also creates substantial forces which may disintegrate delicate samples, however, it must be stressed that spinning frequencies are low (100-1000 Hz) and mechanical problems do not appear as severe as in MASS studies of solids.

^1H nuclei are abundantly present in all biological systems and their detection does not require long measuring times. Spectra of plant seeds (sunflower, corn, and peanut) provide useful information by resolving characteristic signals of major fatty acids (8). Fast and nondestructive measurements of the relative amount of linoleic acid are suitable for selection of new varieties with improved oil quality. Systems which contain several liquid-like components are less amenable to ^1H NMR studies, because the overlap of resonances makes interpretation unreliable.

Despite low natural abundance (1.1%) ^{13}C spins represent a powerful alternative. Dispersion of their chemical shifts is about 20 times larger, and easier separation of responses clearly offsets any disadvantages arising from longer measuring times. Carbon resonances have facilitated determination of oil composition (4, 9), detection of sugars (5), and studies of germination (2).

The present study has utilized direct excitation of ^{13}C spins using radio frequency pulses and broadband decoupling of ^1H spins. The results are semi-quantitative because different Nuclear Overhauser Effect (NOE) enhancements and spin-lattice relaxation times give rise to unequal intensities of protonated and nonprotonated carbons. Slow repetition times and gated decoupling lead to more precise and uniform intensities of

^{13}C signals, but the experiment requires significantly longer measuring times which are not practical for most applications.

Polarization transfer using INEPT (10) or DEPT (11) pulse sequences represents a better alternative because it improves the signal-to-noise ratios. Since nonprotonated carbons are not detected and it is not possible to optimize conditions for simultaneous observation of all CH_n groups (n = 1, 2, 3), we did not use polarization transfer during the present study, which should establish the initial assignment of ^{13}C NMR spectra.

EXPERIMENTAL

Silver fir seeds (*Abies alba* Mill.) were collected in 1986 in the Črna region, Slovenia, Yugoslavia. NMR measurements did not require any sample preparation and single, intact seeds were simply placed into the standard spinner.

All spectra were recorded on a Bruker MSL spectrometer operating at ^{13}C resonance frequency 75.47 MHz. Although the NMR probe was designed for solid-state measurements, the instrument configuration has been changed in such a way that ^{1}H spins were continuously decoupled using broadband irradiation with relatively weak power (approximately 0.5 W). The experiment closely resembled traditional liquid-state ^{13}C NMR, and magic angle sample spinning was added as an essential line-narrowing technique. Since the sample rotation was relatively slow (typical frequency 400 Hz), it did not require balancing of the spinner and it did not cause any obvious damage to samples.

^{13}C signals were detected after 45° radio frequency pulses (duration 5 μs), and sampled into 16K data points with the total spectral width 18.5 kHz. The relaxation delay afer each acquisition was 2 s. After finishing the signal accumulation, exponential multiplication with line-broadening 3 Hz was used. Zero-filling up to 64K points improved definition of line positions as they were determined by Fourier transformation.

Since nondestructive NMR does not allow referencing of chemical shifts with respect to the internal tetramethylsilane, spectra of various samples must be recorded under identical conditions and compared. Precision of measurements did not suffer significantly, because individual differences between seeds or drift of the external magnetic field generally did not change the observed chemical shifts more than 0.1 ppm. Accuracy of results was further improved by using the terminal methyl group in fatty acid chains as a secondary reference at 14.00 ppm, so that experimental error of reported chemical shifts does not exceed ±0.02 ppm.

Signals of some selected seeds (which showed high signal-to-noise ratios) were reprocessed to enhance apparent resolution. Free induction decays were multiplied by a two term exponential of the form $\exp(-at-bt^2)$ with the constants $a = -6.28\ s^{-1}$ and $b = 17.76\ s^{-2}$. This procedure (12) provided better separation of resonances at the expense of intensity distortions.

RESULTS AND DISCUSSION

Figure 1 shows a ^{13}C NMR spectrum of a single fir seed. The 20 minute measuring time lead to signal-to-noise ratio of about 120:1 and a linewidth of about 3 Hz in typical samples. Although the presence of various liquid-like components contributes to the apparently complicated pattern, all major resonances are readily assigned. Limonene has two nonprotonated carbons resonating at 149.10 and 132.84 ppm, while other signals are stronger due to NOE enhancement. Their chemical shifts (120.78, 108.55, 41.10, 30.84, 30.60, 27.93, 23.30, and 20.54 ppm) agree with the previously reported values (13), and small differences are explainable by solvent effects.

