EXPERIENTIA

Vol. XIV - Fasc. 4 Pag. 121-160 15. IV. 1958

The Separation of Fatty Acids[1]

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It is the purpose of this review to discuss methods which are currently being applied to the separation, analysis, and identification of fatty acids. In a brief presentation, it is impossible to treat all methods or to give examples of all techniques. The author has therefore chosen to outline the newer physical techniques and to give emphasis to those methods with which he has had some experience. Separations are performed either for preparative or for analytical purposes. Methods applicable for the one purpose may or may not be applicable for the other. Therefore, each method will be discussed from both points of view wherever possible.

It was but a short time ago that the chemist interested in lipides had at his disposal only a few crude techniques for the separation of their components. Most of the early methods for the separation of fatty acids depended upon the formation of derivatives which were poorly soluble in some solvent. The separation of 'saturated' and 'unsaturated' acids as their lead salts, the fractionation of lithium salts of unsaturated acids, and the isolation of linoleic and linolenic acids via the bromides depended upon chemical alterations of the fatty acids prior to separation. The common methods which attempted the measurement of individual fatty acids or groups were based largely upon chemical reactions. Thus, the determination of unsaturation by iodine value, of linoleic and linolenic acids by means of the thiocyanogen value, or saturated acids via oxidative procedures involved reactions by which the sample was essentially destroyed. The undiscriminating chemical methods were really group determinations, and no methods for the measurement of specific fatty acids were available.

Two physical methods earliest in use were fractional distillation and fractional crystallization. These have both grown in importance to fat chemistry and are indispensable today. Crystallization at low temperatures, carefully worked out by BROWN and his students[2], proved to be a great improvement over the salt separations. Distillation separates acids according to differences in one set of molecular properties and crystallization according to another, and thus the two have supplemented each other.

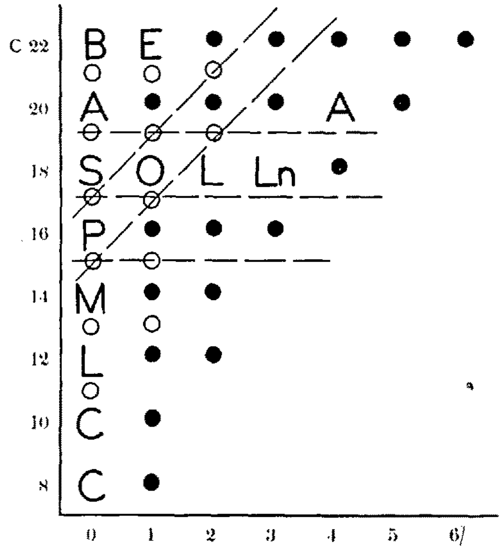

Fig. 1.—A simplified representation of the variety of naturally occurring fatty acids. The most abundant acids are represented by initials, less abundant acids by black circles, and branches and odd-numbered acids by open circles. Horizontal lines indicate separations by chain length (distillation) and diagonal lines represent possible separations by crystallization, chromatography, etc.

Before listing and discussing some modern separation procedures, it would be well to discuss the problem that faces the chemist. The naturally-occurring fatty acids differ by chain length and by degree of unsaturation. The problem is even more complex than was formerly thought, because odd-numbered and branched-chain acids have recently been found to be minor constituents of natural fats. Some of the complexity of the problem of separation can be illustrated by the chart shown in Figure 1. The most abundant acids are indicated by their initials. Less common acids are shown by solid circles. Some of the branched acids and acids of odd chain length have been indicated in the diagram by open circles. Isolation of a single acid thus requires a methodology which has a very high ability to discriminate.

Fractional distillation, usually carried out with the fatty acid esters, separates according to boiling point, a property related to the molecular weight. Ideally,

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[1] Presented before the symposium on Chemistry and Physiology of Fats at the meeting of the Chemical Institute of Canada, Ottawa, October 9, 1957. Hormel Institute publication No. 168.

[2] J. B. BROWN and D. K. KOLB, *Progress in the chemistry of fats and other lipids*, Vol. 3 (Ed. by R. T. HOLMAN, W. O. LUNDBERG and T. MALKIN, Pergamon Press, London-New York 1955), p. 57.

this method fractionates a mixture into groups which have a uniform chain length but branched fatty acids present in natural mixtures complicate the problem. This is represented in Figure 1 by horizontal zones. Crystallization separates according to solubility, which is influenced both by chain length and by unsaturation in the fatty acid series. In practice, this technique is used to make a rough separation of saturated acids from unsaturated acids, but in fact the liquors would contain considerable amounts of the short chain saturated acids, so the separation cannot be represented as a strictly vertical line in Figure 1. By the appropriate use of both types of fractionation, the intersection of the zones permits the isolation of individual components in reasonable purity.

