illustrated in Figure 11 involves setting the monitor analogue on mass 15 (that mass due to methyl ion) and plotting its intensity on one channel of a 2-channel recorder. Plotted alongside on the second channel is the conventional curve for the thermal conductivity current. The mass spectrometer sensitivity is comparable with that of thermal conductivity feeding a 10-mv. recorder if a 1,000 fold attenuation of the analogue signal is used.

New developments in the technique of mass spectrometry, such as heated inlets, ion sources, flight tubes and increased speeds of recording spectra, should open new areas of applicability for the comparatively old tool of mass spectrometry in the field of lipid research.

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Nuclear Magnetic Resonance in Lipid Analysis[1]

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NUCLEAR magnetic resonance was detected in 1945 and has been used in organic chemistry problems since about 1953. Its applications have increased rapidly, coincidental with improvement in design of equipment and familiarity with the technique of its operation.

The phenomenon is based on the fact that the nuclei of many atoms have a spinning motion which causes them to behave as tiny magnets. Thus they are affected by any applied magnetic field. If, in addition to this magnetic field, there is applied an oscillating field in the radio frequency range, the nucleus will resonate between different energy levels at a definite frequency. In doing so, it absorbs a little of the r.f. energy. This minute change in energy can be amplified and recorded on a chart.

Although a number of different atoms exhibit nuclear magnetic resonance, the hydrogen atom has been the subject of most investigations and it is hydrogen or proton magnetic resonance that is dealt with here. The resonance frequency of the hydrogen atom is altered by a change in its chemical environment. Thus, if the resonance frequency of an isolated hydrogen atom is taken as a reference point, the corresponding frequency for a hydrogen atom in a given compound, e.g. chloroform, will differ by a measurable value and this value is called the chemical shift. If there are several hydrogen atoms in the compound, every one that has an appreciably different environment will produce a separate peak in the nmr spectrum of the compound. Thus there is a measurable chemical shift for each kind or species of hydrogen in a compound, depending on the influence of the neighboring atoms or groups.

Units of Measurement

The terminology and units of measurement are more involved than those in other types of spectra. Two parameters are utilized, the chemical shift and the coupling constant. Briefly it may be said that the chemical shift is the position of the peak in the spectrum, while the coupling constant is the spacing between two peaks arising from a single proton or group of closely-related protons (as a result of spin-spin coupling). It is not feasible to measure either of these two parameters in absolute values, hence they are usually reported as parts per million of the total magnetic field (in gauss or milligauss) measured from a reference point in the spectrum. The coupling constant is often expressed as frequency in cycles per second. This value can be calculated from the relation $C = SF$, where C is the coupling constant, S is the peak spacing in parts per million and F is the frequency of the applied field in megacycles per second.

The reference material commonly used is tetramethylsilane, $Si(CH_3)_4$. Its hydrogen atoms are practically equivalent so that it produces only one sharp peak. The position of this peak is arbitrarily taken as 10 ppm. The remainder of the scale is calibrated each time; there is no printed scale on the chart. The horizontal axis of the chart represents magnetic field strength and the vertical axis represents the amplitude of the response or signal.

The area under the peak constitutes another measurable value, being proportional to the number of hydrogen atoms in the particular group. Hence area measurements can be employed, with some reservations, to aid in the identification of functional groups or to make quantitative estimates of the proportion of a component in a mixture. However this phase of the subject is still under study and all of the factors involved in quantitative measurements are not fully understood.

Apparatus and Sample Preparation

The apparatus consists of a very powerful magnet, a radio frequency generator, and a radio frequency detecting system with a recorder. If the magnetic field is held constant and the frequency of the oscillating field is varied, resonance signals from the sample will be detected and recorded. In practice it is

[1] Issued as N.R.C. No. 6464.

more convenient to maintain the oscillating field at constant frequency and to vary the current on the large d.c. magnet, but the result is the same.

The sample is placed in a glass tube of 5 mm. diameter and placed between the poles of the magnet. The tube is spun by an air jet in order to average out variations in the magnetic field. The current to the magnet is then varied so as to "sweep" the desired portion of the field and the resultant tracing on the recorder constitutes the nmr spectrum of the sample. A larger sample is required for nmr than for infrared or ultraviolet examination; the minimum size for long-chain fatty acids is at present about 0.1 g.

