Review

Some new methods for separation and analysis of fatty acids and other lipids*

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The separation and analysis of lipid compounds are currently of wide interest and the subject of intense research. The purpose of this paper is to review some of the methods presently in use and to discuss their relative merits. A recent two-volume work by Kaufmann (1) gives detailed information in most fields of lipid analysis, and a series of reviews of specific fields has recently been published (2). Emphasis here is placed upon fatty acids and, to a certain extent, upon methods with which the authors have some personal experience.

The great variety of fatty acids found in nature makes the total analysis of fats a major problem which has not yet been solved satisfactorily despite enormous progress toward that end. Table 1 attempts to illustrate this variety, showing the fatty acid composition of four different fats. The first three represent analyses by gas-liquid chromatography by Ahrens et al. (3, 3a), and the fourth is taken from a recent compilation of fatty acid composition of fats by the U.S. Department of Agriculture (4). These and other recent analytical studies have shown that most fats contain a great variety of fatty acids occurring in a concentration range below 0.5%. The structures of most of these minor components are unknown but they are considered to include isomers of the common unsaturated fatty acids, and acids having odd-numbered chain lengths or branched chains. Accurate analyses

of all fatty acids in a fat cannot be expected from one method only. Even gas-liquid chromatography, with its extraordinarily good separations, should not be considered the ultimate answer to all analytical problems. Combinations of methods are required, and group separations consequently play an important part in the development of fatty acid analyses.

CRYSTALLIZATION METHODS

Group separations generally depend either upon degree of unsaturation or upon chain length, and a suitable combination of methods should ideally yield fractions containing single substances. Separation according to unsaturation by the differential solubilities of the lead or lithium salts of fatty acids is a technique now used infrequently. Its place is usually taken by the techniques of low temperature crystallization and of complex formation and precipitation with urea. Silk et al. (5), however, have recently made a comparison between fractionation of lithium salts in acetone and fractionation by urea complex precipitation, and have found that the former technique has an advantage in removing C_{16} and C_{18} acids of relatively low unsaturation from C_{20} and C_{22} unsaturated acids.

Low temperature crystallization of fatty acids has been worked out largely by Brown and his students (6). Its main advantage lies in the simplicity of the technique. Facilities for working at $-20°$ to $-70°$ are, however, required. No reagents except the solvent are involved and oxidation or other changes in the fatty acids are unlikely. One inherent disadvantage of the

method is the co-crystallization of fatty acids, which leads to less perfect separations than solubility data would predict. While it is normally possible to obtain quite good separation of saturated from unsaturated fatty acids by this method, a separation of monoene from polyene acids is less precise, and in this instance urea fractionation may give better results. Because low temperature crystallization demands concentrations of 5% to 15%, the method is not practical for analytical separations on a microscale. In such cases one may substitute paper chromatography at low temperatures. This technique has been used by Kaufmann and Mohr (7) and by Schlenk *et al.* (8) in fatty acid analyses, and can be applied to other lipids as, for example, the separation of cholesterol from other non-saponifiable material.

The recent technique of zone-melting may possibly prove useful in the preparation of highly purified fatty acids. In this technique, crystalline material in a tube is melted in a narrow zone which moves from one end of the tube to the other in cycles. The impurities tend to remain in the melt and will eventually be concentrated at one end of the tube. The technique was originally developed to produce inorganic products of high purity for the electronics industry, but may also be applied to the purification of organic substances (9, 10). The separation of eicosanol and hexacosanol (20:80) was achieved on a milligram scale by Schildknecht and Vetter (11). They obtained 9.6 mg of pure hexacosanol from 28.5 mg of the original mixture, but were unable to isolate any pure eicosanol. Figure 1

TABLE 1. FATTY ACID COMPOSITION OF FOUR DIFFERENT TYPES OF FATS *

Fatty Acid Designation †	Menhaden Oil	Human Milk Fat	Corn Oil	Lard
<12:0		1.9		
12:0	0.1	6.8	0.1	
12:br?		.04		
13:0	0.1	.06		
13:br	tr			
13:br	tr			
14:0	7.2	8.5	0.3	
14:br	0.1	.08		
14:1	0.1	0.2		
15:0	0.5	0.5		
15:br	0.2	0.4		
15:br	0.2			
16:0	17.0	20.9	12.7	32.2
16:1	9.8	2.4		
16:2	2.0			
16:3	1.3			
16:4	2.0			
17:0	0.4	0.5		
17:br	0.3			
17:br	0.4	0.7		
18:0	3.1	7.3	2.7	7.8
18:1		6.5		
18:1	14.5	29.3	30.7	48.0
18:2	2.0	7.1	53.5	11.0
18:2	0.7			
18:3	1.3	0.9		0.6
18:4	3.2			
19:0	tr			
un		0.7		
19:br	0.4			
19:un	0.4			
20:0	0.1	1.3		
20:1	2.1	0.9		
un		0.3		
20:2	0.6	0.3		
20:2	0.6			
20:3	2.0	0.4		
20:4	0.6	0.6		0.4
un		0.1		
22:1?		.07		
un		0.2		
20:5	12.5	0.08		
un		0.2		
20:un	0.7			
un		0.1		
20:un	0.5			
un		0.2		
21:0	tr	0.5		
20:un	1.7			
22:un	tr			
un		0.1		
22:5	2.0	0.1		
22:6	8.9	0.3		
24:un	tr			

* Values in columns 1 and 3 from Ahrens *et al.* (3) expressed as weight per cent acid; values in column 2 from Insull and Ahrens (3a) expressed as weight per cent methyl esters; values in column 4 from Goddard and Goodall (4) expressed as weight per cent acid. All values in excess of 1% in italics.

† In the shorthand designation (3), the first numeral indicates chain length, the second indicates number of double bonds, br = branched, and un = unknown.
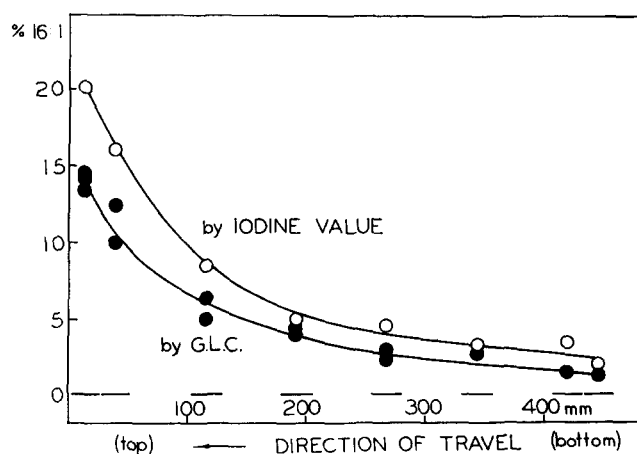

FIG. 1. Composition of the contents of a zone-melting tube plotted against its length. Separation of approximately 5 g of a mixture of 7% methyl palmitoleate in methyl palmitate in a column 4 × 450 mm at 4° to 5° after 24 passes. o-o = Composition determined by iodine value. ●-● = Composition determined by area proportionation of gas-liquid chromatograms. Short horizontal bars represent lengths of samples taken for analysis.

shows the effect of zone-melting on a mixture of methyl palmitate and methyl palmitoleate. The fractionation shown is far from ideal, but is given here as an example of the application of a new principle to the purification of lipids.

Schlenk (12) has reviewed the use of inclusion complexes of urea with straight-chain organic substances. Because urea readily forms inclusion complexes with straight-chain molecules, it is useful for separating these from branched-chain or cyclic molecules. The separation of saturated and unsaturated fatty acids by urea is less sharp. The tendency of unsaturated fatty acids to form complexes with urea decreases with increasing unsaturation, and progressively more unsaturated components can be precipitated by serial crystallization (13). The method is very useful for preparative purposes, and has been used as a preliminary step in the preparation of methyl arachidonate (14). Compared to low temperature crystallization, the urea method has the advantage of more convenient working temperatures, usually above 0°, and it is easily adapted to both small-scale and large-scale work. The method must be considered, however, more as a concentration step than as a quantitative separation between groups.