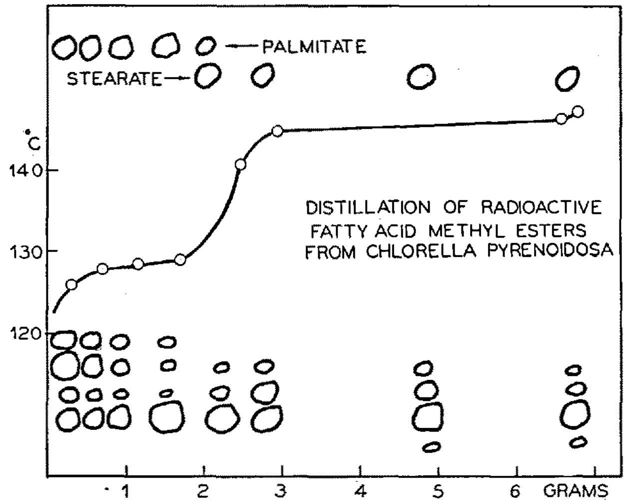

Fig. 2.—Fractional distillation of radioactive fatty acid methyl esters derived from Chlorella lipides using a spinning band column. Paper chromatograms of the fractions are shown below, and of hydrogenated fractions above. Redrawn from the data of MANGOLD and SCHLENK[3].

A combination of distillation and crystallization is still the best means to prepare pure fatty acids in quantity. All methods for the preparation of more than a few grams of pure acid or ester must use these methods or variants of them. The weakness of these methods is that they are not readily applicable to separations of small samples such as is often necessary in the analysis of biological samples.

Equipment has been designed specifically for fractional distillation of long chain esters in quantities greater than 5 g, and modern apparatus can achieve surprisingly clean separations[4]. For fractional distillation of small amounts of esters the spinning band column has proven to be very efficient. Separation often is better than can be judged by examination of the curve of boiling points, because unsaturated esters predominate at the beginning, and saturated esters at the end of fractions of uniform chain length. An example[3] of fractional distillation in a spinning band column monitored by paper chromatography, is shown in Figure 2. The separation between 16 and 18 carbon acid esters is quite sharp, because fraction 5, the transition fraction, is the only one which contains esters of both chain lengths. The proportion of unsaturated esters is seen to decrease as the distillation of each chain length proceeds, and stearate appears only in the last two fractions.

Amplified distillation was introduced by WEITKAMP as an attempt to reduce the amount of sample needed for a fractionation according to chain length[5]. The remarkable success with which he and his colleagues separated 32 component acids of wool wax indicates the potential of this technique of fractional distillation. A small sample of fatty acid esters is distilled with a much larger amount of hydrocarbon mixture, the boiling point of which has a smooth gradient spanning the range of boiling points of the esters. When the boiling point of each of the individual esters is reached, it distills with hydrocarbons of similar boiling point, and the individual esters are then spaced by large amounts of hydrocarbons. This method has not been generally adopted, perhaps because the recovery of fatty acids from a large amount of unsaponifiable matter is difficult.

Fractional crystallization at low temperatures is almost indispensable for the preparation of large amounts of fatty acids. Through systematic studies of the solubilities of acids and esters in a variety of solvents and in wider ranges of temperature, the scope of its applications is continually being extended. One inherent disadvantage of the method is the co-crystallization of fatty acids which leads to less perfect separations than solubility data would predict.

A modern variation on the theme of crystallization is fractionation of fatty acids and esters by urea complex fractionation[6]. When urea is allowed to crystallize in the presence of straight chain compounds, it crystallizes in a hexagonal form, incorporating the aliphatic substance within its crystal lattice. The tendency of saturated acids to form complexes is greater than that of unsaturated acids or branched substances, and therefore the formation of urea complexes from mixed fatty acids enriches the saturated components in the solid complex and the unsaturated and branched components in the liquor. Urea complex fractionations find their best use in preparative procedures, because the process is easily adapted to large quantities. In effect, by the use of urea, the

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[3] H. K. MANGOLD and H. SCHLENK, J. biol. Chem. *229*, 731 (1957).

[4] K. E. MURRAY, *Progress in the chemistry of fats and other lipids*, Vol. 3 (Ed. by R. T. HOLMAN, W. O. LUNDBERG, and T. MALKIN, Pergamon Press, London-New York 1955), p. 245.

[5] A. W. WEITKAMP, A. M. SIMLJANIC, and S. ROTHMAN, J. Amer. chem. Soc. *69*, 1936 (1947).

[6] H. SCHLENK, *Progress in the chemistry of fats and other lipids*, Vol. 2 (Ed. by R. T. HOLMAN, W. O. LUNDBERG, and T. MALKIN, Pergamon Press, London-New York 1954), p. 243.

temperatures needed for the separation of fatty acids by crystallization are raised from sub-zero to room temperature. Therefore, separations can be achieved without elaborate equipment.