The spectra are nearly always determined in a solvent, which must be chosen to have as little interference as possible with the spectrum of the sample. Carbon tetrachloride, having no protons, gives no peaks. Chloroform, carefully purified, is a good solvent. It gives a single peak at 2.75 parts per million which is usually clear of the sample peaks. Ethyl alcohol is present in many grades of chloroform and must be removed. Other solvents are benzene, toluene, and water. The characteristic peaks of these liquids are easily recognized. However water gives a broad band which may hide the sample peaks. Deuterium oxide may be used in place of water. It gives no peaks in the region of proton resonance. Deuterated acetone, chloroform, etc. are also available.

The solution strength may be from 5 to 30% by weight. The lower concentrations give more reliable values for the chemical shift. However they require greater signal amplification with consequent baseline instability and prominence of any impurities in the solvent.

A drop of the reference standard, tetramethylsilane, may be added to the solution or it may be placed in a closed capillary tube in the sample tube. The sample under test is not affected by the analysis and can be recovered completely. When carrying out a series of nmr analyses, it is desirable to use the same solvent, the same concentration, and the same settings of the equipment, as far as possible. This makes it easier to interpret and compare the spectra. The sample should be as pure as possible, although small amounts of impurities are less apt to cause trouble than in infrared or UV analyses. Any substance present in an amount less than 2% of the total sample is not readily detected by the equipment now in use.

Spectra of Typical Fatty Acids and Related Compounds

In general, the spectrum of a fatty acid consists of a number of peaks, each produced by a hydrogen nucleus or proton of different character. The position of each peak provides evidence for the chemical environment of that proton and the area under the peak gives an indication of the number of protons of the same kind.

The spectra of some simple esters, determined with low resolution equipment, are shown in Figure 1. The three peaks at the right show the typical pattern of a straight chain fatty acid or ester. In the methyl oleate spectrum one sees the terminal methyl peak at 9.1 ppm., the large peak which represents the ordinary CH_2 groups of the chain at 8.7, and the third peak due to the αCH_2 groups at about 8.0. The last includes the CH_2 adjacent to the carbonyl group and

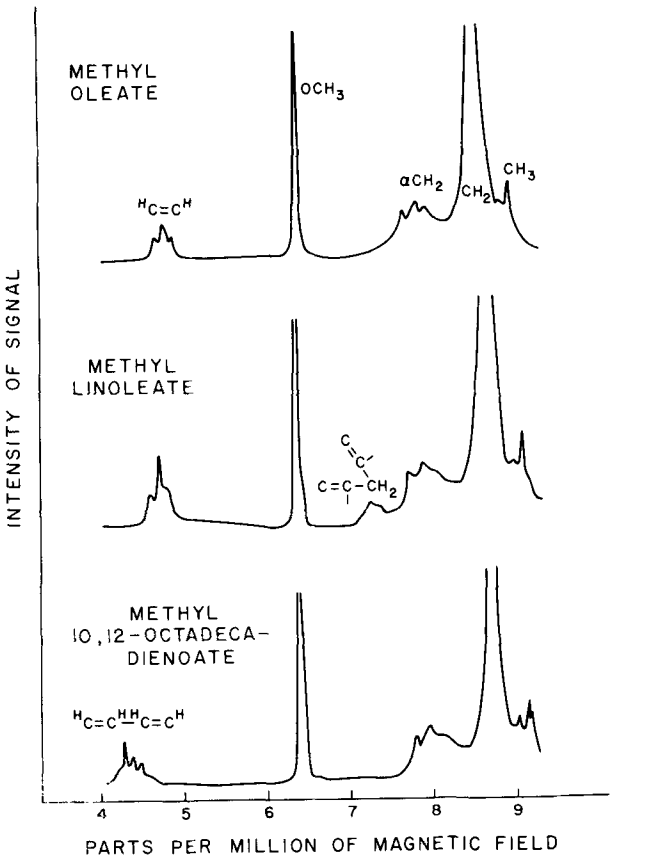

FIG. 1. Proton resonance spectra of methyl esters, determined in chloroform solution at low resolution. Oscillator frequency 40 megacycles/sec.

the two CH_2 groups adjacent to the double bond carbons. The remaining peaks represent the CH_3 of the methyl ester grouping (a very sharp peak at 6.3), and the olefinic (CH = CH) group at 4.7. The position of these peaks may be somewhat different in acids of short chain length.