Chemical modification of the fatty acids has been widely used to increase differences between homologues and to make their separation easier. Formation of salts, hydroxamic acids, and other carboxyl derivatives have been used to achieve this end. Unsaturated acids have traditionally been isolated as their polybromides, but this procedure induces a certain amount of cis-trans isomerization. A parallel procedure which does not induce isomerization has been introduced recently. Jantzen and Andreas (15, 16) have studied the reaction kinetics between unsaturated fatty acids and mercuric acetate in methanol. Mercuri-methoxy addition compounds form easily at room temperature. Cis-unsaturated acids react and precipitate much more rapidly than trans acids, thereby allowing separation of these isomers. The addition compounds may then be decomposed to yield the original unsaturated acids. The mercuri-methoxy derivatives of various polyenoic acids have also been used for their separation by chromatography (vide infra).

DISTILLATION METHODS

In contrast to the foregoing methods of separation, fractional distillation separates primarily according to chain length. Murray (17) has reviewed its uses in lipid analysis and has commented upon the design of

suitable equipment. A variety of very efficient stills is now available, among which brush stills and whirling-band columns have particular applicability to the distillation of long-chain methyl esters. They provide a minimal pressure drop from pot to head of column. This has the practical effects of lowering the pot temperature necessary to drive a distillation and, consequently, of minimizing thermal polymerization of the charge. However, the low pressure which is necessary to distill the longer esters at temperatures below 200° substantially decreases the fractionating efficiency. Conditions for distillations should be chosen as a compromise between nonalteration of the sample and maximum efficiency of separation. Overlapping zones must be expected between fractions of different chain lengths, and only partial separation according to unsaturation may be achieved. Table 2 shows an example of the results that can be obtained, with reasonable care, by using the whirling-band column as a preparative tool (14). This study gives the practical details of the distillation procedure that are essential for high efficiency. The so-called amplified distillation of methyl esters in a larger amount of hydrocarbons has been useful for the fractionation of samples of less than 1 g (18). However, since the advent of the several chromatographic techniques now in common use, distillations are no longer needed except for the concentration of minor components and as steps in isolation procedures. Preparative gas-liquid chromatography may supplant small-scale distillation because of the sharpness of separation it offers (vide infra).

A pot residue of polymerized material is normally found after fractional distillation of highly unsaturated fatty acid esters. To avoid residues it may be desirable to work at the highest possible vacuum and speed, even if a good fractionation must be sacrificed. Molecular distillation allows minimum pressures and temperatures, and the rotary still developed by Biehler et al. (19) minimizes the time of exposure to heat, thereby further decreasing oxidation or polymerization of highly unsaturated esters, yet retaining a useful degree of chain-length separation. Hektogram or kilogram quantities may be distilled with laboratory models of rotary molecular stills. Figure 2 shows the results of such a molecular distillation of the methyl esters of the fatty acids of cod liver oil.

ADSORPTION METHODS

Adsorption chromatography, usually performed on columns of alumina, silica, or other inorganic material, has been a valuable tool in lipid analysis for several

decades (20). Good examples of its use are the separation of free acids from neutral lipids, the fractionation of nonsaponifiable lipids on alumina columns (21), and the many good adsorption chromatographic methods now in use in the field of steroids (22). Chromatography in columns has been used with good results for the separation of cholesterol esters, triglycerides, free cholesterol, and phospholipids of serum (23, 24). Hirsch and Ahrens (25) have reviewed this field, given illustrations of the potentiality of the technique, and suggested a standardized procedure for separating these classes of compounds. Wren and Mitchell (26) have extended the use of silicic acid columns to the separation of complex lipids, many of which are unidentified and some of which contain amino acid residues.

Adsorption chromatography is not easily adapted to paper because paper adsorbs polar solvents sufficiently to make the system separate partly by partition, even after impregnation of the paper with the adsorbent. Some suitable adsorbents may need treatment at high temperatures to give the desired activity, thereby excluding the use of paper. Recently, however, a successful application of adsorption chromatography to the separation of the tocopherols on paper impregnated with zinc carbonate has been reported (27).

Two relatively recent techniques employ adsorption chromatography on a microscale. Glass-fiber sheets are able to withstand the high temperatures necessary for activation and can be used as support for thin layers of common adsorbents. Dieckert and Reiser (28) have developed systems of glass-paper chromatography and methods of detecting spots after develop-
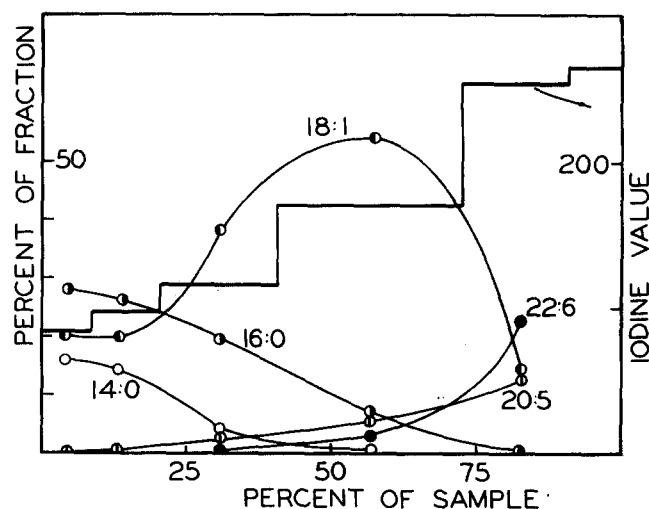

FIG. 2. Composition of serial fractions obtained during distillation of cod-liver oil fatty acid methyl esters in a rotary molecular still. The stepwise curve indicates iodine value and the size of the cuts. The smooth curves indicate the variation in content of representative esters in the several fractions. Composition of fractions was determined by area proportionation of gas-liquid chromatograms in this laboratory. Fractionation and determination of iodine values were performed at Johan C. Martens and Co., Bergen, Norway, by courtesy of Robert Nergaard.

ment of the papers. These techniques have been used for the separation of lipid classes and bile acids and their metabolic products (Fig. 3). Recently the method has been used as part of a quantitative microdetermination of cholesterol in serum (28a).

Thin-layer chromatography on glass plates also allows adsorptive separation of small quantities of

TABLE 2. FRACTIONATION OF METHYL ESTERS OF POLYUNSATURATED FATTY ACIDS OF PORK LIVER BY WHIRLING-BAND DISTILLATION (14) *

Fractions	1–3	4	5–9	10	11–17	18–20	21–30	31–33	34–38	Residue
Pot temperature	150°–155°		160°–165°		170°–178°		183°–195°		200°	
C_{14}	+	+								
C_{16}	+	+	+	+						
C_{18}				+	+	+				
C_{20}						+	+	+		
C_{22}								+	+	
Weight per cent of original sample	1.0	0.5	4.8	1.0	37.3	3.0	37.5	3.1	9.8	3.4

* Chain lengths present in each fraction determined by paper chromatography.