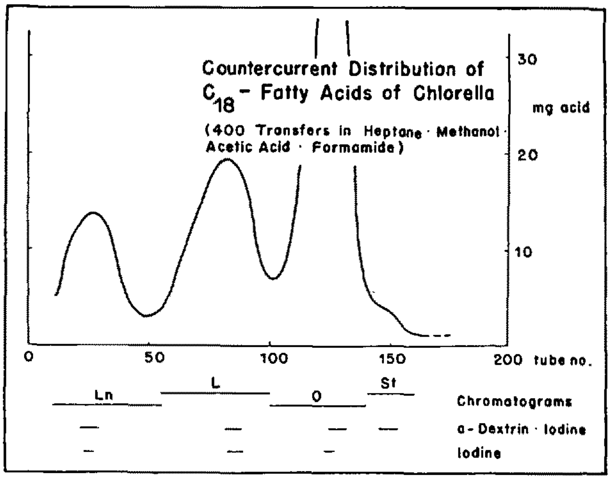

Fig. 3.—Countercurrent distribution of the 18-carbon fatty acids from Chlorella. Separations were monitored by spotting equal volumes on paper and developing with iodine and α-dextrin-iodine, indicating positions of the maxima. Paper chromatograms indicated the extent of each zone. From Mangold and Schlenk[3].

Countercurrent distribution has been worked out in detail in the last decade for the separation of fatty acids[7]. In this technique, use is made of the differing solubilities of the fatty acids in two immiscible solvents. If a sufficient number of extractions is made, the fatty acids of a single chain length, but differing in unsaturation, can be separated from each other. Acids having the same degree of unsaturation and unequal chain lengths are also separable. In several careful investigations, the acids of 16, 18, and 20 carbon atoms having 1 to 5 double bonds have been isolated by this means. Figure 3 shows one such separation[8]. The 18-carbon acids of *Chlorella* fat were separated to obtain stearic, oleic, linoleic and linolenic acids. Countercurrent distribution equipment currently available allows the separation of samples ranging from milligrams to grams in a single run.

Chromatography is perhaps the most widely used general method for lipide and fatty acid separations[9]. In its broadest definition, chromatography is the separation of substances by virtue of the different concentrations of these substances in two phases which are in equilibrium and one of which is mobile. The stationary phase may be a liquid or a solid, and the mobile may be a liquid or a gas. The separation may be achieved by adsorption on a solid phase or partition between two fluid phases. The separation of homologs or of vinylogs can be easily achieved by chromatography on a microgram to a gram scale, but large scale preparations are not yet feasible by this method.

In most chromatographic methods, lengthening the carbon chain by two carbon atoms and introducing one additional double bond have opposite and almost equal effects upon the adsorptive properties of the fatty acids. Thus, acids lying on an approximately diagonal line running from lower left to upper right of Figure 1 will migrate very closely together. For example, palmitic and oleic acids are such an 'inseparable pair'. Therefore, and accurate analysis or an isolation by this method requires another separation according to chain length.

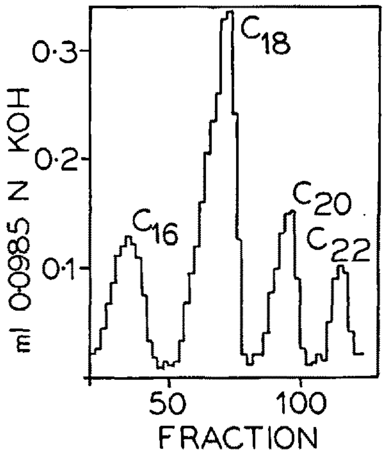

Fig. 4.—Separation of fatty acids by reversed phase column chromatography. The unsaturated fatty acids from depot fat of rats which chromatographed with linoleic acid were hydrogenated and chromatographed again. Zones were located by titration. Mead[10].

Elution chromatography in columns is the original form of chromatography. However, it seems not to have been used as widely as other techniques for the separation of fatty acids. This method has been employed for the isolation of highly unsaturated fatty acids in a high state of purity. A very recent and illustrative example of the potency of the method is taken from the work of Mead[10] and is shown in Figure 4. By reversed phase partition chromatography the unsaturated acids from the depot fat of rats were separated into groups. The group migrating with linoleic acid was hydrogenated and rechromatographed. The zones shown in Figure 4 thus are saturated acids which represent the palmitoleic, linoleic, arachidonic and docosapentaenoic acids originally present in the fatty acid mixture.

Displacement chromatography has been used for the separation of fatty acids, their esters, and other lipides[9]. In this form of adsorption chromatography, the column is developed by saturating it with a substance

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[7] H. J. Dutton, *Progress in the chemistry of fats and other lipids*, Vol. 2 (Ed. by R. T. Holman, W. O. Lundberg, and T. Malkin, Pergamon Press, London-New York 1954), p. 292.