Methyl linoleate has the additional peak at 7.2 due to the CH_2 group between the two double bonds. In the conjugated ester, methyl 10,12-octadecadienoate, this peak is of course absent. Further, the signal due to the conjugated double bond grouping is at 4.2, not 4.7 (1).

It is not possible to determine the position of a double bond in the chain by nmr unless it is very close to one end or the other of the chain. Similarly, there would be no appreciable difference between the spectra of linoleic acid and 6,9-octadecadienoic acid. However it would be possible to distinguish between a 1,4-diene acid, such as linoleic, and a diene acid with more widely separated double bonds, e.g. 5,11-octadecadienoic acid. The latter would have a peak at 4.7 equivalent in area to two olefinic groupings but there would be none at 7.2 since there is no activated CH_2 as in linoleic acid.

In linolenic acid, the ordinary terminal methyl signal is altered by the proximity of the 15,16-double bond (2) so that this grouping ($CH_3CH_2CH=CH-$) can be recognized from the spectrum.

Triglyceride oils, shown in Figure 2, have the basic pattern of three peaks at the right due to the fatty acid part of the molecule. The glycerol part gives rise to two peaks, one at 4.8 (CH–O) and one at 5.8

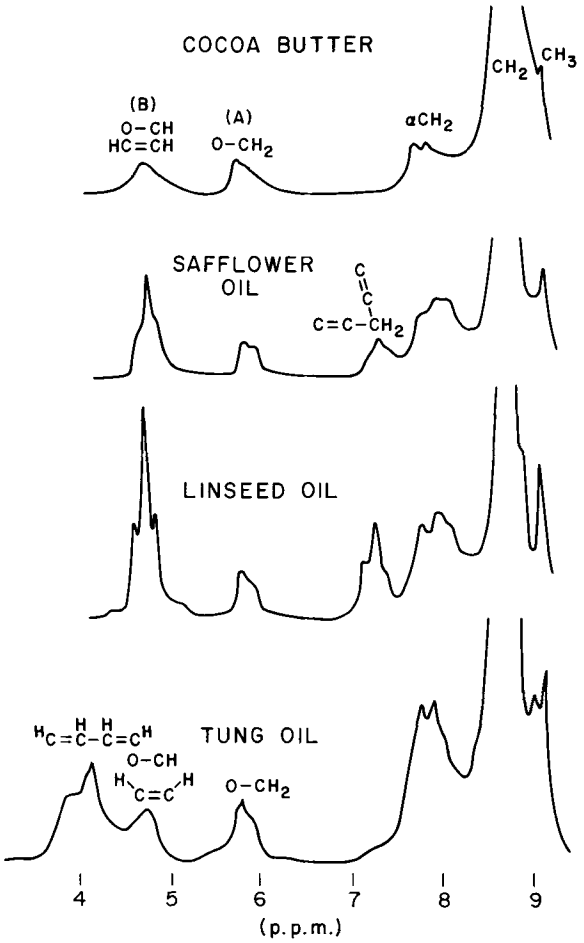

FIG. 2. Spectra of glyceride oils, determined under the same conditions as in Fig. 1.

(CH_2–O). The latter is useful in comparing the areas of peaks since it represents 4 hydrogens, i.e. the two CH_2 groups of the glycerol (1). The CH–O at 4.8 overlaps the olefinic peak. If there were no olefinic acid in the oil, peak A would have 4 times the area of peak B. The spectra for safflower and linseed oils show the large content of 1,4-diene acid (linoleic) by the peak at 7.2. Tung oil displays its large proportion of conjugated acid at 4.1 and a small proportion of olefinic acid at 4.7.

Mono- and di-glycerides have not been studied thoroughly as yet, partly because of the difficulty of obtaining pure samples. In the spectra of 1,2-distearin (Figure 3), the peaks for the glycerol groups are apparently at 5.9 (position 1), 5.2 (position 2), and 6.4 position 3, unesterified). The OH signal may be off-scale or hidden by one of the other peaks.

The hydrogens of COOH and OH groups do not give peaks in specific positions. Their peaks vary in position depending on the concentration of the sample solution. This is a result of association by hydrogen bonding. In most spectra of acids, the carboxyl peak is at very low field and is not shown. 12-Hydroxystearic acid (Figure 4) shows the hydroxyl OH peak at 3.3 at the concentration used in this determination. It is convenient to identify an OH signal by determining the spectra at two different concentrations. The peak which shifts is due to OH. It will be noted that the CH to which the OH is attached gives a distinctive signal at 6.4.

