Applications of Mass Spectroscopy in Fatty Acid and Lipid Chemistry

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SPECTROSCOPY is now a word familiar to almost all fatty acid and lipid chemists. Probably somewhere in a majority of reports of research in the field of fats and oils the word spectroscopy will be found. But while readily recognized, the exact connotation is not the same to all research workers. To the chemist concerned with oil or soap color, spectroscopy means visible absorption of a liquid or visible reflectance of a solid. To one interested in autoxidation effects, spectroscopy may mean spectrochemical analysis by UV emission spectroscopy or by atomic absorption spectroscopy or by X-ray fluorescence spectroscopy, to determine the extent or confirm the absence of trace metals which promote oxidation. Until a few years ago, spectroscopy to most oil chemists meant UV absorption, a means for detecting and measuring the extent of conjugation effects. More recently IR absorption spectrophotometry has become the concept oil chemists associate with the word spectroscopy. Following its extremely effective use by organic chemists in many areas, we may expect nuclear magnetic resonance (NMR) spectroscopy to become, in its turn, the popular use of the word spectroscopy. If we consider each of the subdivisions of the electromagnetic spectrum from long, low-energy radio waves to the short high-energy gamma radiation, and if we consider the ways in which these radiations may be used in analytical spectroscopy, i.e. emitted, absorbed, reflected, scattered, etc., it can be shown that there are some 30 divisions of spectroscopy, each of potential value to the research worker. Thus, spectroscopy means many things to many fatty acid and lipid chemists.

NMR spectroscopy is the newest division of spectroscopy to gain consideration by analytical chemists and by organic chemists. Its success, to augment the better-known IR and UV spectrophotometry in organic chemistry, means that a continual growth in popularity by the oil chemist interested in analyses of mixtures or in molecular structures of components is assured. Along with this increased emphasis on applications of NMR spectroscopy, there has been a parallel interest by the oil chemist in one of the older branches of spectroscopy, which heretofore has played little or no role in the rapid technological advances in fatty acid chemistry which have occurred during the past few decades. Mass spectroscopy has been used for over 50 years and has attained in some applications a position of considerable dependence in solving problems in analytical chemistry. However, it has not heretofore been used by oil chemists, except in very isolated experiments. The reasons for this slow development will appear when we consider the techniques and the development of mass spectroscopy as another tool for the analytical chemist.

In mass spectroscopy, the molecules of the sample being analyzed are bombarded by a stream of electrons. The sample must be in the gaseous phase, must be at low pressure, and the energy of the electrons must be far greater than that needed for the mere removal of an electron from the molecules. The impact energies usually employed are far in excess of the ionization potential of the molecule or for any probably excitation. Under these conditions, the Franck-Condon Principle calls for the least change in the positions and momenta of the nuclei at the time of impact. In general then, as a result of electron bombardment, there may be neutral molecules in an excited state, neutral fragments, negative ions and positive ions. These products may be stable or metastable and may have excess electronic, rotational, vibrational or kinetic energy. Of all these products, mass spectrometry ordinarily deals only with the ions with a positive charge.

The processes by which the fragments are formed by bombardment of organic molecules by a stream of high energy electrons is very complex and in many cases not entirely understood. In the case of vibrational-rotational spectra of IR spectrophotometry relatively simple formulas give the exact number of fundamental absorption bands. However, in any actual IR spectrum, the appearance of numerous combination bands, difference bands and overtone bands make any application of such a simple calculation of no particular value to the analytical chemist. So in mass spectroscopy, the fragments which might be expected from ionization and breakage of chemical bonds are augmented by ions formed by rearrangements at the moment of fragmentation or by the spontaneous decomposition of metastable ions. Again, as in IR absorption spectra, these rearranged and metastable ions have, when once properly interpreted, led to conclusions of particular value in elucidation of molecular spectra.

The positive ion fragments are separated, either by the path they follow in a magnetic field (magnetic spectrometer) or by the time they require to traverse a free field (time-of-flight spectrometer), according to their mass, or more strictly according to the ratio of their mass to their change, m/e, known in mass spectrometry as the specific mass. If fragments with different specific masses are brought successively to a slit, and their intensities measured with a suitable detector, a spectrum with one parameter the value of the ratio m/e and the other the intensity at this specific mass will be obtained. Although not employing radiation in the electromagnetic spectrum, such a pattern, intensity as a function of specific mass, is known as a mass spectrum and the process has always been considered a branch of spectroscopy.