[8] H. K. Mangold and H. Schlenk (unpublished data).

[9] R. T. Holman, *Progress in the chemistry of fats and other lipids*, Vol. 1 (Ed. by R. T. Holman, W. O. Lundberg, and T. Malkin, Pergamon Press, London-New York 1952), p. 104.

[10] J. F. Mead, J. biol. Chem. *227*, 1025 (1957).

which is more strongly adsorbed than any component of the sample. Thus, as the displacer substance moves through the column, it displaces the sample substances from the adsorbent, and these arrange themselves in an adsorption series ahead of the displacer. In order that the fronts of zones be as sharp as possible, a coupled segmented column is used in which the separation is sharpened by serial chromatography.

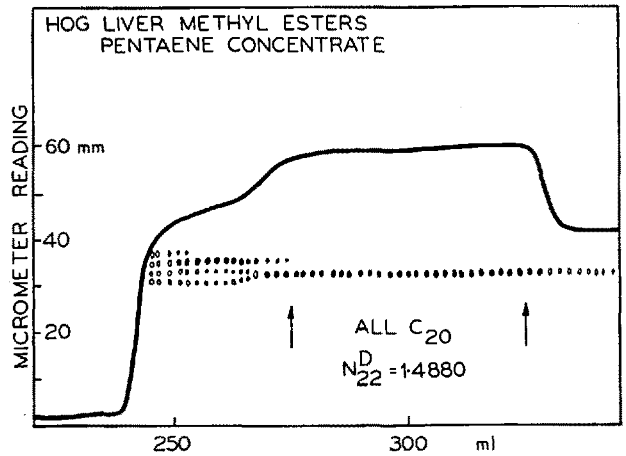

Fig. 5. – Isolation of methyl eicosapentaenoate by displacement chromatography on charcoal in 95% ethanol using 1% ethyl stearate as displacer. Paper chromatography was used to monitor fractions. Holman and Hayes[11].

The isolation of methyl eicosapentaenoate, using displacement chromatography on charcoal with ethyl stearate as displacer, is shown in Figure 5. A concentrate of polyunsaturated esters was chromatographed, ·5 ml fractions of eluate were collected, and an interferometric measurement of the refractive index of the effluent was made.

Paper chromatography was then used to assess the complexity of each fraction. It is seen that the first major zone indicated by the interferometer consisted of four unsaturated substances only partially segregated. The major substance was found by alkaline isomerization[12] to be a pentaene. The fractions between the arrows were combined, and a small portion was hydrogenated. Paper chromatography revealed only methyl eicosanoate to be present. Thus, the main fraction was identified as methyl eicosapentaenoate.

The isolation of ethyl octadecatetraenoate from cod liver oil esters by displacement chromatography is shown in Figure 6. The separation was performed and monitored in the same manner as described above. Representative fractions were also isomerized to identify the type of polyene unsaturation present. This revealed that the early fractions contained largely triene, and the latter fractions largely pentaene. The center cut was the ethyl octadecatetraenoate.

A similar experiment performed on glyceryl ethers shows the applicability of the method on lipides other than fatty acids and esters[13]. An ostensibly pure selachyl alcohol was found by paper chromatography to be contaminated with another unsaturated substance. The sample was also subjected to displacement chromatography, and the interferometric curve and paper chromatograms indicated the presence of three components. The first fraction (Fig. 7) was found to be a glyceryl ether of hexadecenol, and the final fraction is thought to be its C_{20} homolog. The main fraction was selachyl alcohol. Isomerization of representative fractions revealed that the glyceryl ethers were not polyenes.

Carrier displacement chromatography is a variation of the above technique in which a small amount of sample is chromatographed with an admixture of several unrelated substances to act as spacers. By this means the otherwise undetectable components of the sample are separated by a sufficient volume of effluent to make collection of fractions feasible. In Figure 8 such a separation is shown. 10 mg each of three branched isomers of stearic acid were added to 50–100 mg amounts each of methyl laurate, myristate and palmitate. This mixture was then chromatographed on carbon in ethanol using 1% methyl stearate as displacer. The individual branched isomers of stearic acid were located by titration of 1 ml fractions. They were found to appear just ahead of each of the carrier substances, clearly separated. It is apparent that the type of branching of the chain strongly affects the adsorbability of these isomers of stearic acid.