Stearolic acid (Figure 5) has no peak corresponding to the acetylenic group since there are no hydrogens attached to the C ≡ C carbons. A terminal acetylene group (C ≡ CH) has a signal at about 7.5. A terminal olefinic group (Figure 6) appears in the usual olefine region, 4–5 ppm., but separate signals are recorded for the CH_2 and the CH of this grouping. At high resolution, the complex coupling forces are indicated by the splitting of the peaks. Absence of the usual terminal methyl group is apparent in this spectrum. The minor signal at 9.1 may be due to a small amount of saturated acid as impurity. The sharp peak at 10.0 in these spectra is produced by the reference material, tetramethylsilane, which has been added to the sample.

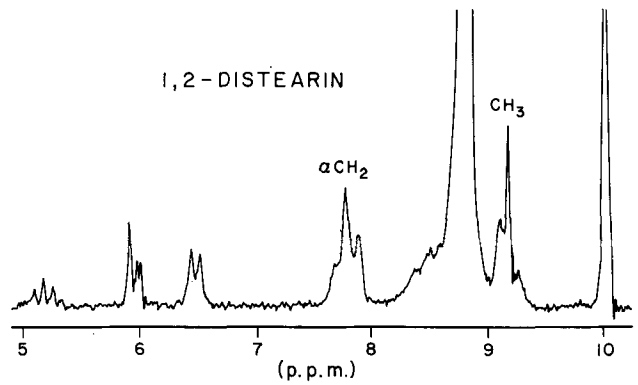

FIG. 3. Spectrum of 1,2-distearin, determined at high resolution. Oscillator frequency 60 megacycles/sec.

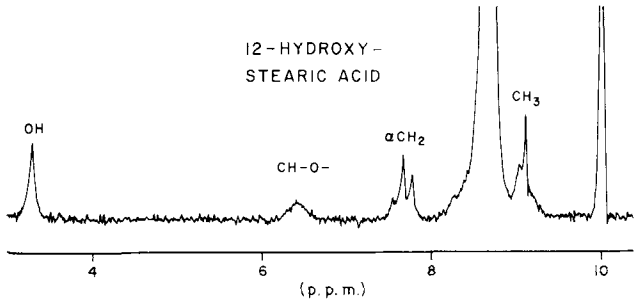

FIG. 4. Spectrum of an hydroxy acid, showing two peaks produced by the two different hydrogens of the CHOH group.

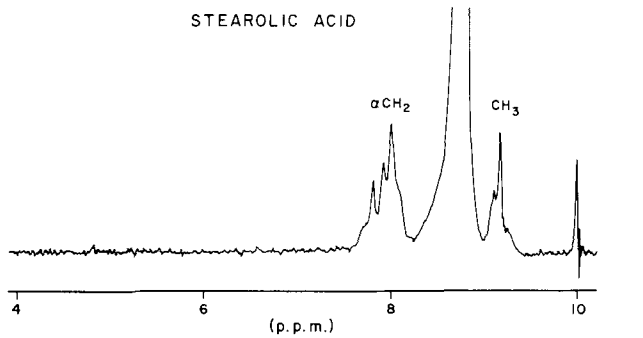

FIG. 5. Spectrum of an acetylenic acid. Methylene groups adjacent to the triple bond grouping are included in the αCH_2 band.