The second major component is oil, which contributes to the following signals: carbonyls are found at around 171 ppm; -CH=CH- groups resonate between 127 and 131 ppm; glycerol carbons CH-O- and CH_2-O- are shifted to 69.0 and 61.9 ppm; $-CH_2-$ groups cover the range from 34 to 22 ppm; and the terminal methyl group gives rise to the last signal at 14 ppm.

Detailed studies of single seeds require longer measuring times (about 10 hours). Improved signal-to-noise ratio allows resolution enhancement by digital apodization (Figure 2) and detection of other liquid-like substances. The presence of α-pinene has been confirmed and one of its resonances appears at 26.25 ppm (13). Figure 2 also shows several additional peaks which definitely exceed the noise level, but we did not attempt to assign such weak responses in the present study.

So far, ^{13}C spectra of about 80 single fir seeds have been recorded and we noticed obvious differences between individual samples. Oil or limonene is always the major component, but the relative quantities show significant variations. About two-thirds of the seeds contained both substances simultaneously, thus giving rise to the spectrum (Figure 1) which might be considered "typical". Other samples had a very small or negligible amount of oil, therefore, resonances of limonene dominated in their spectra. However, we found

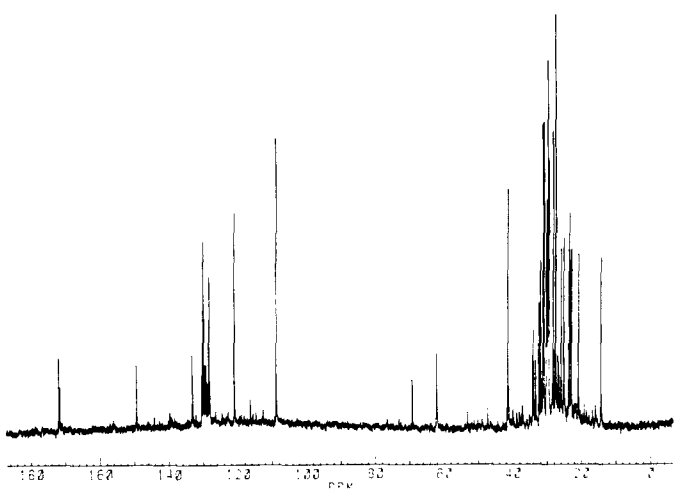

FIG. 1. ^{13}C NMR spectrum of a single fir seed showing signals of limonene and oil as major liquid components. Since MASS eliminated line-broadening arising from intrinsic differences in magnetic susceptibility, superior resolution and sensitivity facilitate nondestructive determination of composition.

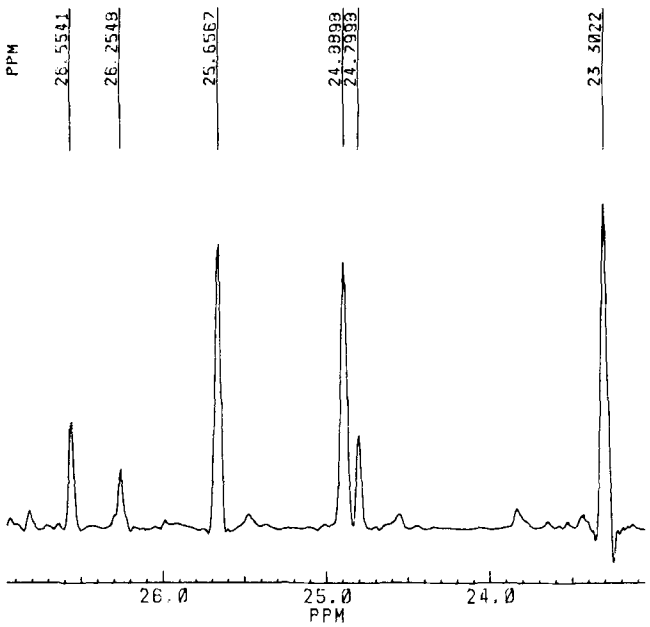

FIG. 2. Part of the ^{13}C spectrum of a single fir seed as obtained by long-term signal acquisition (10 hours). Signals of oil (26.55, 25.66, 24.89, and 24.80 ppm) and limonene (23.30 ppm) represent the major liquid-like components. Additional peaks arise from α-pinene (26.25 ppm) and smaller quantities of other substances.

