Some Applications of Mass Spectrometry to Lipid Research[1]

HERBERT J. DUTTON, Northern Regional Research Laboratory,[1 e] Peoria, Illinois

THIS PAPER reviews recent applications of mass spectometry to lipids and projects certain possible future applications. Since an excellent review (1) emphasizing structural applications to mass spectrometry in lipid research by foremost contributors to the field has recently appeared, this topic will be given only cursory treatment and recent applications complementary to those already reviewed will be included.

Operation of Mass Spectrometer. Briefly stated, mass spectrometry consists in (a) volatilization of the sample into vacuum, (b) conduction of the sample to the ion source of the mass spectrometer where it is bombarded with a stream of electrons to produce ionized molecules and fragments of molecules, (c) electrostatic acceleration and dispersion of the ions with or without magnetic deflection, and (d) detection of the kinds and amounts of ions dispersed. For illustrative purposes an outline of operation for the "Time-of-Flight" type of mass spectrometer (2), which does not employ magnetic deflection, will be given (Fig. 1).

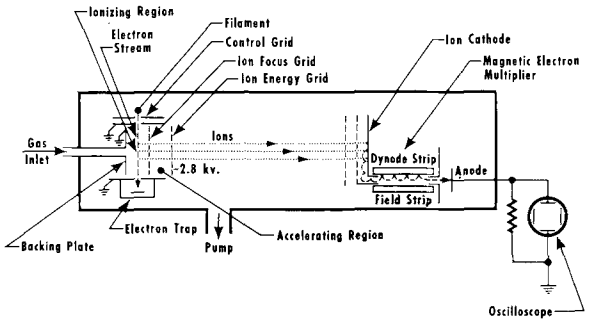

FIG. 1. Schematic of "Time-of-Flight" mass spectrometer.

The lipid sample is vaporized into vacuum to give a pressure of about 0.1 mm. and is led through a leak or pinhole in a gold leaf foil into the ion source region where pressures of 10^{-6} to 10^{-7} mm. are maintained. As shown in Fig. 1, a narrow beam of electrons from a heated filament bombard the stream of incoming molecules to produce ionized molecules and fragments. In the time-of-flight optics an accelerating voltage of 2,800 volts is used to accelerate a pulse of ions down the flight tube. Since all ions experience the same electrostatic field, it is apparent that the lighter ions (more correctly those with the lower mass-to-charge ratio) will be given a greater acceleration than the heavier ions. Having been imparted their respective velocities, the ions are now permitted to "float" down the flight tube, thereby achieving their dispersion in terms of time of arrival at the receptor. The number of ions arriving at the end of the flight tube is detected by a magnetic type of electron multiplier. This information is displayed on an oscillograph screen with time of ion arrival, which is in proportion to the square root of the mass ($\sqrt{m}$), impressed on the horizontal plates (abscissa), and ion current or number of ions impressed on the vertical plates (ordinant). Alternatively, or coincidentally, the same mass spectral data may also be presented through analogue amplifiers into standard strip-chart recorders. A photograph of the time-of-flight-type mass spectrometer as installed at the Northern Regional Research Laboratory is shown in Fig. 2. At the left

FIG. 2. Installation of mass spectrometer at the Northern Laboratory.

is a heated inlet system, which provides for the expansion of, for example, 5 μl. of fatty acid methyl ester, into 5-l. volume at 200°C. to produce the desired pressure of 0.1 mm.

After passing through the molecular leak made of gold leaf foil, the gas stream is led to the mass spectrometer proper, shown in the center of the photograph. At the extreme right is the oscilloscope, equipped with a Polaroid camera for rapid recording of mass spectra. Between the oscilloscope and the mass spectrometer is the two-channel, strip-chart recorder used for presenting mass-spectral information produced by the analogue amplifiers.

Application of mass spectrometry may be classified as to its use in structural determination, qualitative identification, and quantitative analysis. Usually, however, two or three of these elements are present in any given application, and no attempt will be made here to separate them in the following examples.