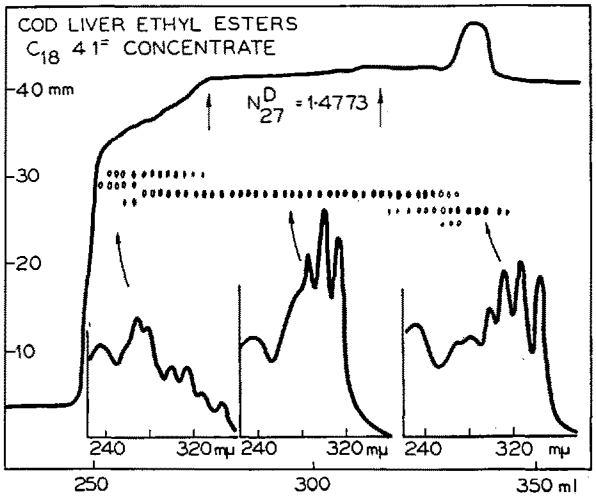

Fig. 6. – Isolation of ethyl octadecatetraenoate by displacement chromatography using paper chromatography to monitor fractions. Inserts are ultraviolet spectra of three representative fractions after alkaline isomerization. Holman and Hayes[11].

Paper chromatography of fatty acids developed in recent years, is now used routinely as a test of purity

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[11] R. T. Holman and H. Hayes (unpublished data).

[12] R. T. Holman, *Methods of Biochemical Analysis*, Vol. 4 (Ed. by D. Glick, Interscience, New York 1957), p. 99.

[13] H. K. Mangold, H. Hayes, and R. T. Holman (unpublished data).

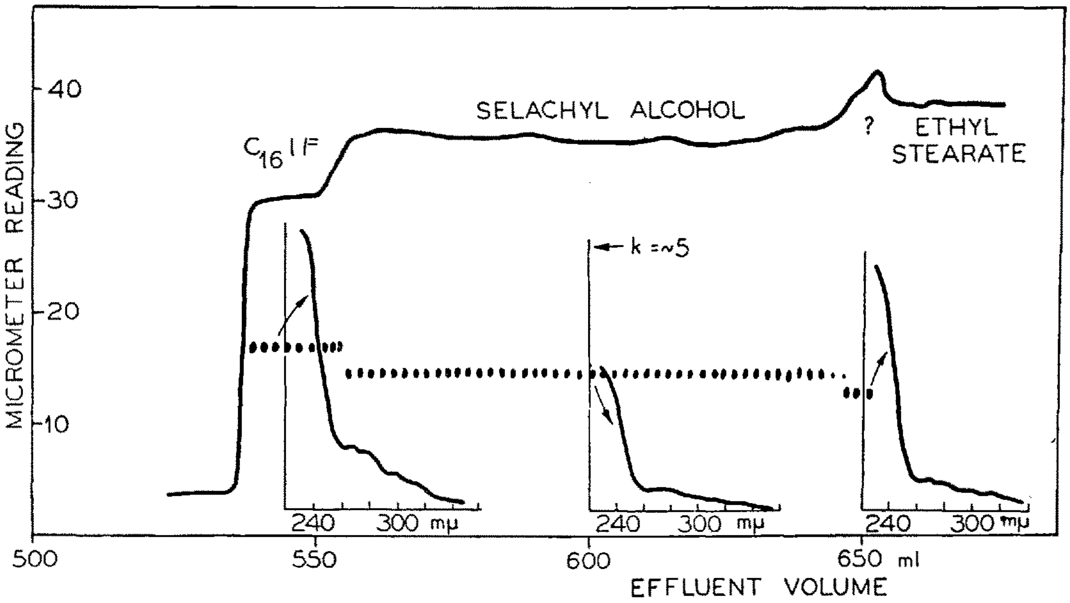

Fig. 7.—Separation of glyceryl ethers by displacement chromatography using paper chromatography to monitor fractions. Inserts are ultraviolet spectra of selected fractions after alkaline isomerization. Mangold, Hayes, and Holman[13].

and as a means of monitoring other separation procedures. Several techniques have been used in different laboratories, but comments here will be confined to the techniques developed in The Hormel Institute. In the techniques developed by Schlenk and his group[14,15] the fatty acids are spotted on siliconized paper and developed by a solvent chosen for the separation desired. For many fatty acid separations, 85% acetic acid is a good solvent. Spots on the developed chromatograms are made visible by spraying the paper with α-cyclodextrin and exposing to iodine vapor. This treatment develops light spots on a blue background, and both saturated and unsaturated acids and esters can be detected. If one is interested only in unsaturated substances, the use of α-cyclodextrin is not necessary, because unsaturated lipides yield yellow or brown spots when exposed to iodine vapor.

Figure 9 shows two paper chromatograms in which the unsaturated acids having 18 and 20 carbon atoms were separated. It will be seen also that a mixture containing acids of several chain lengths could not be clearly separated by this method. A prior separation according to chain length prevents the dilemma of inseparable pairs such as oleic and eicosadienoic acids. For establishing the purity or identity of unsaturated acids, paper chromatography of a hydrogenated sample is very helpful, because chain length can thereby be determined.