A mass spectrometer consists essentially of four related components. First is the inlet or sample handling system. If the sample is a gas, this part of the instrument can be quite simple. However, if solid samples of relatively high volatility are to be analyzed, the inlet system becomes a somewhat complex device to vaporize the sample and to effect its flow into the low pressure ionization chamber. The ionization chamber is the second main component of the spectrometer. The gas or vapor reaches this area by pressure differential through a leak orifice. Within the ionization chamber, the molecules of the sample are bombarded by an electron beam formed by electrons emitted from a hot filament. By a voltage impressed upon them, the positive ions are then drawn out of this chamber and further accelerated by a relatively high voltage between plates within the chamber. The third component of the spectrometer is the analyzer tube where the accelerated postive ions are separated either by the influence of a uniform magnetic field or by the time required for them to traverse a free field. The overall effect is to segregate the heterogeneous ion beam into a fan of ion beams, each of which contains ions of one specific mass only. These beams are swept across an exit slit in such a manner that each beam in succession impinges on a target or collector where it gives up its charge, producing a weak current which is first amplified and then

(Continued on page 14)

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Applications of Mass Spectroscopy in Fatty Acid and Lipid Chemistry . . .

(Continued from page 4)

conducted to a recorder system. The recorder thus indicates the intensity of the amplified current, dependent in turn on the number of ions of specific mass impinging upon the target at any given time, as ions of increasing specific mass successively reach the target. Thus, a record of intensity of beam of a specific mass is plotted as a function of the specific mass. This is the mass spectra of the sample.

While the pattern of the nature of the ions and of their relative abundance is complex, for a given value of the uniform magnetic field and for a specific value of the voltage impressed between the plates within the ionization chamber, it is dependent upon the molecule bombarded. Thus, the mass spectrum of a molecule is uniquely characteristic of the molecule and, in general, two compounds that are structurally different will give different fragmentation patterns, or different mass spectra. The mass spectrum is, therefore, somewhat like an IR spectrum, a fingerprint of the specific molecule.

As mass spectrometry has been employed in specific applications, i.e., analysis and control of petroleum distillates, for decades, and as it obviously appears to offer advantages to research and analysis of fatty acids and their derivatives, the reader will question why we have not long ago seen specific applications to fatty acid chemistry. The answer lies in the fact that highly precise instruments, adopted for use with high-mass ions are necessary to investigate long-chain fatty acids, esters, triglycerides and their various derivatives. As the sample in mass spectroscopy must be in the gaseous phase, the techniques, quite naturally, were first adapted to investigations of gases and later to materials which volatilize readily. Only within about the last 10–12 years has the technique been extended successfully to the analysis of compounds of mol wt in the range 300 of the long-chain fatty acids and esters to 600 of the triglycerides. A first requirement was the introduction of a heated inlet system which would permit a solid sample to be vaporized and allow a small quantity of the resulting vapor to be conducted through the gold leak system to the low pressure ionization system. Instruments, with innovations which included heated inlet system designed to permit this operation, were first described in the early 1950's but they did not become immediately commercially available. Nor were the problems of dealing with molecules of high mass solved solely with the introduction of successfully heated inlet systems. At higher masses, resolution becomes more difficult. In the specific mass range to 100, to separate each specific mass unit requires a resolution of only 1%. At specific masses of 600, one part in 600 is necessary to obtain the same resolution, separation of one specific mass unit. An additional difficult problem is the slow outgassing of the source after each sample is loaded when samples of low volatility are analyzed. So-called memory effects, from accumulated residues in the ion source from previous samples, create a serious problem in applications of mass spectrometry to molecules of high mass. It is mainly advances in these areas which have recreated an interest in the potential applications of mass spectrometry to problems in the field of vegetable and animal fats, lipid chemistry and problems concerning the composition of natural oil-containing commodities.

In 1959, Ragnar Ryhage with the Laboratory for Mass Spectrometry, Kemiska Institutionen I Karolinski Institutet in Stockholm, described a mass spectrometer which made use of an inlet system which could be operated at temp up to 400C, permitting measurement of compounds with mol wt up to 619 (*n*-tetratetracontane). Provisions for adjustment of the magnetic current over the range of 0.3–4 amp, changing the magnetic field from 650–7700 gauss, provided a range sufficient to cover a mass number from ca. 4–580 with satisfactory resolution. At the time the description of the instrument was published, the authors claimed to have measured 450 mass spectra of long-chain compounds, including a number of long-chain alcohols, a few fatty acids, rosin acids, bile acids and sterols. Based on this experience, the the instrument was improved by redesign of the sample inlet and the vacuum systems and by improvements in the ion source, the collector and the analyzer tube.

Ryhage collaborated with Einar Stenhagen, a colleague from the Department of Medical Biochemistry, Institute of Medical Chemistry, University of Uppsala, in a systematic survey of the mass spectra of pure compounds of interest to the fatty acid chemist. They found that while they could obtain satisfactory spectra of the free long-chain fatty acids, the methyl esters were more volatile and less subject to thermal decomposition than the corresponding free acids and are, therefore, better suited for mass spectrometric analysis. Triglycerides have been investigated only in a preliminary manner, as considerable experimental difficulties are encountered. These compounds are difficult to pump out from the mass spectrometer and tend to give persistent "memory effects." However, as satisfactory methods are now readily available to convert either the free fatty acids or the glycerides to their methyl esters, most mass spectroscopy studies have been made using the methyl esters. One point of particular interest should perhaps be mentioned which has been obtained from only the preliminary investigations of the direct measurement of the mass spectra of

triglycerides. In these spectra, intense peaks are found at specific mass numbers m/e = M − 171 and M − 199 (where M is the specific mass of the molecule-ion or parent peak, that is the mol wt of the compound under investigation). These two peaks arise from ions formed with loss of acyloxy groups from the molecule-ion. A peak is also found at specific mass m/e = M − (171 + 14), but no corresponding peak is found at m/e = M − (199 + 14). Loss of acyloxymethylene thus occurs from positions 1 and 3, but not from position 2 of the glycerol moiety. Thus, mass spectra, obtained directly from the triglycerides, appears to be a potential means of differentiating the acyl groups attached at position 2 from those at positions 1 and 3.