lipids. This technique, developed by Kirchner and Miller (29) and by Stahl (30), employs layers of silica or other adsorbents, approximately 0.25 mm thick, on glass plates. Spots may be developed by techniques similar to those used for glass-paper chromatography. Glass-fiber and glass-plate methods have the advantage that they allow the use of a sulfuric acid spray and charring as a general purpose detector

of organic materials as well as other specific reagents. Both methods tend to give more discrete spots than paper chromatography techniques, allowing the separation and detection of very closely related substances. Mangold and Malins (30a, 30b, 41) have investigated the separation of lipid substances by thin-layer chromatography, and Figure 4 shows a separation of some unsaponifiable substances using their
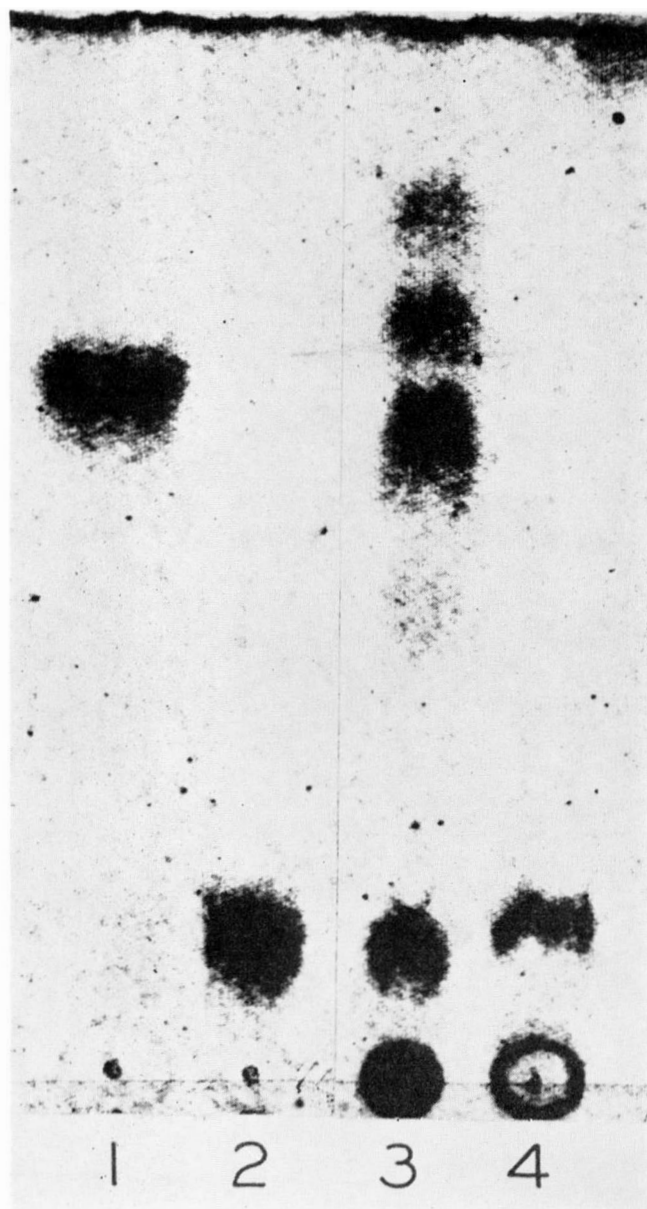

FIG. 3. Separation of blood lipids on glass paper coated with 1% silicic acid. The solvent was isooctane; the time of running was 9 minutes. The samples were (1) 2 μg cholesterylacetate; (2) 2 μg cholesterol; (3) 10 μl of a Bloors extract of serum representing a 1:5 dilution of the serum; and (4) 10 μl of the same extract hydrolyzed with KOH. Illustration by courtesy of James B. Hamilton. Developed with H_2SO_4.
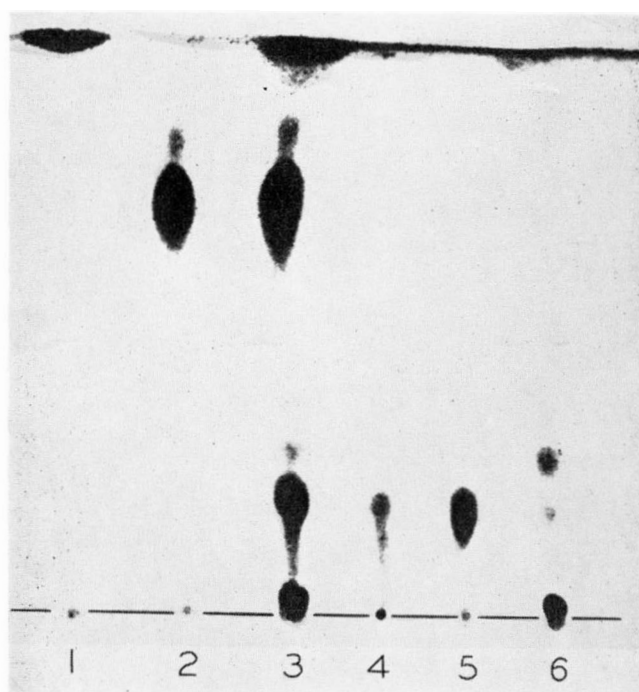

FIG. 4. Thin-layer silicic acid chromatography according to the techniques of H. K. Mangold and D. C. Malins, using vertical ascending chromatography for 50 minutes. The solvent was ethyl acetate: light petroleum ether, 20/80(v/v). The samples were (1) squalene; (2) α-tocopherol; (3) mixture of 1, 2, 4, 5, and 6; (4) vitamin A; (5) cholesterol; and (6) selachyl alcohol. Developed with H_2SO_4.

technique. The esters of epoxy, hydroxy, conjugated, and nonconjugated unsaturated fatty acids are similarly separable (31, 32). The separation and identification of some positional isomers of unsaturated oxy acids have been accomplished by thin-layer chromatography, although separation of these substances could not be demonstrated by paper or gas-liquid chromatography. Thin-layer and glass-paper chromatography are a useful adjunct to adsorption chromatography on columns because they allow rapid scanning of the gross separation by a micromodification of the same procedure. Modern methods of monitoring adsorption chromatograms reveal the separation of such closely related substances as structural and stereo isomers and lipids that differ only by a functional group.

For separation of large amounts of substances having greatly different adsorptive properties, batch adsorption may be more convenient than column chromatography. For example, this laboratory has employed batch adsorption for the isolation of pure oxy fatty acid esters from natural oils and autoxidation mixtures.

A special technique of displacement chromatography on carbon columns was introduced by Tiselius and his students and has been applied to fatty acids and esters (33). In this technique the zones of substances migrate, one immediately following and displacing the other, and therefore the method demands very sharp fronts of the eluting zones. A very efficient column is required, and one consisting of a series of segments of decreasing volumes, joined by capillaries, has been developed for this purpose (34). Displacement chromatography has been used for the separation of certain unsaturated acids, as illustrated in Figure 5.
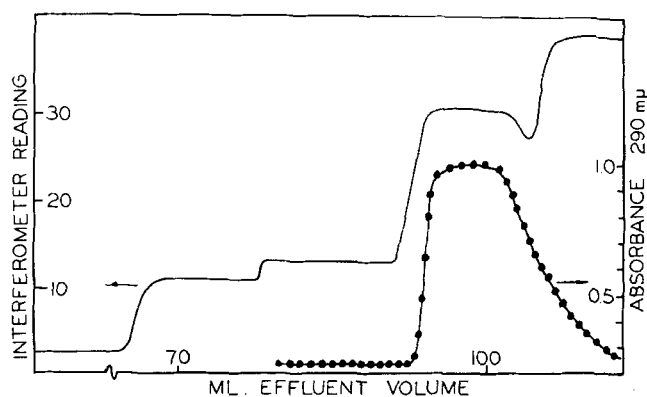

FIG. 5. Separation of 400 mg fatty acids of *Apodenthria undulata* by displacement chromatography using light absorbance at 290 mμ to detect the conjugated triene component. The solid curve represents the micrometer readings (a measure of refractive index) plotted against effluent volume. The dotted curve represents a plot of relative absorbance at 290 mμ versus effluent volume. The displacer was 1% ethyl stearate in 95% ethanol and adsorbent was Darco G60 charcoal.

Carrier-displacement chromatography, a modification of this technique, has given good separations of homologous series. Small amounts of the acids to be separated, plus larger amounts of their respective esters, are chromatographed together, the latter acting as carriers. Thus the sample acids emerge spatially separated by their esters. Displacement chromatograms have been monitored by paper chromatography as well as by refractometry. As in most chromatographic methods, overlapping zones occur, and displacement techniques inherently cannot give as good separations as can some other recent chromatographic techniques because the

zones are adjacent and displace each other. However, the multiple column designed to exploit displacement chromatography is theoretically and practically the most efficient column available. It can be used to advantage with other forms of column chromatography (34).