The problem of inseparable pairs has been partially solved in other ways by Schlenk and his coworkers[15]. Paper chromatography at low temperatures, e.g. separates the saturated acids as a group which does not migrate. Thus, palmitic and oleic acids which migrate together at room temperature are clearly separable at −5°. Myristic and palmitic acids remain

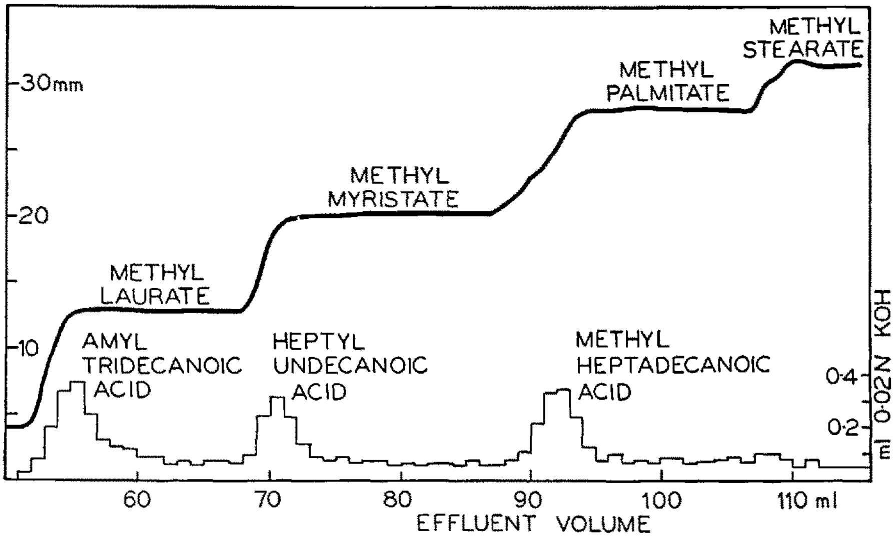

Fig. 8.—Carrier displacement separation of four branched isomers of stearic acid.

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[14] H. K. Mangold, B. G. Lamp, and H. Schlenk, J. Amer. chem. Soc. 77, 6070 (1955).

[15] H. Schlenk, J. L. Gellerman, J. A. Tillotson, and H. K. Mangold, J. Amer. Oil Chemists' Soc. 34, 377 (1957). – H. K. Mangold, J. L. Gellerman, and H. Schlenk, Federation Proc. (in press).

at the origin, whereas the migration of oleic and linoleic acids is only somewhat reduced. Thus, it appears that by proper choice of conditions, otherwise inseparable pairs need not longer be a problem in paper chromatography.

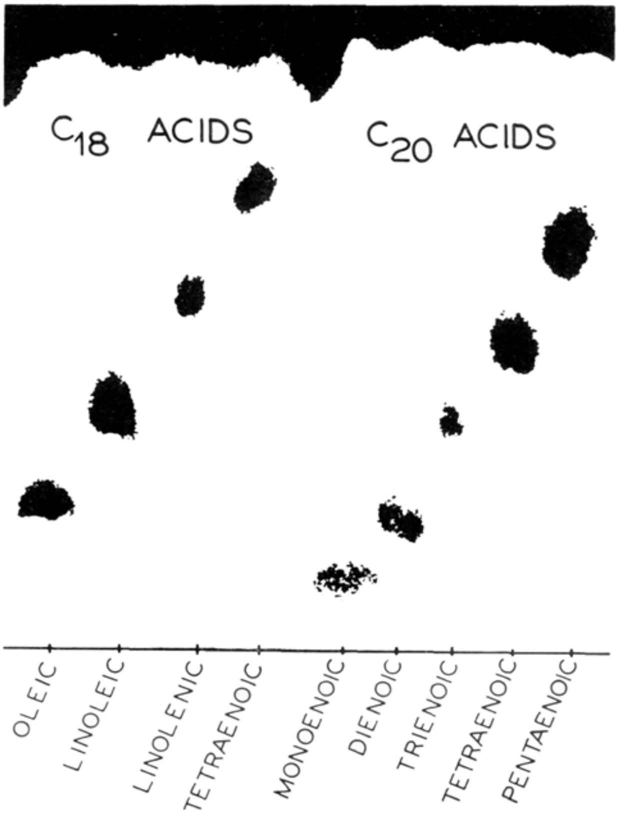

Fig. 9. — Separation of unsaturated fatty acids by paper chromatography. HOLMAN[15].

A series of densitometer curves from radioautographs of paper chromatograms[15] is shown in Figure 10. After spotting the papers with methyl esters from radioactive *Chlorella* fatty acids, one paper was developed immediately and the others were stored dry for varying times prior to development. The developed chromatograms indicate that autoxidation is no problem under normal conditions, but that prolonged exposure of unsaturated substances on dry paper allows autoxidation. This experiment also illustrates the possible conversion of paper chromatograms to numerical data by means of densitometer curves. Analyses of fatty acids can by this method be made with an error of a few percent.

