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Paper Chromatography of Lipides[1]

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INDICATORS for the paper chromatography of lipides should detect not only one type of chemical compound, say, fatty acids, but should be universal enough to detect also esters, glycerides, and other lipides. This implies that indicators should respond directly to the constituents of a mixture rather than to their derivatives formed chemically before or after chromatographing. Chemical reactions might be selective and so leave the indicator "blind" to some of the components. In addition, a classification of the substances detected should be afforded to help in their final identification.

These requirements are met to a large extent with the indicators that have been reported previously from this laboratory (1). Because their mode of functioning has already been discussed, only their applicability and the demonstrated effects are listed in Table I.

TABLE I

Indicators for Paper Chromatograms of Lipides

Reagent	Compounds detected	Appearance
1. I_2 vapor	Unsaturated lipides	Yellow or brown spots on light background
2. α-Cyclodextrin, then I_2	All lipides except di- and triglycerides	White spots with saturated, blue to dark brown spots with unsaturated lipides, purple background
3. Lead tetra-acetate	α-Monoglycerides	White spots on brown background
4. Lipase, then 2 or 3	Di- and triglycerides, preferably saturated	As 2 or 3

One can expect these indicators to give only a wide classification since they react with virtually all nonphospholipides. Lead tetra-acetate can be considered as specific for α-monoglycerides only when all other compounds having 1,2-glycol or α-amino-alcohol structure can be safely ruled out. Some work with radioactive lipides is reported here, using β-radiation of C^{14} for their detection. This is a most universal indicator method, but it does not elucidate the chemical nature of the components.

The solvent systems reported earlier for the chromatography of lipides have been extended and are summarized in Table II.

An attempt is made to give a detailed account of the experiences which have accumulated in our laboratory since the initial publication. Under General Procedures preparation of the chromatograms is described first, followed by discussions of superposition and chain-length analysis. Under Special Experiments low temperature chromatography and autoxidation are discussed. Other sections deal with quantitative work (2), analysis of glycerides, and the application of paper chromatography to the analysis of menhaden oil.

TABLE II

Solvents for Developing Paper Chromatograms of Lipides

Acetic acid + water	Tetrahydrofuran + water	Chloroform + methanol + water	Carbon tetrachloride + methanol + water
Alcohols Aldehydes Acids Esters Other monochain lipides	Monoglycerides	Diglycerides, triglycerides, lipide contaminants of synthetic glycerides	

A revised and enlarged list of R_f values of monochain lipides is given. These values were obtained by using the better-defined conditions which proved to be necessary in further developing the methods. Chromatography of radioactive lipides occurred in different phases of the investigations and is discussed in the corresponding sections.

General Procedures

Preparation of the Paper. Whatman No. 1 paper for chromatographic purposes was cut across the fiber into strips of about 11.5 x 46 cm. The strips were dried at 120°C. in an oven for 2 hrs. and stored over $CaCl_2$. A 5% weight/volume solution of silicone (Dow Corning fluid 200, 10 cs. at 25°), in ether was prepared and placed in a chromatographic jar. The papers were dipped into the solution and hung for aeration until the ether was evaporated, after which they were stored without special precautions. Approximately 500 strips could be impregnated with 5 liters of liquid. For quantitative paper chromatography it was often of advantage to wash the coated dry papers before running the chromatograms. The solvent was permitted to ascend the paper to a point above the anticipated length of the chromatogram, *i.e.,* when 16–20 hrs. are required for development, the time for washing should be 24–30 hrs. The washed papers were air-dried and stored in a jar fitted tightly with a cover plate. Chromatograms developed on such papers have a particularly light and even background after staining with I_2. Silicone is not appreciably soluble in aqueous acetic acid. The quality of the paper is maintained so that two-dimensional chromatograms in reversed phase can also be made.

Solvent System for Monochain Lipides. A mixture of acetic acid + water in a ratio of 85:15[3] covers the broadest spectrum of monochain lipides in regard to chain length, unsaturation, and functional groups. Therefore it is advisable to use it first for any unknown mixture of fatty acids, esters, etc. Some highly unsaturated acids migrate too close to the front line

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for good separation. Unsaturated C_{16} acids, for example, can be chromatographed better with acetic acid + water in a ratio of 75:25. Mixtures of formic acid, acetic acid, and water were sometimes found advantageous for the analysis of acids having more than 3 double bonds or for quantitative analysis of unsaturated C_{18} acids. The optimal resolving power of 42% formic acid, 40.5% acetic acid, and 17.5% water[4] is focused to about the same area in the chart of fatty acids as that of 75% acetic acid plus 25% water. With the ternary mixture the R_f values are somewhat more widely spread, and the amounts of sample which can be chromatographed without streaking appear to be larger than with acetic acid containing a higher percentage of water. On the other hand, the applicability to a broad range of fatty acids is lost. Formic acid of 88% concentration, which is the usual concentration commercially available, gives a dark yellow background and too low R_f values with C_{18} acids. The above ternary composition can often be a good compromise.