PARTITION METHODS

Countercurrent distribution was first investigated by Craig and his colleagues for the separation of fatty acids and esters, and the field has been expanded by Dutton (35). Use of a strongly polar solvent to overcome association of fatty acids is necessary to obtain good separations of these compounds, but less polar solvents are suitable for the separation of esters (36), particularly from mixtures containing oxygenated components (32). In countercurrent distribution, as in other methods of pure liquid-liquid partition, the partition coefficients are influenced by unsaturation as well as by chain length. The effect of increasing the chain length by two methylene groups is opposite to, and nearly equal to, that of increasing the unsaturation by one double bond. Thus eicosadienoic, oleic, and palmitic acids migrate as one group, and linoleic, palmitoleic, and myristic acids as another (Table 3).

TABLE 3. ANTICIPATED SEPARATION OF FATTY ACIDS BY PARTITION METHODS USING PAPER CHROMATOGRAPHY

R_f Value						
			16:4			
10:0	12:1	14:2	16:3	18:4	20:5	22:6
12:0	14:1	16:2	18:3	20:4	22:5	
14:0	16:1	18:2	20:3	22:4		
16:0	18:1	20:2	22:3			
18:0	20:1	22:2				
20:0	22:1					
22:0						
0						

The groups of fatty acids having higher R_f values at the top of the table are potentially more complex if the natural mixtures are not subjected to prior group separation as, for example, by chain length (diagonal cuts).

After preliminary group separations, countercurrent distribution has its greatest usefulness in the preparation of pure acids in quantities above the capacity of chromatographic columns (37). It has the disadvantage that a large, delicate, and complicated apparatus is required.

Partition chromatography is being applied to nearly every field of lipid research, the solvent composition being governed by the particular substances handled. The less polar solvent is customarily used as the stationary phase, and may be a high boiling hydrocarbon, silicone, or similar substance adsorbed on diatomaceous earth (kieselguhr, Celite®), or cellulose powder. The eluting solvent usually contains a high proportion of acetic acid, acetonitrile, or similar polar solvent. Contrary to adsorption chromatography, partition methods usually require some form of temperature control to obtain sharp separations because the phase equilibrium is affected by temperature changes.

Hirsch (38) has applied Boldingh's rubber columns to the separation of fatty acid esters (Fig. 6) and one of two techniques. Kaufmann (1) and his students have developed methods using undecane as the stationary phase, and aqueous acetic acid as moving solvent. The spots are detected by converting the fatty acids to heavy metal salts, which are treated with reagents that give colored products with the metal ion. Different paraffins may be used, but undecane has been preferred because it is easily removed by heating the paper before developing the spots. Quantification may be obtained by spot density measurements or by polarography of the metal ion eluted from the spot. Schlenk et al. (8) used siliconized papers, and aqueous acetic acid or acetonitrile as solvents. They developed the spots by complex formation of the fatty acids with α-cyclodextrin followed by exposure to iodine vapor.
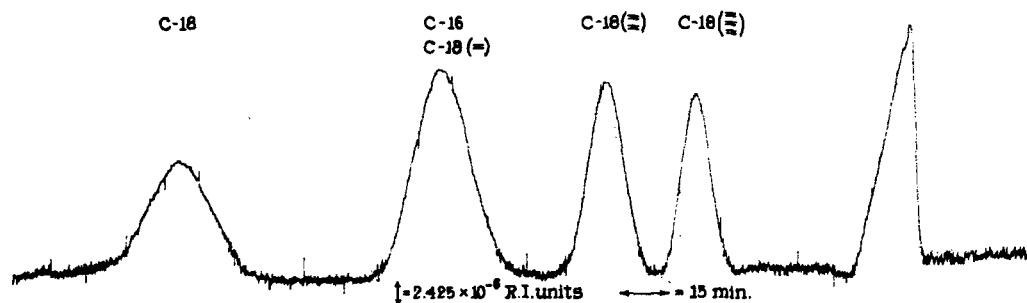

FIG. 6. Separation of fatty acid methyl esters on a rubber column monitored by a differential refractometer. The tracing reads from right to left and the first peak is the solvent front. The abscissa is time and the ordinate is refractive index. Courtesy of Hirsch (38).

Gellerman and Schlenk (39) have reported the use of silicone-Celite® columns for the same purpose. Although a nonpolar column has usually been used in lipid work, some recent applications using a more polar stationary phase show great promise. The separation of certain steroids on columns containing propylene glycol or ethylene glycol (22), as well as the separation of fat-soluble vitamins on polyethylene glycol-Celite® columns (40), are typical examples. These columns are very stable, are less subject to bleeding as a consequence of temperature variation, and have the advantage that they may be eluted at a higher rate.

Reversed-phase partition chromatography on paper is a convenient tool in microscale lipid work. Separation of fatty acids is usually performed according to

The α-cyclodextrin will stain with iodine, whereas the complex will not react, thus giving white spots on a violet background. Alternatively, unsaturated substances may be stained by iodine vapor alone. (Humidification is necessary before exposure to iodine vapor.) This method of detection has the advantage over those using metal salts, in that no washing of the papers is necessary and the whole development may take only a few minutes. Quantitative radiometry in paper chromatography of fatty acids has been reported. Mangold et al. (43) methylated the acids with radioactive diazomethane and measured the activity in the paper, whereas Kaufmann and Budwig (42) used salts of radioactive cobalt.

Several techniques have been devised to overcome the overlapping of groups encountered in partition.

Removal of either the saturated or the unsaturated acids may sometimes solve the problem. Saturated acids may be removed by running the papers at low temperatures to freeze out those acids at the starting point of the chromatograms. This technique has certain restrictions, as, for example, the residual solubility of the saturated lauric acid and the limited solubility of the unsaturated erucic acid at a given low temperature. Unsaturated acids may be removed by running the chromatograms in peracetic acid, thereby oxidizing the unsaturated acids, with the result that these migrate with the solvent front (43). Further modifications of fatty acid paper chromatography include hydrogenation of unsaturated acids on the paper (1), and the addition of mercuri-methoxy groups to the double bonds of the esters (15, 16, 44). The latter derivatives are more polar than the original esters and otherwise inseparable groups may thus be separated by partition methods. Saturated fatty acids of 20 to 30 carbon atoms have been separated on paper as the mercuric acetate addition compounds of their allyl esters (45). Recently a technique for the saponification of small amounts of fats on the paper prior to paper chromatography of their fatty acids has been reported (46).

Two-directional chromatography (adsorption in one direction; partition in the other) has been successfully applied to the separation of tocopherols (27), and circular-paper chromatography may be convenient in the separation of closely related compounds. Paper chromatography cannot be expected to give the separation efficiency of gas-liquid chromatography. It is, however, a convenient, quick, and inexpensive method, and a valuable tool in monitoring preparations.

GAS-LIQUID CHROMATOGRAPHY

Gas-liquid chromatography has lately drawn the interest of the lipid chemists and is a technique undergoing rapid development. The method was introduced by James and Martin (47) when they separated the normal saturated carboxylic acids up to 12 carbon atoms in chain length. Cropper and Heywood (48) extended the method to the separation of the methyl esters of even-numbered fatty acids up to behenic acid, and since then there has been a rapid expansion in the use of this method in the lipid field.

The general theory and practice of the gas chromatographic methods are extensively treated in recent textbooks and reviews (49 to 56), and the problem of presenting the increasing amount of data in a consistent way has led to the recommendations of the Amsterdam Committee (57, 58). Some developments in the field of separation of fatty acid methyl esters have been described by Farquhar et al. (59). The emphasis in recent studies of gas-liquid chromatography in this particular field has been in improving separation by finding more suitable stationary phases and in improving the instrumentation. It has thus been possible to extend the working range for methyl esters of fatty acids as far as 34 carbon atoms (60) and to detect and estimate components below 0.05% of the original sample (59).

In the last few years a number of instruments for gas-liquid chromatography have become available commercially. In order to reduce the working temperature, to speed up the chromatograms, and to improve the separation, it is common practice to convert the substances to lower boiling derivatives. The fatty acids are usually separated as methyl esters, although sometimes higher esters are used.