Gas phase chromatography is a technique for fatty acid analysis which is attracting much attention currently. To date, conditions have been found for very elegant separations of fatty acid esters up to 18 or 20 carbon atoms long. A column packed with inert material impregnated with a grease of very low vapor pressure is commonly used. An inert gas is passed through the heated column and the sample is injected into the stream of hot gas ahead of the column. The substances are retarded in the column more or less according to their vapor pressures and their affinities for the grease. The emerging vapors are detected by a katharometer, gas density balance, ionization chamber or other device. The gas phase chromatographic separation of the methyl esters of the fatty acids from human milk fat shown in Figure 11 is taken from the work of INSULL *et al.*[16]. The chromatogram indicates that several previously unsuspected acids are present in the fat.

There is great current interest in separation of saturated and unsaturated esters from 18 to 22 carbon atoms in length, and conditions for better separation of these substances by gas phase chromatography are constantly being sought by many investigators, already with some encouraging results. These advances will require redesign of columns and detectors, and involve newer and more efficient packing materials, for the requirements of the problem appear to be just beyond the limits of current equipment.

The esters of mono- and dicarboxylic acids are easily separated and identified in the system of gas phase chromatography developed by JAMES and WEBB[17]. This work illustrates the potential of gas phase chromatography in determination of structure. By oxidative degradation of polyenoic acids, and chromatography of the esterified mono- and dicarboxylic acids derived from them, the positions of the double bonds in the unsaturated acids are readily deduced.

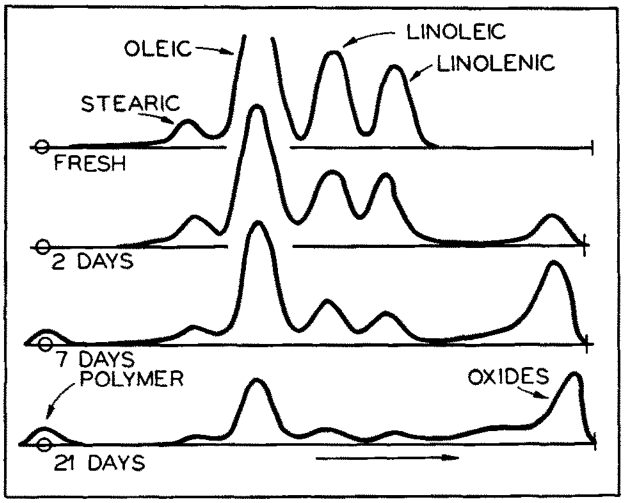

Fig. 10. — Paper chromatography of radioactive 18-carbon fatty acid methyl esters from Chlorella, showing the effect of autoxidation. From SCHLENK *et al.*[14].

Mass spectrography should be mentioned here as a new physical method useful for identification and determination of structure. The method is currently being applied to substances with molecular weights as

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[16] W. INSULL, Jr., J. HIRSCH, A. T. JAMES, and E. H. AHRENS, Jr. (unpublished data).

[17] A. T. JAMES and J. WEBB, Biochem. J. *66*, 515 (1957).