Interpretation of the spectra of methyl stearate is essential to understanding of subsequent applications involving isotopic labeling and will serve to illustrate

[1] This is a laboratory of the Northern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

the type of structural interpretations, which are made (1). The first point of interest (Fig. 3) is the presence of the parent peak at mass 298. Fortunately, in many lipid materials of interest, parent peaks are present and immediately provide the investigator with the molecular weight of this material. This parent ion results from an electron "hit" on the molecule, this interaction releasing two electrons and giving the molecule a net positive charge of one. Splitting also occurs under electron bombardment, and the

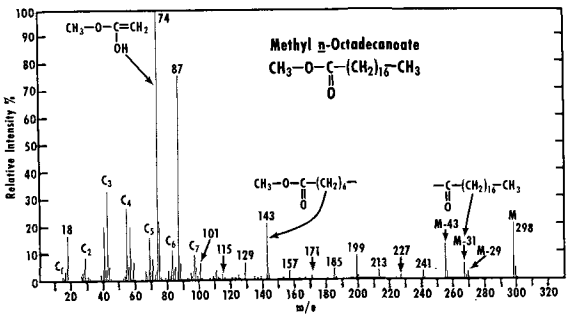

FIG. 3. Mass spectrum methyl octadecanoate.

fragmentation pattern observed is characteristic of the molecule being broken. This pattern may be used for fingerprint type of empirical comparison and identification of compounds. However, these spectral data in the hands of experienced mass spectroscopists frequently may permit resynthesis of the parent compound from its observed fragments.

In general, the most prominent peaks to be observed in the mass spectra of fatty acid methyl esters are those of the oxygen-containing fragment which has the formula:

$$[CH_3-O-\overset{\overset{O}{\|}}{C}-(CH_2)_n-]^+ \qquad (n>1)$$

Superficially at least, this is indicative of a simple cleavage type of fragmentation (see below).

The prominent peak at mass 267 (M-31), is that assigned to the fragment generated by the loss of an acylium ion. The ion of greatest abundance, referred to as the base peak, comes from the 2,3-cleavage and loss of one hydrogen.

$$CH_3-\overset{\overset{O}{\|}}{\overset{1}{C}}-\overset{2}{C}H_2-\overset{3}{C}H_2-\overset{4}{C}H_2R \longrightarrow CH_3-\overset{\overset{O}{\|}}{\overset{1}{C}}-\overset{\overset{H}{|}}{O}+\overset{2}{C}H_3+\overset{3}{C}H_2=\overset{4}{C}H-R$$

In a manner similar to that described for methyl stearate, the dominant peaks of methyl oleate, methyl linoleate, and methyl linolenate (Fig. 4) are those of the oxygen containing fragments and are indicative of simple rupture of the carbon-carbon bonds. Of course, these three spectra differ significantly in the position of the parent peaks that are found at mass 296, 294, and 292, corresponding to their respective molecular weights.

It has been established for C_{18} monoenes, unfortunately, that *trans* and *cis* isomers and that isomers of double bond position, other than in the α and β position, give practically identical spectra. It is postulated that in its activated state before bond rupture and fragmentation, the monounsaturated molecule must assume a generalized statistical structure that is independent of the initial position or configuration of the double bond.

From these considerations, the potential application of mass spectrometry to the important problem of locating double-bond positions would seem to be obviated. However, Dinh-Nguyen, Ryhage and Stenhagen have recently shown, by first saturating the double bonds with deuterium, that location of the double bonds may be deduced at least in petroselenate, oleate, and elaidate (3). Because of the uncomplicated nature of the hydrazine reduction reaction (4), the use of tetradeuterohydrazine deuterate affords an excellent procedure for adding deuterium double bonds. In deuterated petroselenate (methyl 6,7-dideuterostearate), the fragment corresponding to 6 carbon atoms of the acid now contains one deuterium atom and is therefore at mass 130 rather than 129 when derived from stearate (Fig. 5). In a similar manner the C_7 fragments and those higher contain two deuterium atoms and are therefore two mass units higher than the corresponding stearate fragments. This "two-higher mass" also applies to the