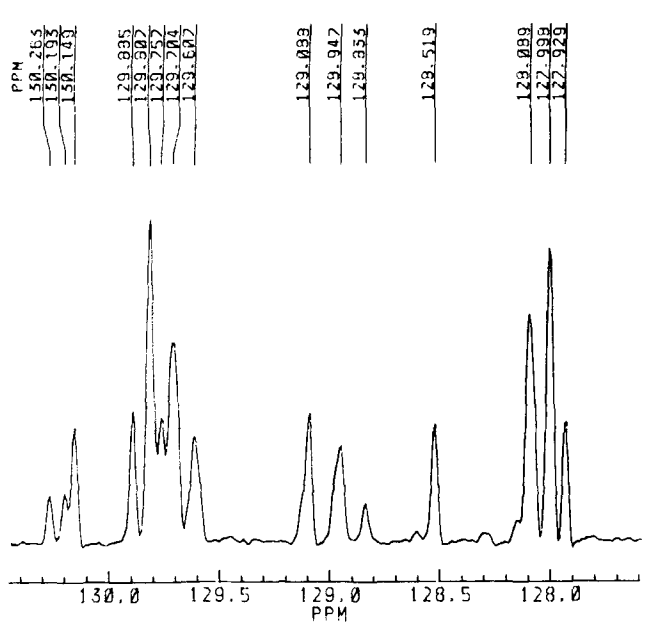

FIG. 3. Part of the ^{13}C spectrum of a single fir seed showing signals of -CH=CH- groups. Comparison with chemical shifts of model substances and other plant seeds identified all unsaturated fatty acids (see text).

one particular seed which contained oil as the only NMR detectable substance (8).

Although oil signals show significantly different intensities, the shape of the pattern does not change considerably. This observation (roughly speaking) indicates that individual characteristics of seeds have contributed to various quantities of oil, while the fatty acid composition has remained almost the same.

Relative abundances of fatty acids in simple lipids extracted from fir seeds have been previously studied using chromatographic separation. Results have shown small amounts of saturated fatty acids (4.1% palmitic and 2.6% stearic), traces of palmitoleic fatty acid (0.7%), and major contributions coming from oleic (30.0%) and linoleic fatty acids (43.7%). Two additional constituents were present and they were labeled X_1 (5.1%) and X_2 (13.8%) because they could not be identified completely (14).

^{13}C NMR is not very suitable for discrimination between saturated fatty acids which predominantly contain CH_2 groups separated by more than three bonds from each end of the chain. Such carbons give rise to unresolvable resonances at 29.7 ± 0.2 ppm. On the other hand, signals of -CH=CH- groups are shifted to 127–132 ppm and they contribute to a characteristic pattern (Figure 3) which clearly identifies unsaturated fatty acids.

Linoleic fatty acid has 18 carbons and two double bonds between positions 9 and 10, and 12 and 13. Chemical shifts of the four largest peaks at 129.81, 129.70, 128.09, and 128.00 ppm are compared with the comprehensive collection of ^{13}C spectra of model compounds (1, 15). The resonances are assigned to C_{13}, C_9, C_{10}, and C_{12}, respectively. Small differences arising from solvent effects or steric hinderance are noticeable, because signals of C_{13} and C_9 are shifted upfield for about 0.4 ppm with respect to the values obtained by Bus *et al.* (15). Therefore, the accuracy of the assignment is tested in a better way if the spectrum of oil in the intact fir seed is compared with the spectrum of a sunflower seed which contained predominantly linoleic acid (75%). Chemical shifts agreed within the experimental accuracy ± 0.02 ppm because these two systems were studied under identical conditions. Similarly, peaks at 129.76 and 129.61 ppm represent C_{10} and C_9 in the oleic acid which has one double bond. The assignment is based on the comparison with the model substance (oleic acid methyl ester) and further supported by a perfect agreement with the nondestructive study of single peanut seeds.

Remaining resonances reflect the presence of additional fatty acids X_1 and X_2. X_2 is more abundant and it has three double bonds, contributing to six peaks with chemical shifts 130.15, 129.88, 129.09, 128.95, 128.52, and 127.93 ppm. Signals at 129.88 and 127.93 ppm show an obvious similarity with resonances of C_{13} and C_{12} in the linoleic acid, therefore, one double bond is expected between C_{12} and C_{13}. Other chemical shifts have also been compared with ^{13}C results in possible model compounds (15), and X_2 has been identified as *cis*-5, *cis*-9, *cis*-12-octadecatrienoic fatty acid. Finally, chemical shifts of three well separated smaller resonances at 130.26, 130.19, and 128.83 reveal X_1 as *cis*-5, *cis*-9-octadecadienoic fatty acid (15), while the fourth peak coincides with the stronger response at either 129.09 or 128.95 ppm. Definite determination has not been possible because intensities of peaks are distorted as a result of extensive resolution enhancement. In any

case, the uncertainty of the chemical shift is only 0.14 ppm and it does not preclude the identification of the fatty acid.