The supporting materials for the packed columns are kieselguhr (Celite®) or ground firebrick, although some experiments with sodium chloride (61), and even with rings of stainless steel gauze, have been reported (62). The stationary liquid phases used in work with fatty acid methyl esters and similar compounds are either of the nonpolar type such as the paraffin greases and silicone products, or of the polar type such as polyesters. With nonpolar phases, unsaturated and branched components emerge before the corresponding saturated ones, whereas with polar phases, unsaturated acids emerge after their saturated analogues. Late-emerging components of one chain-length group may be retained in the column so long that they overlap with components from the following chain-length group.

The efficiency of separation in a column is the result of at least two factors: column efficiency and solvent efficiency. Column efficiency is often expressed as the height equivalent of a theoretical plate and is measured by the spread of a component when it passes through the column. This is related to many factors associated with the packing and design of the column. The solvent efficiency, or separation factor, depends on the distribution coefficients of the different solutes between the liquid and the gas phases. It is expressed by the ratio of the retention volumes of two peaks. The choice of stationary phase depends in each case on the particular separation desired. The plate efficiency of a polar column is usually less than that of a nonpolar column. Experienced investigators have been able to obtain columns of the latter type with a height equivalent of a theoretical plate as good as 0.3 mm (63, 64, 65).

The capillary columns introduced by Golay (66) have height equivalent of a theoretical plate of the same order as packed columns but they give better separation because of the extreme length of the column and the very small charge used. These columns therefore demand very sensitive detectors. Capillary columns have been used by Lipsky and co-workers (67, 68) and promise separation of isomers of fatty acid esters (84).

The carrier gases preferred in work with fatty acid esters have been argon, helium, hydrogen, and nitrogen. Argon is used in instruments equipped with β-ray argon ionization detectors. Helium is commonly used in the United States in instruments with thermal conductivity detectors, radio frequency detectors, and thermionic ionization detectors, while the less expensive gases, hydrogen and nitrogen, are in common use with thermal conductivity detectors elsewhere. Hydrogen alone or mixed with another gas is used in connection with flame- and flame-ionization detectors.

The practical identification of components in a chromatogram is achieved by the use of standards. Members in a homologous group may be identified by plotting the logarithms of retention times, or retention volumes, versus some group quality, such as chain length or degree of unsaturation. However, care must be exercised because different components may behave similarly on a given column. Either operation of the oven at two temperatures or use of two columns based upon different separatory principles is mandatory in making identification certain.

The concept of "carbon number" is a rather new method of presenting qualitative data from gas-liquid chromatography of fatty acid esters (69, 70). Carbon numbers represent no difference in principle from relative retention times but are perhaps of practical value because they indicate more directly and vividly the position of a component in the elution pattern.

James (64) has devised a method to determine the degree of unsaturation of straight-chain and simpler branched-chain components. By plotting the logarithm of the retention time measured on one stationary phase against similar values from a different stationary phase, saturated components lie on one straight line and monoenes, dienes, trienes, etc., lie each on a separate parallel line.

The quantitative aspect of gas-liquid chromatography has been studied by several investigators. A serious problem arises from the fact that the detectors do not always give a response proportional to concentration. The detectors most frequently used in the lipid field have been the gas-density balance, the thermal conductivity cell, and the β-ray argon ionization cell. The gas-density balance has the simplest relationship between signal and effluent composition. The response depends only on the difference in concentration and molecular weight between the component and the carrier gas. This detector was introduced in gas-liquid chromatography by Martin and James (71) and has been used by several investigators (3a, 69, 72 to 76).

For other detectors the response is not a simple function of concentration and molecular weight. The thermal conductivity cell, first used in gas-solid chromatography by Claesson (77), is perhaps the most widely used. Its response varies not only with the amount but also, as a rule, with the type of compound being detected (78). However, with hydrogen or helium as carrier gas, the response of a thermal conductivity cell is less dependent upon the nature of the components than when nitrogen is used. Therefore one may omit calibration when using one of the two light gases if the constituents do not vary too much in type. Calibration is always essential to ensure results of high accuracy. The practice of comparing peak areas with weight per cent for one homologous group and with mole per cent for another closely related group is the result of the omission of the necessary calibration. Rosie and co-workers (79) claim that the response of a thermal conductivity detector with helium is independent of the individual sensing unit (hot wire or thermistor), the concentration, and the temperature of the detector, and depends only on the molecular weight and class of the compound. They have published a list of relative response factors for some hydrocarbons, ketones, alcohols, etc. (80). However, Sørensen and Søltoft (81) have shown that the relative response for the methyl esters of fatty acids varies with amount as well as with chain length and unsaturation when nitrogen is used as carrier. Jart (82) reported similar findings and found nonlinearity of the response using helium (83). Beerthuis and co-workers (84) have avoided these difficulties by converting the components to CO_2 before passing the gas stream into the detector.

Lately, the β-ray argon ionization detector of Lovelock's design (85, 86) has attracted considerable interest. This and the flame ionization detector have a very high sensitivity and may therefore be employed in connection with capillary columns. Both detectors are also commonly used with ordinary packed columns. Linearity of response with vapor concentration is claimed for these detectors but the proper conditions for linearity must be ascertained (59, 87).

However, in spite of the difficulties in making quantitative measurements, variation between the lipid

compositions of similar biological samples is often so great that the inaccuracies of the detector are not apparent to the investigator and are small compared to the biological comparisons to be made. By gas-liquid chromatography it is possible to get qualitative and quantitative information impossible to collect by other methods.

Another question in connection with the qualitative and quantitative interpretation of the chromatogram is the stability of the substances inside the column. It is well established that esters of polyunsaturated acids are astonishingly stable at temperatures up to 200° (88). Transesterification, however, may take place in columns packed with polyesters. Orr and Callen (89)
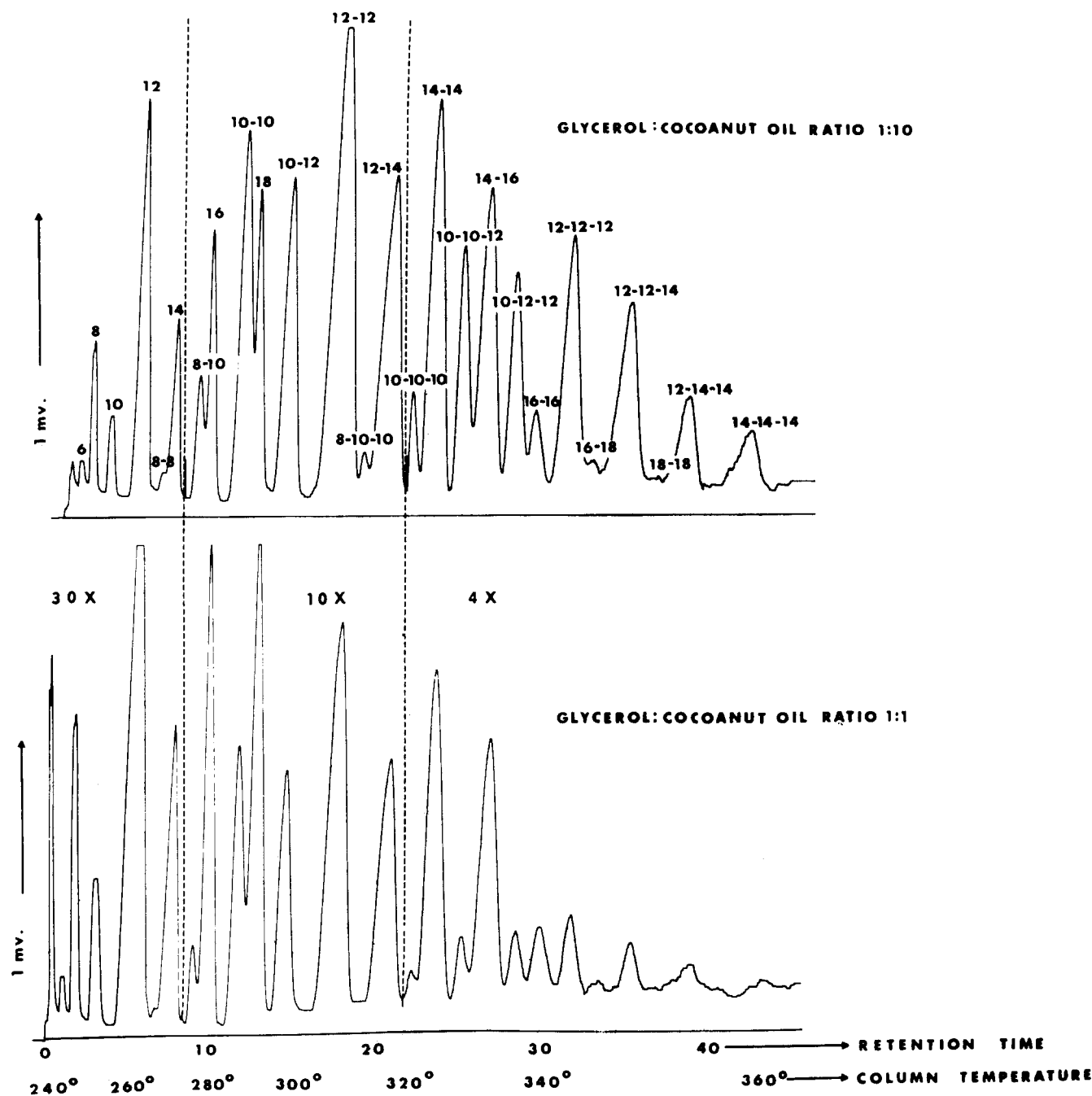