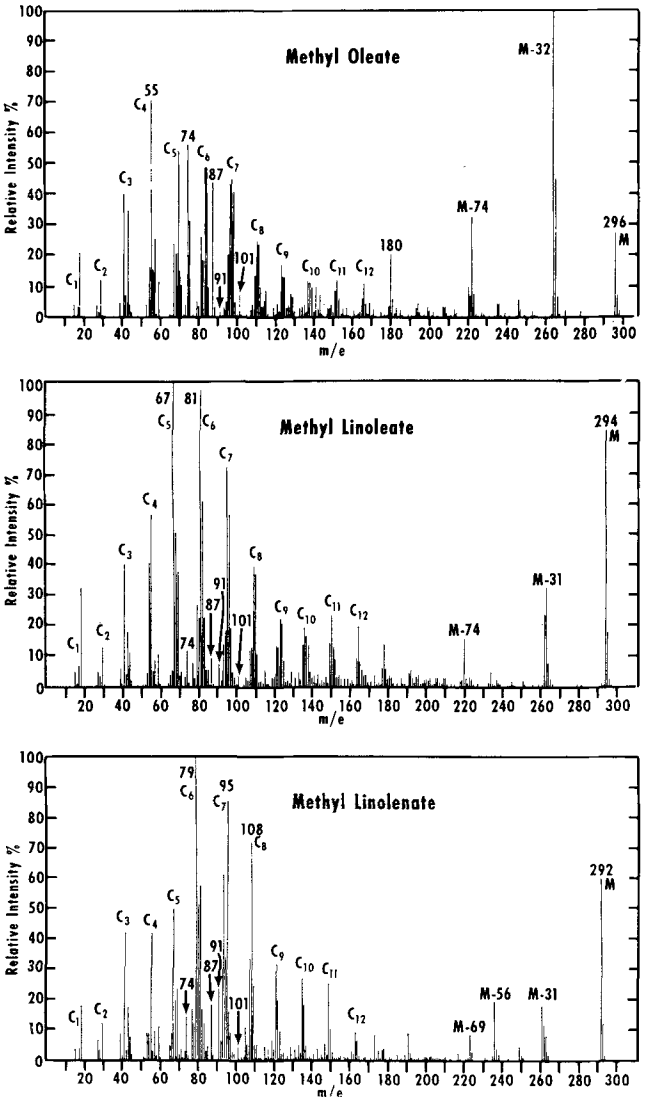

FIG. 4. Mass spectra of methyl oleate, methyl linoleate, and methyl linolenate.

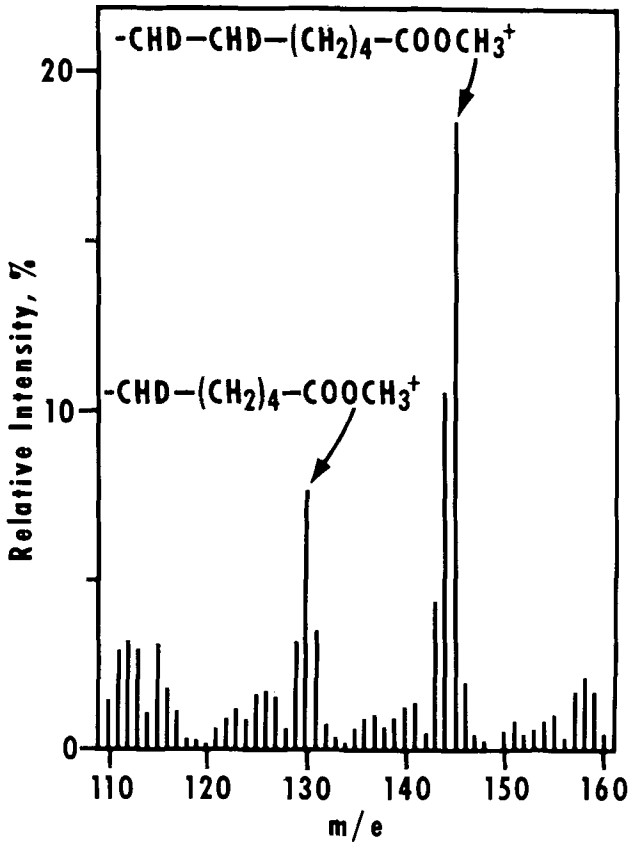

FIG. 5. Central portion of the mass spectrum for methyl 6,7-dideuterostearate.

parent ions for methyl stearate and dideuterostearate at mass 298 and 300, respectively.