Temperature. The migration of lipides in such a reversed phase system is greatly influenced by temperature. The chromatographic jars were placed in an incubator, routinely at 30 ± 1° but sometimes at 40°C. In quantitative paper chromatography, where optimal reproducibility is required, the rigid control of temperature becomes of crucial importance.

Regulating the Migration. Though R_f values are discussed more fully later in this paper, general rules are given here for regulating migration of spots to a practical distance from the front area or the starting point: to lower R_f values decrease the temperature or increase the concentration of the more polar component of the solvent, *i.e.,* water; to raise R_f values take the opposite measures. By these procedures the migration of any lipide having one chain of 10–22 carbon atoms can be controlled to have an R_f value between 0.2 and 0.8, which corresponds to the useful region of the chromatogram, especially in quantitative work.

The Sample. The material to be chromatographed is applied as a 1% solution in ether, methanol, acetone, or acetic acid. In chromatographing C_{18} acids or their methyl esters, a sample of about 50 γ is suitable while with saturated C_{20} or C_{22} acids this amount causes streaking. Larger amounts must be used with mixtures, especially when minor components are to be detected. The sensitivity of iodine vapor for chromatograms of pure linolenic acid permits detection of 5 γ of acid. It is possible to detect qualitatively, with satisfactory separation, 17 γ of linolenic acid in the presence of 340 γ of linoleic acid. Chromatograms of mixtures of these acids are represented in Figure 1. For the reversed ratio 17 γ of linoleic acid could be detected in the presence of 340 γ of linolenic acid. It is obvious that chromatograms must be overloaded when searching for contaminants. Other examples of minor component analysis are listed under Quantitative Paper Chromatography (Table IV, No. 8), and under Paper Chromatography of Glycerides.

Curves shown in Figure 1 relate to a rather simple pair of substances in which the minor component is clearly set apart from the major one. When pairs are not set apart, optical density measurements, described below, still can indicate a contaminant by

[3] Solvent ratios are given by volume throughout.
[4] Approximate composition obtained by mixing HCOOH, reagent grade, 88%, with CH_3COOH, C.P., 85%, and H_2O in a ratio 50:50:5.

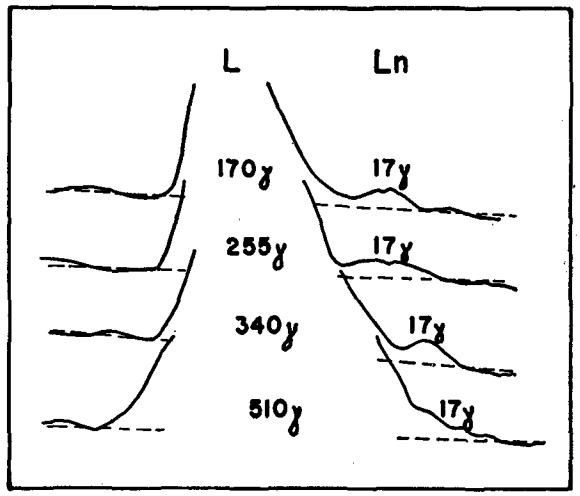


FIG. 1. Chromatography of linoleic acid contaminated with different amounts of linolenic acid (L and Ln). Procedure: 85 CH_3COOH + 15 H_2O, 30°, I_2 indicator. The optical density is plotted over the background (broken lines).

yielding a curve which is not smooth. This was often encountered with impure preparations of C_{20} and C_{22} unsaturated acids. The samples have not been more closely studied for separation and identification of the minor component by paper chromatography, but their impurity was verified by chromatography after hydrogenation (chain-length analysis).