Although signals of CH_2 groups are not essential for assignment, they can be utilized for further verification of the presence of various fatty acids. Figure 2 shows a characteristic peak at 25.66 ppm which arises from C_{11} in the linoleic acid and it serves as a relevant indicator. Carbons C_4 and C_3 in acids X_1 and X_2 also have very unique chemical shifts (15), thus giving rise to the signals at 26.55 and 24.80 ppm. These peaks, however, must be regarded only as supporting evidence for the correct assignment, because they do not distinguish between X_1 and X_2. It should also be noted that nondestructive NMR identification of fatty acids agrees with the results of the chemical analysis in similar systems (16).

Esterification of fatty acids to the CH_2-O- or CH-O- groups of the glycerol backbone has a pronounced effect on chemical shifts of carbons C_1 and C_2 which are close to the formed chemical bond. If the spectra of naturally occurring triglycerides in plant seeds are compared with previously assigned resonances of fatty acids (1) or their methyl esters (15), interesting information can be deduced.

Let us first consider a sunflower seed which contained predominantly linoleic acid (75%). The chemical shift of the carbonyl carbon C_1 in the methyl ester is 174.10 ppm (15), while the spectrum of oil in the seed shows two peaks at 171.74 and 171.47 ppm with the intensity ratio 2:1 (Figure 4). The result reveals that esterification of linoleic fatty acid to the CH_2-O- and CH-O- group shifts resonances upfield for 2.36 and 2.63 ppm, respectively. Since two CH_2-O- sites are available, the observed intensity ratio verifies the assignment. Similar effects are also observed at the next carbon C_2 in the chain (Figure 5). Esterification to the CH_2-O- group shifts the resonance upfield for 0.39 ppm and the larger peak is detected at 33.71 ppm. The other resonance at 33.88 ppm reflects attachment to the CH-O-group and an upfield shift of 0.22 ppm. Next carbons (C_3, C_4, etc.) do not reveal any measurable shifts, because substituent effects generally decrease with the increased number of chemical bonds.

The study of the sunflower seed revealed chemical shift differences which are induced by the esterification of linoleic fatty acid. We assumed that the result can be extended to similar systems. Carbons C_2 in *cis*-5, *cis*-9- octadecadienoic and *cis*-5, *cis*-9, *cis*-12-octadecatrienoic acid methyl ester have a distinct signal at 33.55 ppm (15), and esterification to the CH_2-O- group should raise to the resonance at 33.16 ppm. Similarly, the attachment of the CH-O- group should induce an upfield shift of 0.22 ppm and the signal is expected at 33.33 ppm. Experimental results (Figure 5) show only one signal at 33.05 ppm, thus indicating selective esterification to the CH_2-O- group.

This conclusion is firmly supported by additional

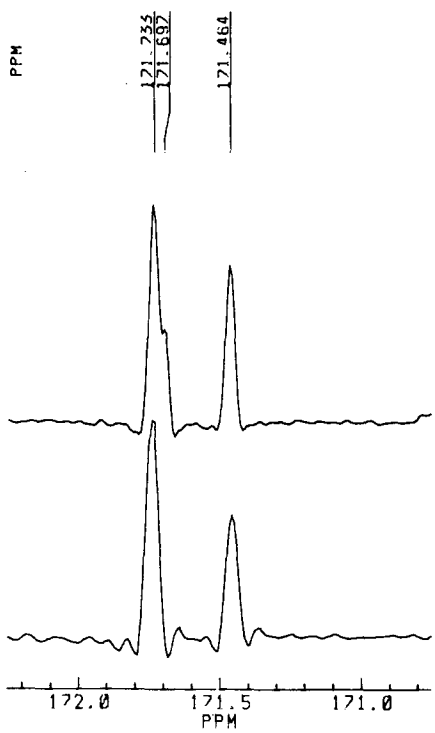

FIG. 4. Signals of carbonyl carbons as obtained in fir (upper trace) and sunflower seeds (bottom trace). Since two CH_2-O- sites are available for esterification, signals on the left are twice as large as the signals at 171.46 ppm which represent carbons C_1 attached to the CH-O- group.