An analogue tracing from the time-of-flight spectrometer on a mixture of methyl stearate and methyl 6,7-dideuterostearate is given in Fig. 6, and the corresponding photograph of the oscillographic pattern is given in Fig. 7. These data confirm, with a nonmagnetic type of spectrometer, the prior obesrvations (3) of workers in Sweden. Figures 6 and 7 also give the spectra for a mixture of methyl stearate and methyl 6,7-dideuterostearate. Parent peaks at masses 298 and 300, C_7 fragments at 143 and 145, and C_6 fragments at 129 and 130 derived from methyl stearate and methyl 6,7-dideuterostearate, respectively, are readily apparent.

Two factors may modify the simple application of this isotope-labeling procedure for the location of double bonds. One is the relatively low production of ions corresponding to C_{10} (mass 199) and higher fragments; the second is the observation (1) that two and three carbon atom fragments are known to be lost from the middle of the chain, and recombination gives the long-chain fragments observed. While the latter factor does not necessarily vitiate the possibilities of developing an analytical method it does require the synthesis and studies of the individual members of the isologous series of monoenes after deuteration. This research is currently underway (5).

A long-range expectancy for mass spectrometry would be, for example, the analysis of an 11-component mixture of monoenes such as has been isolated by countercurrent distribution from hydrogenated soybean oil (6). Since 10 to 30 component mixtures are routinely analyzed by mass spectrometry in the petroleum industry, this expectancy may still have a reasonable chance for realization.

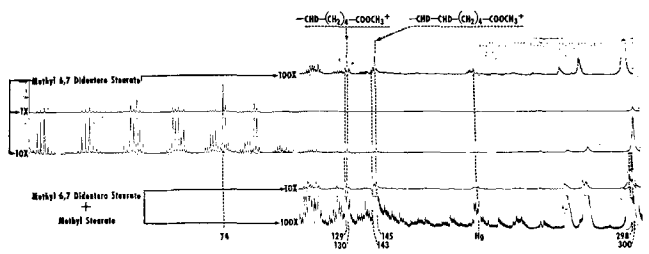

FIG. 6. Analogue tracing of mass spectra for methyl 6,7-dideuterostearate (topmost 3 curves) and a mixture of methyl 6,7-dideuterostearate (lower 2 curves).

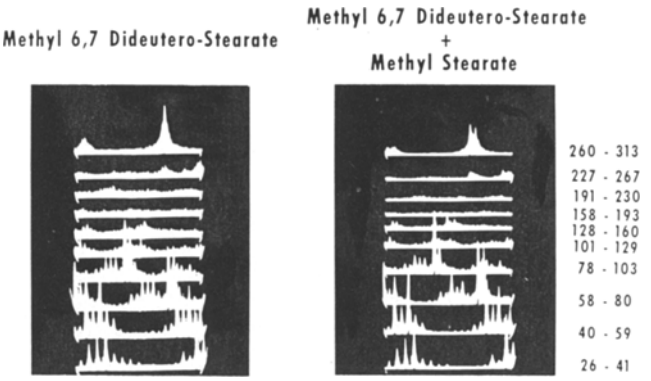

FIG. 7. Photograph of oscillographic tracings for methyl 6,7-dideuterostearate and a mixture of methyl 6,7-dideuterostearate and methyl stearate.

The complexity of reactions involved in catalytic hydrogenation has been effectively demonstrated (7). Catalytic deuteration of methyl oleate with platinum catalyst gives the expected parent peak at 300 but gives in addition the succession of unit higher mass peaks of decreasing intensities, which result from substitution of deuterium for hydrogen by a dehydrogenation-deuteration process (Fig. 8).