FIG. 7. Gas chromatogram showing the separation of mono-, di-, and triglycerides on a temperature-programmed silicone rubber Celite® column. The numbers on the peaks refer to the chain lengths of the constituent fatty acids. Upper curve: rearranged mixture of glycerol: cocoanut oil, 1:10. Lower curve: rearranged mixture of glycerol: cocoanut oil, 1:1. Courtesy of Huebner (95).

have shown that this is the case for at least one of the polyesters at 240°, but their finding is questioned by Link *et al.* (90). Work recently done in this laboratory indicates that esters of some unsaturated hydroxy and hydroperoxy acids will undergo dehydration to more highly unsaturated derivatives, and that conjugated polyunsaturated esters may undergo *cis-trans* isomerization (32). In addition to the possibility of alterations during the run, gas-liquid chromatography suffers the disadvantage that some components of the sample may never emerge from the column, and therefore will be undetected. This may cause significant errors in calculations of composition since these are often based on emerging components only.

The preparation of methyl esters of fatty acids on a microscale is often necessary prior to analysis by gas-liquid chromatography. The esterification, although a simple procedure on a large scale, presents some difficulties when milligram or microgram quantities must be handled. Three micromethods have been offered recently for the conversion of lipids to methyl esters of their component fatty acids. Stoffel *et al.* (91) employed catalyzed transesterification in methanol followed by short-path distillation of the methyl esters to free them from nonsaponifiable matter. Luddy *et al.* (92) used sodium and potassium methylate in excess methanol to convert sterol esters, glycerides, and phospholipids to methyl esters of their fatty acids, followed by separation of the esters from other substances by silicic-acid chromatography. These two methods were devised to avoid the saponification of lipids and the extraction of nonsaponifiable matter, which is often an unsuitable procedure with minute samples. By studying the conditions of the reaction, Schlenk and Gellerman (92a) have removed many of the objections to the use of diazomethane for conversion of free acids to methyl esters. By use of 10% methanol in ether as solvent, and by avoiding an excess of diazomethane, they were able to prepare methyl esters without demonstrable formation of polymers or volatile side products. Their method is proposed as a microesterification suitable for preparation of C^{14} methyl esters and is performed on such a small scale that the hazard of explosion caused by mishandling diazomethane is removed.

The use of the gas-liquid chromatographic technique in the lipid field has not been restricted to the fatty acids and their esters. Cropper and Heywood (48) have separated fatty alcohols; Link and co-workers (90, 93) have separated fatty alcohols and nitriles; and McInnes *et al.* (94) have estimated monoglycerides quantitatively. Huebner, using temperature programming, has separated mono-, di-, and triglycerides

on a short column of silicone rubber on Celite®, as shown in Figure 7 (95, 96). Glyceryl ethers have been separated as their acetates on a polyester column by Blomstrand and Gürtler (97), and Figure 8 shows a
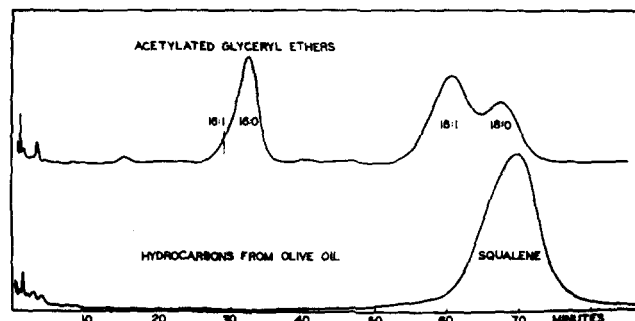

Fig. 8. Separation of glyceryl ethers and elutions of squalene using a 0.35 × 60 cm column packed with 5 g 80 to 100 mesh Celite® coated with Apiezon L. Temperature 220°, flow rate 80 ml argon per minute β-ray argon ionization detection.

similar separation on a nonpolar column, as well as the elution of squalene from the hydrocarbon fraction from olive oil.

Gas-liquid chromatography has a great potential usefulness in combination with other chemical and physical methods of investigation. Passing the effluent gas through such instruments as infrared and ultraviolet absorption spectrometers, emission spectrographs, mass spectrometers, etc., are possibilities. Little work has been done along along these lines as yet. James and co-workers (98) have, in a study of the metabolism of propionic acid, collected radioactive fatty acids and esters from the gas chromatograph, and Popják *et al.* (99) have connected their column directly to a radioactivity counter. Fractions may be collected from the gas stream for further study, provided the components are not destroyed by the method of detection. In our laboratory, ultraviolet and infrared spectra of the zones from gas chromatographs have been used for partial identification of substances (31). Moreover, components separated on thin-layer chromatographs provide sufficient material for identification by gas chromatography and ultraviolet and infrared spectra. Conversely, the zones from gas chromatographs may be rechromatographed on thin-layer plates for verification of identity and uniformity (32).

It is always possible to collect microgram quantities from an analytical gas chromatograph, but specially designed columns and detectors are usually needed in order to allow collection of significant amounts. Preparative columns have generally been constructed by

the individual investigators, but recently preparative instruments or additions to older models have appeared on the market. Most of these are designed for samples up to one milliliter, but one manufacturer claims that his instrument has a capacity of 50 ml, and that it has separated fatty acid methyl esters up to C_{14} (100). Fulco and Mead (101) have prepared as much as 90 mg of an eicosatrienoate by gas-liquid chromatography and liquid-liquid column chromatography. One serious disadvantage of preparative gas-liquid chromatography is the possible contamination of products due to bleeding of the stationary phase, and some subsequent purification is often necessary.

COMPARISON OF METHODS OF ANALYSIS

In an attempt to compare analytical methods, Schlenk et al. (101a) applied gas-liquid chromatography, several versions of paper chromatography, and alkaline isomerization to one preparation of methyl esters of fatty acids from *Chlorella pyrenoidosa*. Gas-liquid chromatography was found to be more rapid and reproducible and to have greater resolving power than the other methods, but it may give erroneous results because of the presence of nonvolatile components. The quantitative results from the three methods were in general agreement for the major components (C_{16} and C_{18}). Results obtained by alkaline isomerization of the C_{18} fraction differed from those by the other methods, probably traceable to impure linolenate standards. The error was increased when C_{18} standards were used for C_{16} fractions. With these fractions the use of 16:2 and 16:3 standards yielded data in better agreement with those obtained by paper chromatography and gas-liquid chromatography.