Ryhage and Stenhagen, with colleagues from their respective institutions and in collaboration with Bo Halgren and Nguyen Dinh-Nguyen from the Institute of Medical Biochemistry, Gothenburg, published during the period 1957 to the present approx 40 papers dealing with fatty acid compounds, mostly as methyl esters. These papers constitute a comprehensive survey of the mass spectra characteristic of fatty acid derivatives, illustrate the advantages of mass spectroscopy investigations in research on fatty acid materials, and open up the potentials of this tool to research investigations of natural commodities in the areas of fatty acid and lipid chemistry.

Before commenting further on these outstanding contributions of the Swedish workers, it is interesting to note that one of the early applications of mass spectroscopy which has been quoted to illustrate the usefulness of the technique involves fatty acid chemistry. Twenty years ago S. Weinhouse, G. Medes and N. F. Floyd used mass spectroscopy to settle an important question regarding the mechanism of fatty acid metabolism. As high mass spectrometers were not available, the work involved mass spectroscopic analysis of pyrolysis products. Up to the time of these experiments there were three proposed mechanisms of fatty acid metabolism, each giving rise to the same end products, the ketone bodies. The first of these was the classical theory of β-oxidation which assumed successive β-oxidations along the fatty acid chain, the last four carbons giving rise to acetoacetic acid. The first step in the oxidation of octanoic acid, studied by Weinhouse and his colleagues, according to this theory would be the splitting off of the carboxyl group and the adjacent carbon atom. If the carboxyl group carbon were labeled with C^{13}, the acetoacetic acid formed by this proposed mechanism would contain no excess C^{13} (above natural abundance). A second hypothesis, known as the theory of multiple alternate oxidation, assumed that oxidation occurs at alternate carbon atoms throughout the fatty acid chain. According to this theory, if again the carbon of the carboxyl group is labeled with C^{13}, the acetoacetic acid resulting would contain excess C^{13} in its carboxyl group, but nowhere else in the acetoacetic acid formed. Finally, a third hypothesis called the β-oxidation condensation theory assumed that the ketone bodies are formed by the condensation of some 2-carbon intermediate resulting from β-oxidation of fatty acids. According to this hypothesis, if the carboxyl carbon of normal octanoic acid is labeled with C^{13}, the acetoacetic acid formed would have excess C^{13} equally distributed between its carboxyl and carbonyl groups.

To determine which of these three hypotheses was the correct one, carboxyl labeled normal octanoic acid was incubated with liver slices from fasted rat. The resulting solution, containing the newly synthesized acetoacetic acid from the fatty acid, was decomposed into acetone and CO_2, according to the procedure of Van Slyke. The CO_2 liberated was analyzed for its excess C^{13} content by mass spectroscopy. The acetone was precipitated as the mercury complex, and further degraded. The two end carbons of the acetone, isolated from the mercury complex, were differentiated from the central carbon by chemical means and were then oxidized to carbon dioxide and analyzed by mass spectrometry for excess C^{13}. The results, compared with those calculated from values expected on the basis of each of the proposed theories:

	Atom % C^{13} excess	
	Carbonyl	Carboxyl
Observed	0.84	0.83
Oxidation-condensation theory	1.10	1.10
Multiple alternate oxidation theory	0	2.20
Classical oxidation theory	0	0

led the authors to report "When *n*-octanoic acid labeled by the incorporation of C^{13} in the carboxyl group was incubated *in vitro* with liver slices from fasted rats, the resultant acetoacetic acid contained all excess C^{13} equally distributed between the carbonyl and carboxyl carbon atoms. The results offer unequivocal evidence that the ketone bodies are formed by condensation of a 2-carbon intermediary resulting from β-oxidation of the fatty acid."

The detailed survey by Ryhage, Stenhagen and their colleagues has made available a wealth of mass spectra data, particularly on methyl esters of long-chain fatty acids. The published descriptions contain actual mass spectra illustrating the type of methyl ester investigated, and, in addition, analyses of the spectra include identification of the base (most intense) peak, the parent (molecule-ion) peak, most of the more intense peaks and explanations for the formation of characteristic peaks from fragment rearrangements.

The mass spectrum of methyl esters of long-chain carboxylic acids are dominated by peaks due to ions containing oxygen. The hydrocarbon peaks of the spectrum can be almost com-

(Continued on page 20)