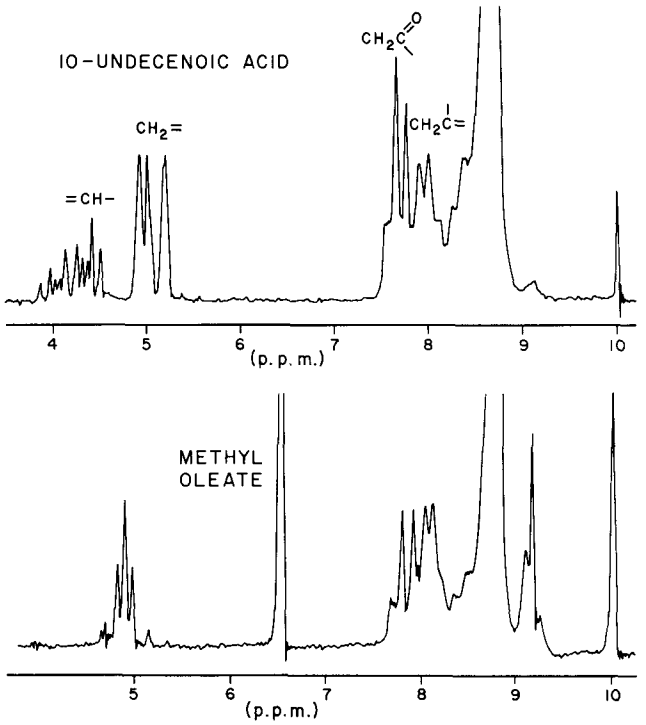

FIG. 6. Spectrum of an acid with a terminal vinyl group and a spectrum of methyl oleate, both at high resolution.

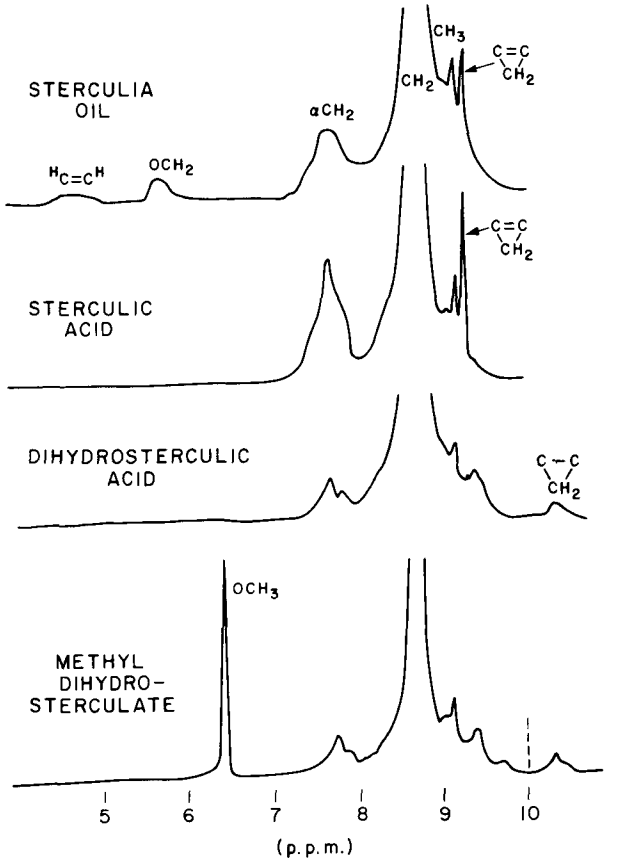

FIG. 7. Spectra of sterculic acid, sterculia oil (from seed of *Sterculia foetida*), and derivatives.

Detection of Functional Groups

NMR was utilized in determining the structure of sterculic acid (3,1). Although formula I was believed to be correct, there was some evidence for formula II.

$$CH_3(CH_2)_7C{=}C(CH_2)_7COOH. \qquad \text{I}$$
$$\diagdown\diagup$$
$$CH_2$$

$$CH_3(CH_2)_5CH-CH\,CH{=}CH(CH_2)_7COOH. \qquad \text{II}$$
$$\diagdown\diagup$$
$$CH_2$$

The nmr spectrum of sterculic acid (Figure 7) shows no peak for ordinary olefinic unsaturation at 4.7 and none for the cyclopropane ring at 10.3, hence structure II cannot be correct. The spectrum does show a sharp peak at 9.2, indicative of the CH_2 of a cyclopropene group. Thus the nmr evidence provided convincing proof for structure I and this has since been confirmed by synthesis.

The seed oil of *Hibiscus syriacus* shows a peak at 9.2 in its nmr spectrum (Figure 8), suggesting the presence of a cyclopropene group. The occurrence of the cyclopropenoid acid, malvalic, in this species has been demonstrated by C.R. Smith and co-workers (4).

Examples of peak positions for various groups in fatty acid molecules are given in Table I. Some variation in these values is to be expected, depending on chain length and neighboring groups. In addition to those shown, there would be peaks for the hydrogen of amine, mercaptan, aldehyde, aromatic rings, and in fact for hydrogen in any combination.