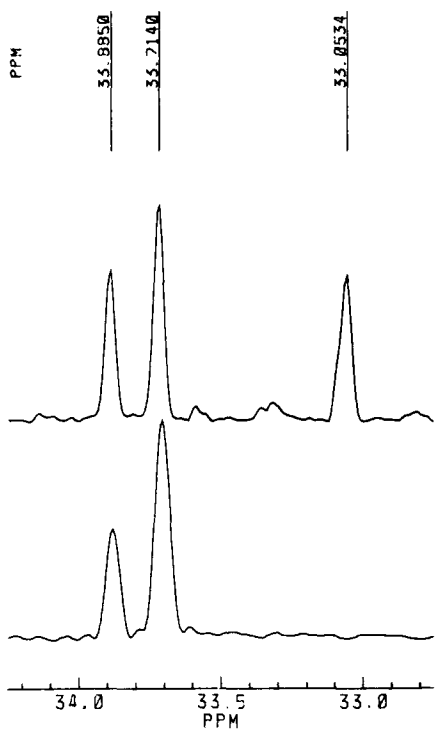

FIG. 5. Comparison of signals C_2 as obtained in fir (upper trace) and sunflower seeds (bottom trace). Esterification of linoleic, oleic, and saturated fatty acids which are found in sunflower gives rise to two peaks representing those carbons which are close to the CH-O- or CH_2O-group, respectively. The upper spectrum shows that the fir seed contains fatty acids X_1 and X_2 with double bonds between C_5 and C_6. Carbon C_2 has a distinctive chemical shift (15) and the resulting peak at 33.05 ppm reveals selective esterification.

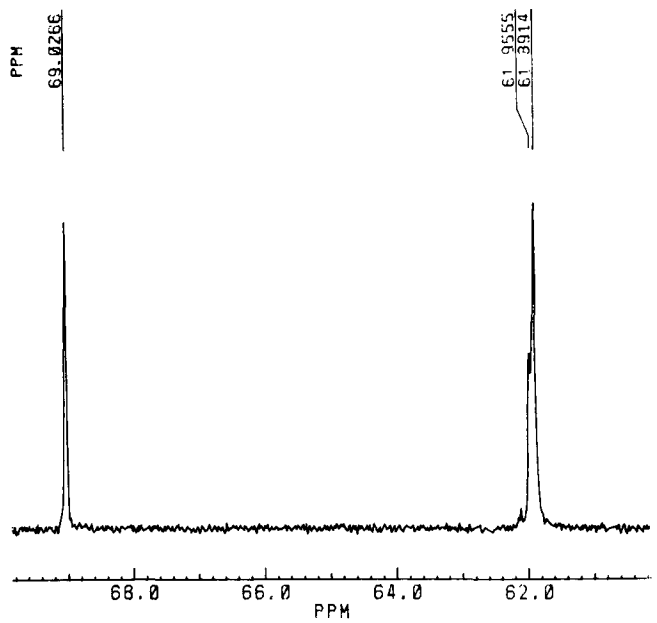

FIG. 6. Signals of carbons CH-O- and CH_2-O- as recorded in a fir seed. Additional hump at 61.96 ppm shows that fatty acids X_1 and X_2 are esterified to the CH_2-O-group. A double bond between carbons C_5 and C_6 produces a small, but measurable difference of chemical shifts. The signal of CH-O- groups at 69.03 ppm is sharp.

experimental evidence. Comparison between the fir and sunflower seeds shows that C_2 resonances of other fatty acids (linoleic, oleic, palmitic, etc.) appear at exactly the same position in both systems. However, the intensity of the peak at 33.71 ppm in fir seeds is reduced because fatty acids X_1 and X_2 misplaced other constituents from the CH_2-O- site.

Carbonyl carbons are also a relevant probe. The upper trace in Figure 4 reveals a slight chemical shift difference for C_1 attached to CH_2-O- groups in fir seeds. The appearance of the smaller peak at 171.70 ppm can be explained by the selective esterification of fatty acids X_1 and X_2.

Finally, the glyceride carbons can be observed (Figure 6) and spectra of fir seeds confirm that different types of fatty acids must be attached to the CH_2-O- group. A small hump (61.96 ppm) is detected on the left side of the resonance at 61.89 ppm, while the signal of the CH-0- group at 69.03 ppm remains very sharp.

Successful application of ^{13}C NMR has revealed preferential esterification of fatty acids X_1 and X_2. It appears very tempting to generalize the above approach by using NMR (including the superior "liquid-state" version) in studies of other oils and fats. Unfortunately, carbons C_2 in many fatty acids (linoleic, oleic, palmitic, stearic, etc.) have exactly the same chemical shift, because double bonds or terminal methyl groups are too far away. Substitution effects are beyond the current detection limit and ^{13}C NMR should not be considered as a generally useful technique for studies of preferential esterification. There are, however, some special systems where the presence of double bonds at "unusual" positions creates sufficient dispersion for those carbons (C_1 and C_2) which are mostly affected.

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