Analytical applications of mass spectroscopy to lipids are largely yet to be made. Hallgren has suggested (8) the possibility for the analysis of simple mixtures of methyl esters of the naturally occurring

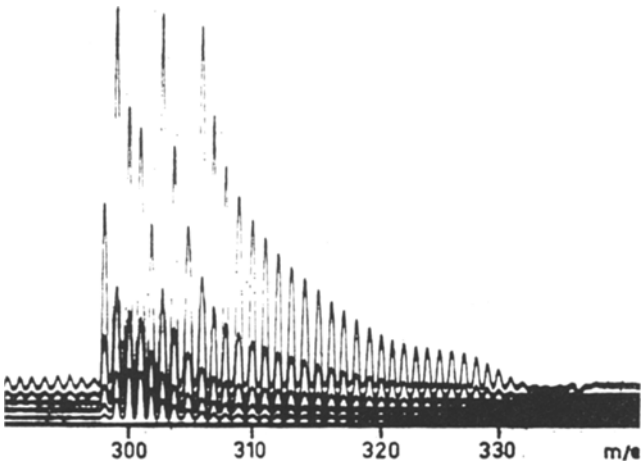

FIG. 8. High mass end of mass spectrum of catalytically deuterated methyl oleate.

C_{18} fatty acids. In systems such as this the intensity of the parent peak multiplied by the appropriate factor characteristic for the individual ester gives its molar percentage. It is not necessary, although desirable, that parent peaks occur, nor even that peaks unique to a compound occur. The intensity at a given mass number contributed by the several compounds present is individually independent in formation and therefore is additive. This property of mass spectra allows one to set up linear simultaneous equations such that when the number of mass intensity observations and equations are equal to the number of components comprising the mixture, the system may be solved. Genge has shown that the analysis of a sample of methyl esters over a period of days is reproducible and accurate (9). Other forms of analysis however are available, adequate, and certainly less demanding in equipment for fatty-acid, methyl-ester analysis. According to unpublished work of Genge, mass spectroscopy may be applicable directly to phospholipids and triglycerides composed of higher fatty acids. Moreover, selective fragmentations observed may provide information on the position of specific fatty acids in the glyceride molecule. Sterols, mono- and diglycerides, and terpenes are among the high-boiling compounds not normally thought to be amenable to mass spectrometric analysis, which have been studied (1, 9).

A recent and most promising application of mass spectrometry is coupling it in tandem to gas chromatography (10) (Fig. 9). These two techniques are

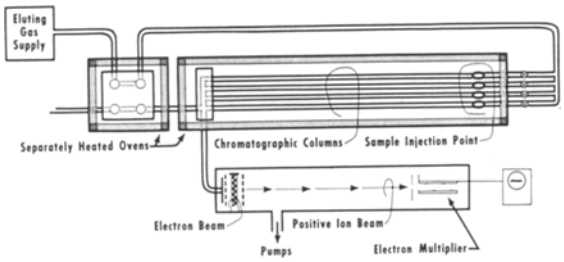

FIG. 9. Schematic view of method of attachment of Time-of-Flight mass spectrometer to gas chromatographic apparatus.

mutually complementary in their resolving power on the one hand, and identification capacity, on the other. The gas chromatographic instrument tells how much of each component and how many components are present, and the mass spectrometric instrument gives qualitative identification data. As Wiley has shown, the time-of-flight instrument providing 10,000 individual analyses per second (2) is particularly adapted to this operation. Use of a standard Polaroid camera allows successive spectra to be photographed with sufficient rapidity for most operations of monitoring and without resort to the movie camera or similar high-speed equipment.

Spectra are frequently photographed for each side of a chromatographic peak as well as at the maximum to test the homogeneity of the peak. One such application has been recorded in our laboratory (11) for the Phillips mixture of hydrocarbons No. 37. Mass spectra recorded the build-up and decrease of the parent peak of mass 56 on the fore side of the chromatographic peak and the build-up and decrease of mass 54 on the down side (Fig. 10). This normal