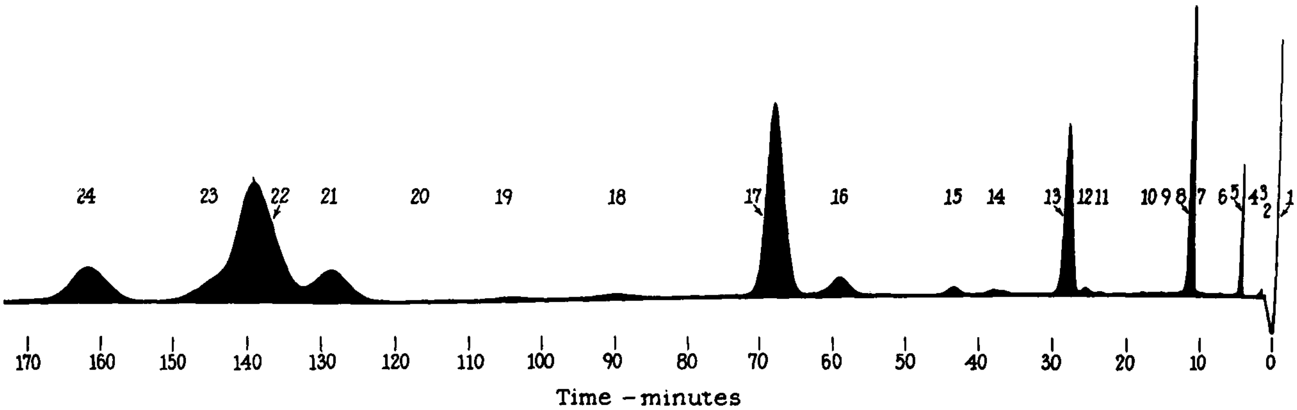

Fig. 11. — Separation of 0·86 mg of methyl esters of human milk fatty acids on a 4-foot column at 197°C with Apiezon M vacuum grease as stationary phase, N_2 pressure, 67 cm Hg and flow rate 54 ml/min. Peaks in order of appearance, right to left: (1) air, (2) two heptanoate esters, (3) two octanoate esters, (4) n-nonanoate, (5) n-decaoate, (6) probably n-undecanoate, (7) probably dodecenoate, (8) n-dodecanoate, (9) probably a branched tridecanoate, (10) n-tridecanoate, (11) probably a branched tetradecanoate, (12) tetradecenoate, (13) n-tetradecanoate, (14) probably two branched pentadecanoate, (15) n-pentadecanoate, (16) hexadecenoate, (17) n-hexadecanoate, (18) probably a branched and an unsaturated heptadecanoate, (19) n-heptadecanoate, (2) probably methyl ester of a branched C_{18} acid, (21) linoleate and linolenate, (22) oleate, (23) probably an isomer of oleate, (24) n-octadecanoate. From INSULL *et al.*[16].

high as 600. It therefore offers great promise in fatty acid chemistry. STENHAGEN[18] has reviewed the mass spectrometry of long chain substances and he and his group are currently studying the method for the determination of structure of branched fatty acids. When molecules are bombarded with electrons and the positively charged molecules and molecular fragments are accelerated by an electric field at right angles to a magnetic field, the ions travel through arcs, the radii of which depend upon the charge and mass of the ions. By proper adjustment of the magnetic field, the ionized particles can be admitted through a slit according to their mass, and thus their relative abundances are measured. Most of the molecules are fragmented by the bombardment by electrons, and the proportions of fragments of each mass is a characteristic of the parent substance. Unsaturation, branching and functional groups all affect the resulting mass spectra, because the fragments are different. The mass spectra of methyl stearate and methyl oleate, shown in Figure 12, illustrate the differences caused by one double bond in a fatty acid molecule.

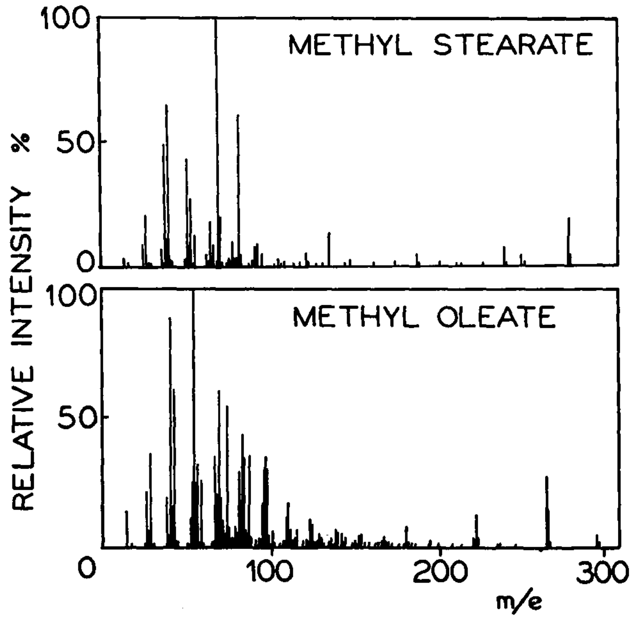

Fig. 12. — The mass spectra of methyl oleate and methyl stearate. Redrawn from STENHAGEN[19].

From the methods of separation and analysis which have been discussed, it will be readily seen that our methodology is now dependent almost entirely upon physical separations. These methods have the inherent advantage that they leave the fatty acids unaltered or require so small a sample that recovery is not a factor. Another advantage of physical separations is that the conditions are subject to infinite variation. One can reasonably expect ultimately to find proper conditions for the separation of any and all fatty acids occurring in nature.

Acknowledgement. — I am indebted to Dr. O. S. PRIVETT for the concentrate of methyl eicosapentaenoate used in one of the experiments reported here, and for reading this paper before the symposium when I became unable to attend.

Résumé

Le problème de la séparation des acides gras est exposé en détail. Les méthodes physiques couramment appliquées à une macro- ou micro-échelle sont discutées et l'on donne des exemples tirés de la littérature ou de méthodes récemment utilisées dans les recherches. Celles-ci comprennent la cristallisation, la distillation fractionnée, la distribution par contre-courant, la chromatographie en phase gazeuse et la spectrographie de masse.

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[18] E. STENHAGEN, Nordiske Kemikermøde i Aarhus, August 1956, p. 59.

[19] E. STENHAGEN (unpublished data).