Alkaline isomerization, iodine value, and gas-liquid chromatography were applied to vegetable oils and synthetic mixtures by Craig and Murty (102). They found that iodine values calculated from gas-liquid chromatography data agreed well with those found by direct measurement. Spectral analysis by the standard methods of the American Oil Chemists' Society gave lower values for linoleate and higher values for linolenate than did gas-liquid chromatography, and this was especially apparent in oils with a high proportion of linolenic acid. Gracián et al. (103) have compared the composition of olive oil as measured by ester distillation, thiocyanogen value, gas-liquid chromatography, alkaline isomerization, and quantitative paper chromatography. Although the methods measured different groups of components, wherever comparison was possible, the several methods yielded very similar data.

For example, the content of monoenoic acids was found to be 75.9%, 75.5%, 74.7%, 76.8%, and 75.7%, respectively, measured by the methods listed above. Tuna et al. (104) used both alkaline isomerization and gas-liquid chromatography for the analysis of the fatty acids of plasma and atheromatous plaques. The results obtained by isomerization fitted well with those of gas-liquid chromatography, and errors in the measurements of the moderately unsaturated acids by isomerization were minimal. Herb et al. (105) have analyzed model mixtures of fatty acid methyl esters and have found good agreement between the known composition and the composition determined by gas-liquid chromatography and by alkaline isomerization. Similar agreement has been demonstrated by Insull and Ahrens (3a) in their studies on human milk fat.

CONCLUSIONS

From the foregoing, it should be apparent that the methods of analysis of fatty acids and other lipid components have been improved considerably in the last decade. The lower limit of detection of individual components has been diminished at least one order of magnitude, and the separation of individual components has been improved. Now even trace components can be detected and measured in the usual lipid sample, and major components can be measured in only trace amounts of material. The amount of a biological specimen now needed for analysis is so small that animals and tissues need not be pooled, and analysis of biopsy samples is readily achieved. Many new biological problems now await solution, and the investigator has at his disposal a choice of rapid and precise methods, the tools for which range from complex electronic equipment down to simple inexpensive implements that can be made in the laboratory.

Current methods of fatty acid analysis give comparable results and each has its advantages and may be applied within its limitations to chemical and biological problems. It appears that the advent of gas-liquid chromatography need not mean the extinction of other methods of lipid analysis. Every man should think for himself.

REFERENCES

1. Kaufmann, H. P. *Analyse der Fette und Fett produkte.* Berlin, Springer-Verlag, 1958.
2. Holman, R. T., W. O. Lundberg and T. Malkin, editors. *Progress in the Chemistry of Fats and Other Lipids.* London, Pergamon Press, 1952–58 (5 vols.).

3. Ahrens, E. H. Jr., W. Insull, Jr., J. Hirsch, W. Stoffel, M. L. Peterson, J. W. Farquhar, T. Miller and H. J. Thomasson. *Lancet* **1**: 115, 1959.

3a. Insull, W., Jr., and E. H. Ahrens, Jr. *Biochem. J.* **72**: 27, 1959.

4. Goddard, V. R., and L. Goodall. *Fatty Acids in Animal and Plant Products*. Washington, D. C., U. S. Department of Agriculture, 1959.

5. Silk, M. H., H. H. Sephton and H. H. Hahn. *Biochem. J.* **57**: 574, 1954.

6. Brown, J. B., and D. K. Kolb. In *Progress in the Chemistry of Fats and Other Lipids*, London, Pergamon Press, 1955, vol. 3, p. 57.

7. Kaufmann, H. P., and E. Mohr. *Fette, Seifen, Anstrichmittel* **59**: 920, 1957.

8. Schlenk, H., J. L. Gellerman, J. A. Tillotson, and H. K. Mangold, *J. Am. Oil Chemists' Soc.* **34**: 377, 1957.

9. Pfann, W. G. *Zone Melting*. New York, John Wiley & Sons, Inc., 1958.

10. Hesse, G., and H. Schildknecht. *Angew. Chem.* **68**: 641, 1956.

11. Schildknecht, H., and H. Vetter. *Angew. Chem.* **71**: 723, 1959.

12. Schlenk, H. In *Progress in the Chemistry of Fats and Other Lipids*, London, Pergamon Press, 1954, vol. 2, p. 243.

13. Abu-Nasr, A. M., W. M. Potts and R. T. Holman. *J. Am. Oil Chemists' Soc.* **31**: 16, 1954.

14. Privett, O. S., R. P. Weber and E. C. Nickell. *J. Am. Oil Chemists' Soc.* **36**: 443, 1959.

15. Jantzen, E., and H. Andreas. *Angew. Chem.* **70**: 656, 1958.

16. Jantzen, E., and H. Andreas. *Chem. Ber.* **92**: 1427, 1959.

17. Murray, K. E. In *Progress in the Chemistry of Fats and Other Lipids*, London, Pergamon Press, 1955, vol. 3, p. 243.

18. Weitkamp, A. W., A. M. Smiljanic and S. Rothman. *J. Am. Chem. Soc.* **69**: 1936, 1947.

19. Biehler, R. M., K. C. D. Hickman and E. S. Perry. *Anal. Chem.* **21**: 638, 1949.

20. Lederer, E., and M. Lederer. *Chromatography*. Houston and New York, Elsevier Press, Inc., 1957, 2d ed.

21. Gridgeman, N. T., G. P. Gibson and J. P. Savage. *Analyst* **73**: 662, 1948.

22. Neher, R. *Chromatographie von Sterinen, Steroiden und verwandten Verbindungen*. Houston and New York, Elsevier Press, Inc., 1958. Also *J. Chromatog.* **1**: 122, 205, 1958.

23. Borgström, B. *Acta Physiol. Scand.* **25**: 111, 1952.

24. Fillerup, D. L., and J. F. Mead. *Proc. Soc. Exptl. Biol. Med.* **83**: 574, 1953.

25. Hirsch, J., and E. H. Ahrens, Jr. *J. Biol. Chem.* **233**: 311, 1958.

26. Wren, J. J., and H. K. Mitchell. *Proc. Soc. Exptl. Biol. Med.* **99**: 431, 1958.

27. Green, J., S. Marcinkiewicz and P. R. Watt. *J. Sci. Food Agr.* **6**: 274, 1955.

28. Dieckert, J. W., and R. Reiser. *Federation Proc.* **14**: 202, 1955.

28a. Swartwout, J. R., J. W. Dieckert, O. N. Miller, and J. G. Hamilton. *J. Lipid Research* **1**: 281, 1960.

29. Kirchner, J. G., and J. N. Miller. *J. Agr. Food Chem.* **1**: 512, 1953.

30. Stahl, E. *Pharmazie* **11**: 633, 1956; *Chemiker-Ztg.* **82**: 323, 1958.

30a. Mangold, H. K., and D. C. Malins. *J. Am. Oil Chemists' Soc.* **37**: 383, 1960.

30b. Malins, D. C., and H. K. Mangold. *J. Am. Oil Chemists' Soc.*, in press.