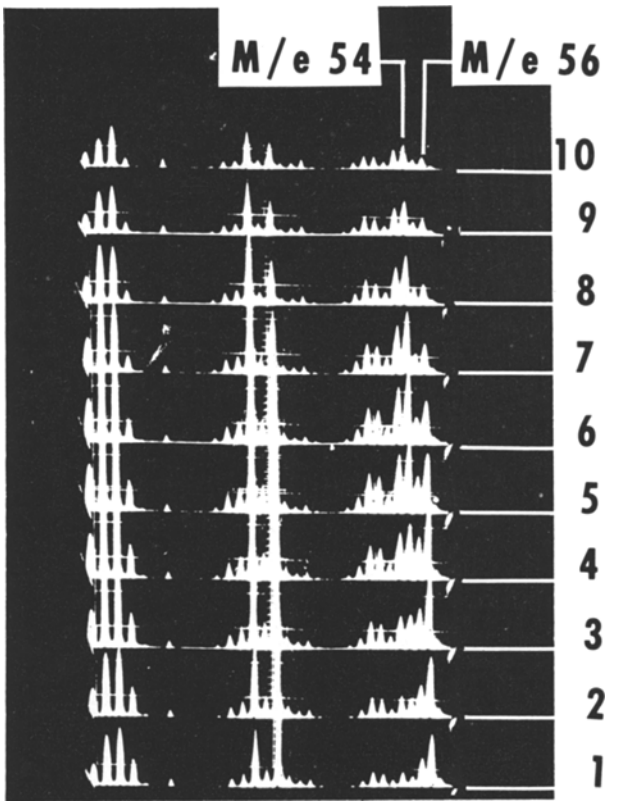

FIG. 10. Mass spectra photographed at successive 15-second intervals during the elution of the *cis*, butene-2 and butadiene peak.

curve for GLC, which seemed to be composed of a single component (Fig. 11), actually was comprised of two overlapping distribution peaks, those for *cis* butene-2 and for butadiene.

An alternative to the simultaneous monitoring of peaks is, of course, the collection of peaks from the chromatogram and their subsequent introduction to the mass spectrometer (12). While advantages may be claimed for each procedure, it is apparent that the advantages of coincident observations during the elution of a peak as just illustrated can only be obtained by the simultaneous tandem-monitoring arrangement. Incidentally, it has been found that the mass spectrograph comprises a chromatographic "detector" of extremely high sensitivity. The system of operation

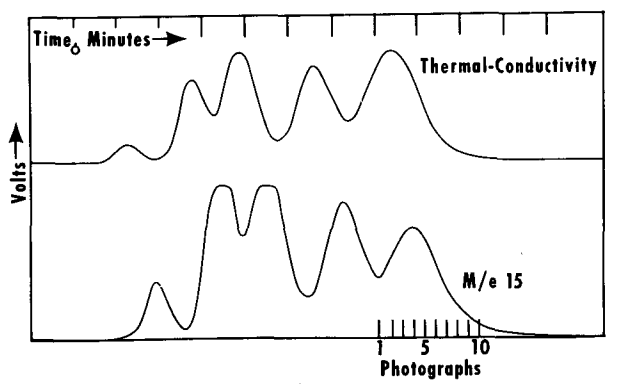

FIG. 11. Chart of simultaneous recording for (a) thermal conductivity detector, (b) mass spectrometric detector (m/e −15).

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.

REFERENCES

1. Ryhage, R., and Stenhagen, E., J. Lipid Res., *1*, 361-390 (1960).
2. Wiley, W. C., and McLaren, I. H., Rev. Sci. Instr., *26*, 1150 (1955).
3. Dinh-Nguyen, N., Ryhage, R., and Stenhagen, E., Arkiv. för Kemi., *15*, 433 (1959).
4. Scholfield, C. R., Jones, E. P., Nowakowska, Janina, Selke, E., and Dutton, H. J., J. Am. Oil Chemists' Soc., *38*, 208 (1961).
5. Stenhagen, E. Private communication.
6. Scholfield, C. R., Jones, E. P., Nowakowska, Janina, Selke, E., Sreenivasan, B., and Dutton, H. J., J. Am. Oil Chemists' Soc., *37*, 579 (1960).
7. Dinh-Nguyen, N., and Ryhage, R., Acta Chem. Scand., *13*, 1032 (1959).
8. Hallgren, B., Ryhage, R., and Stenhagen, E., Acta Chem. Scand., *11*, 1064 (1957).
9. Genge, C. A. Private communication.
10. Gohlke, R. S., Anal. Chem., *31*, 535 (1959).
11. Selke, E., Dutton, H. J., Evans, C. D., and Scholfield, C. R. Communication to the Editor, J. Am. Oil Chemists' Soc. In preparation.
12. Drew, C. M., McNesby, J. R., Smith, S. R., and Gordon, A. S., Anal. Chem., *28*, 979 (1956).

Nuclear Magnetic Resonance in Lipid Analysis[1]

C.Y. HOPKINS, National Research Council, Ottawa, Canada

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.