It is evident therefore that nmr spectra can be employed to detect or differentiate between a number of functional groups such as hydroxy, epoxy, ester, and ether groups; olefinic, conjugated, or 1,4-diene unsaturation; terminal acetylenic or vinyl groups; branched chains, cyclopropane, cyclopropene, and aromatic rings. It is also possible to obtain evidence to distinguish between *cis* and *trans* isomers (5), keto and enol forms, and certain stereoisomers.

Many or most of these determinations can be made by other methods. However nmr has the advantage that the sample is recovered unchanged and in many instances the determination is more rapid, requiring only a few minutes if the equipment is ready to operate. Certain structural problems that are resolvable by nmr would be difficult or impossible to solve by other methods. A further advantage is that several items of information can be obtained from a

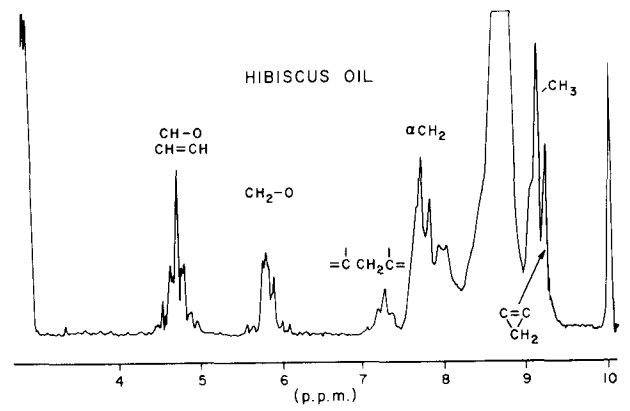

FIG. 8. Spectrum of the seed oil of *Hibiscus syriacus*.

TABLE I

Approximate Peak Positions of Groups in Fatty Acids and Esters Expressed as parts per million relative to tetramethylsilane = 10.00

2–4	α,β-unsaturation
2.75	(chloroform)
4.2	conjugated double bonds in carbon chain
4.7	ethylenic double bond
4.8	CHO of glyceride
5	vinyl CH_2=
5.8	CH_2O of glyceride
6.3	CH_3 of methyl ester
6.4	CH of CHOH in chain (or $\overline{CH}OCOCH_3$)
7	CH—CH in epoxy-ring
7.2	CH_2 of 1,4-diene (=$CHCH_2CH$=)
7.5	CH≡, acetylenic
7.7	CH_2 adjacent to carboxyl
7.9	CH_3 of acetyl group
8	CH_2 adjacent to —CH= in chain
8.2	CH_3 attached to ethylenic C
8.7	CH_2 of carbon chain
9.1	terminal CH_3
9.2	CH_2 of cyclopropene ring
9.4, 10.3	cyclopropane ring

single spectrum. Thus in Figure 8 there is evidence of a cyclopropene group [9.2], which could be confirmed simply by the Halphen test or by infrared. There is seen also the normal terminal methyl [at 9.1]; the 1,4-diene [7.2], probably linoleic; absence of CHOH and OCH_3 groups [about 6.4]; a large amount of olefinic unsaturation [4.7]; and absence of conjugated double bonds [4.2]. A rough quantitative estimate could be made of the proportions of monoenoic, dienoic, and cyclopropene acids by calculation from the areas under the respective peaks.

General Nature of NMR Spectra

The technique can be regarded as supplementing rather than replacing infrared and ultraviolet absorption analysis although it may well have a wider scope than either of these. In its quantitative aspects it is less precise than ultraviolet analysis, but of about the same order as infrared. Interpretation of the spectra is generally not difficult if the spectra of 2 or 3 known related compounds are available. The nmr spectrum is less complex than infrared and much information can be obtained from the structure of the peaks as well as from their position in the field. Although peak position has been emphasized in this review, recent developments in nmr as applied to organic compounds (7,8) show that the splitting of peaks due to spin coupling, with measurement of the resultant spacings, is of at least equal importance.

Another possible field of use lies in the spectra of solid fatty acids and glycerides (without solvent). Some work has been done in this area.

A concise introduction to the subject of nmr spectra is given in the chapter by R.T. O'Connor in Vol. I of Markley's "Fatty Acids" (5). A more extended treatment is offered in Conroy's chapter on nmr in Vol. II of Raphael's "Advances in Organic Chemistry" (6). A recent complete text is (7). Some applications to fatty acid chemistry are described in (1) and (2).

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