31. Morris, L. J., R. T. Holman and K. Fontell. *J. Am. Oil Chemists' Soc.* **37**: 323, 1960.

32. Morris, L. J., R. T. Holman and K. Fontell. *J. Lipid Research* **1**: 412, 1960.

33. Holman, R. T. In *Progress in the Chemistry of Fats and Other Lipids*, London, Pergamon Press, 1952, vol. 1, p. 104.

34. Hagdahl, L. *Acta Chem. Scand.* **2**: 574, 1948; *Sci. Tools* **1**: 21, 1954.

35. Dutton, H. J. In *Progress in the Chemistry of Fats and Other Lipids*, London, Pergamon Press, 1954, vol. 2, p. 292.

36. Scholfield, C. R., J. Nowakowska and H. J. Dutton. *J. Am. Oil Chemists' Soc.* **37**: 27, 1960.

37. Klenk, E., and H. Steinbach. *Z. physiol. Chem., Hoppe-Seyler's* **316**: 31, 1959.

38. Hirsch, J. *Federation Proc.* **18**: 246, 1959.

39. Gellerman, J. L., and H. Schlenk, *Experientia* **15**: B 387, 1959.

40. Theivagt, J. G., and D. J. Campbell. *Anal. Chem.* **31**: 1375, 1959.

41. Mangold, H. K. *Fette, Seifen, Anstrichmittel* **61**: 877, 1959.

42. Kaufmann, H. P., and J. Budwig. *Fette u. Seifen* **53**: 69, 1951.

43. Mangold, H. K., J. L. Gellerman and H. Schlenk. *Federation Proc.* **17**: 268, 1958.

44. Inouye, Y., M. Noda and O. Hirayama. *J. Am. Oil Chemists' Soc.* **32**: 132, 1955.

45. Kaufmann, H. P., and J. Pollerberg. *Fette, Seifen, Anstrichmittel* **59**: 815, 1957.

46. Kaufmann, H. P., and Z. Makus, *Z physiol. Chem., Hoppe-Seyler's* **317**: 39, 1959.

47. James, A. T., and A. J. P. Martin. *Biochem. J.* **50**: 679, 1952.

48. Cropper, F. R., and A. Heywood. *Nature* **172**: 1101, 1953.

49. Keulemans, A. I. M. *Gas Chromatography*. New York, Reinhold Publishing Corp., 1957.

50. Phillips, C. *Gas Chromatography*. New York, Academic Press, Inc., 1956.

51. Pecsok, R. L., ed. *Principles and Practice of Gas Chromatography*. New York, John Wiley & Sons, Inc., 1959.

52. Desty, D. H., ed. *Vapour Phase Chromatography*. New York, Academic Press, Inc., 1957.

53. Coates, V. J., H. J. Noebels and I. J. Fagerson, eds. *Gas Chromatography*. New York, Academic Press, Inc., 1958.

54. Desty, D. H., ed. *Gas Chromatography*. New York, Academic Press, Inc., 1958.

55. Hardy, C. J., and F. H. Pollard. *J. Chromatog.* **2**: 1, 1959.

56. Rose, B. A. *Analyst* **84**: 574, 1959.

57. Ambrose, D., A. I. M. Keulemans and J. H. Purnell. *Anal. Chem.* **30**: 1582, 1958.

58. Johnson, H. W., and F. H. Stross. *Anal. Chem.* **30**: 1586, 1958.

59. Farquhar, J. W., W. Insull, Jr., P. Rosen, W. Stoffel, and E. H. Ahrens, Jr. *Nutrition Revs.* **17**: Supplement, 1959.

60. Kahn, M. A., and B. T. Whitham. *J. Appl. Chem.* **8**: 549, 1958.

61. Cropper, F. R., and A. Heywood. *Nature* **174**: 1063, 1954.

62. Sørensen, I., and P. Søltoft. *Acta Chem. Scand.* **10**: 1673, 1956.

63. Scott, R. P. W., and J. D. Cheshire. *Nature* **180**: 702, 1957.

64. James, A. T. *J. Chromatog.* **2**: 552, 1959.

65. Horwitt, M. K., C. C. Harvey and B. Century. *Science* **130**: 917, 1959.

66. Golay, M. J. E. In *Gas Chromatography*, edited by D. H. Desty, New York, Academic Press, Inc., 1958, p. 36.

67. Lipsky, S. R., J. E. Lovelock and R. A. Landowne. *J. Am. Chem. Soc.* **81**: 1010, 1959.

68. Lipsky, S. R., R. A. Landowne and J. E. Lovelock. *Anal. Chem.* **31**: 852, 1959.

69. Böttcher, C. J. F., F. P. Woodford, E. Boelsma-van Houte, and C. M. van Gent. *Rec. trav. chim.* **78**: 794, 1959.

70. Woodford, F. P., and C. M. van Gent. *J. Lipid Research* **1**: 188, 1960.

71. Martin, A. J. P., and A. T. James. *Biochem. J.* **63**: 138, 1956.

72. James, A. T., and A. J. P. Martin. *Biochem. J.* **63**: 144, 1956.

73. James, A. T., and V. R. Wheatley. *Biochem. J.* **63**: 269, 1956.

74. James, A. T., and J. Webb. *Biochem. J.* **66**: 515, 1957.

75. Insull, W., Jr., and E. H. Ahrens, Jr. *Biochem. J.* **72**: 27, 1959.

76. Boughton, B., and V. R. Wheatley. *Biochem. J.* **73**: 144, 1959.

77. Claesson, S. *Arkiv Kemi, Mineral. Geol.* **23**: A-1, 1946.

78. Keulemans, A. I. M., A. Kwantes and G. W. A. Rijnders. *Anal. Chim. Acta* **16**: 29, 1957.

79. Rosie, D. M., and R. L. Grob. *Anal. Chem.* **29**: 1263, 1957.

80. Messner, A. E., D. M. Rosie and P. A. Argabright. *Anal. Chem.* **31**: 230, 1959.

81. Sørensen, I., and P. Søltoft. *Acta Chem. Scand.* **12**: 814, 1958.

82. Jart, Aa. *Fette, Seifen, Anstrichmittel* **61**: 541, 1959.

83. Jart, Aa. *Acta Chem. Scand.* **13**: 1723, 1959.

84. Beerthuis, R. K., G. Dijkstra, J. G. Keppler, and J. H. Recourt. *Ann. N. Y. Acad. Sci.* **72**: 616, 1959.

85. Lovelock, J. E. *J. Chromatog.* **1**: 35, 1958.

86. Lovelock, J. E. *Nature* **181**: 1460, 1958.

87. Lovelock, J. E., A. T. James and E. A. Piper. *Ann. N. Y. Acad. Sci.* **72**: 720, 1959.

88. Stoffel, W., W. Insull, Jr. and E. H. Ahrens, Jr. *Proc. Soc. Exptl. Biol. Med.* **99**: 238, 1958.

89. Orr, C. H., and J. E. Callen. *Ann. N. Y. Acad. Sci.* **72**: 649, 1959.

90. Link, W. E., H. M. Hickman and R. A. Morrissette. *J. Am. Oil Chemists' Soc.* **36**: 20, 1959.

91. Stoffel, W., F. Chu and E. H. Ahrens, Jr. *Anal. Chem.* **31**: 307, 1959.

92. Luddy, F. E., R. A. Barford and R. W. Riemenschneider. *J. Am. Oil Chemists' Soc.* **37**: 447, 1960.

92a. Schlenk, H., and J. L. Gellerman. *Anal. Chem.*, in press.

93. Link, W. E., H. M. Hickman and R. A. Morrissette. *J. Am. Oil Chemists' Soc.* **36**: 300, 1959.

94. McInnes, A. G., N. H. Tattrie and M. Kates. *J. Am. Oil Chemists' Soc.* **37**: 7, 1960.

95. Huebner, V. R. *J. Am. Oil Chemists' Soc.* **36**: 262, 1959.

96. Huebner, V. R. *Abstracts*, American Oil Chemists' Society, fall meeting, Los Angeles, September 28, 1959.

97. Blomstrand, R., and J. Gürtler. *Acta Chem. Scand.* **13**: 1466, 1959.

98. James, A. T., G. Peeters and M. Lauryssens. *Biochem. J.* **64**: 726, 1956.

99. Popják, G., A. E. Lowe, D. Moore, L. Brown, and F. A. Smith. *J. Lipid Research* **1**: 29, 1959.

100. Johns, T., M. R. Burnell and D. W. Carle. *Second International Symposium Gas Chromatography*, Michigan State University, East Lansing, 1959.

101. Fulco, A. J., and J. F. Mead. *J. Biol. Chem.* **234**: 1411, 1959.

101a. Schlenk, H., H. K. Mangold, J. L. Gellerman, W. E. Link, R. A. Morrissette, R. T. Holman, and H. Hayes. *J. Am. Oil Chemists' Soc.*, in press.

102. Craig, B. M., and N. L. Murty. *J. Am. Oil Chemists' Soc.* **36**: 549, 1959.

103. Gracián, J., E. Vioque and P. de la Maza. *Grasas y aceites* **10**: 286, 1959; *Nature* **184**: 1941, 1959.

104. Tuna, N., L. Reckers and I. D. Frantz, Jr. *J. Clin. Invest.* **37**: 1153, 1958.

105. Herb, S. F., P. Magidman and R. W. Riemenschneider. *J. Am. Oil Chemists' Soc.* **37**: 127, 1960.