The migration of lipides is influenced by the amount being chromatographed. For example, small amounts of linoleic acid and large amounts of linolenic acid, chromatographed separately, often migrate to the same height. Therefore identification of substances is greatly facilitated by applying equal amounts of unknown and authentic samples to the paper. Such an elaborate procedure saves time in the qualitative analysis of a sample. Self-filling micropipettes (5 λ, transfer type, Microchemical Specialties Company) were very satisfactory in qualitative work with solutions of 1% concentration. In quantitative analyses, when chromatograms are compared by means of optical measurements, the solutions must be of equal concentration, *i.e.,* equal amounts of sample must be applied to the paper in identical volumes of solvent (3). Micropipettes controlled with a screw mechanism (blood pipette-suction apparatus, Adams) provided the necessary exactness.

Developing. Using the ascending technique, chromatograms were developed for 17 hrs. (overnight) or up to 24 hrs. in jars 15 x 46 cm. high. The solvent had then traveled to a height of 20–25 cm. The length of the chromatogram influences the R_f values somewhat. Modelling clay is very useful for sealing glass covers to the jars and for fastening strings on which the papers are hung. After development the strips were thoroughly aerated to remove the solvent, particularly the formic acid, as completely as possible.

Staining. Humidity on the paper promotes staining with I_2 and α-cyclodextrin + I_2 (Methods 1 and 2 in Table I). A jar for humidifying the chromatograms is shown in Figure 2. A similar jar, dry and without lining but containing a small dish with I_2 is used for the iodination at 50°. Polyvinyl chloride and lead are resistant enough to the water and the halogen to permit setting up the jars permanently. The papers are humidified and immediately exposed to I_2

vapors for periods of 5 min. When indicator method 2 is to be used, the dry chromatograms are sprayed without lining but containing a small dish with I_2 is with a solution of 1% α-cyclodextrin in water containing 30% ethanol, air-dried, and then processed as above.

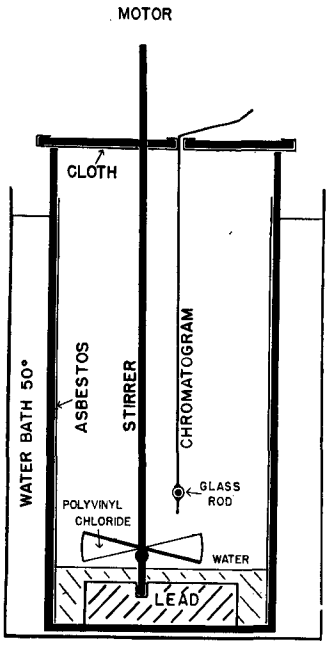


FIG. 2. Humidifying chromatograms. The paper is bent above the front line of the solvent, a glass rod is pinned through the lower end to keep the paper stretched.

Iodine yields yellow or brown spots with unsaturated lipides. Saturated compounds, in large amounts and under highly favorable conditions, are sometimes seen as faint yellow spots that fade completely in a few minutes and never interfere with the interpretation or measurement of unsaturated substances. Weak spots are more easily seen in blue or Hg light. α-Cyclodextrin + I_2 reveals saturated compounds as white spots on a bluish-purple background. With unsaturated compounds this reagent causes spots, either brown or any shade of blue lighter or darker than the background; they appear sometimes only after prolonged aeration, up to 24 hrs. Accordingly chromatograms stained for detecting saturated compounds furnish information about the unsaturated ones too. Aiming only at the latter, Method 1 appears preferable. Acetylenic acids and dicarboxylic acids respond to α-cyclodextrin + I_2 in the same way as the saturated fatty acids. Chaulmoogric and hydnocarpic acids, and selachyl alcohol respond to both methods, batyl and chimyl alcohol only to Method 2. The alcohols are also detectable with lead tetra-acetate.

Not until after our first publication did we find that Inouye and Noda first suggested the use of iodine dissolved in ethanol or the use of Wijs reagent for detecting unsaturated fatty acids in paper chromatograms (4). We found I_2 vapor a far more satisfactory indicator when applied to papers coated with silicone instead of with the hydrocarbons which Inouye and other authors have used in their developing systems (5, 6, 7). Residual hydrocarbon on the paper interfered with the detection of lipides with I_2. This shortcoming possibly explains why Inouye and co-workers developed procedures for separating and detecting unsaturated fatty acid esters in the form of their mercuric acetate complexes (8).

Other Indicator Methods. α-Cyclodextrin, the reagent for saturated lipides, is not readily available, and its preparation (9, 10), although not inherently difficult, will call for special effort in most laboratories concerned with lipides. The principle of distinguishing between host molecules and their complexes (11) on paper could not be expanded to host compounds other than α-cyclodextrin. When applied to different starches or to alcohol-precipitated fractions obtained from them (12), the iodine method showed the same effect as with α-cyclodextrin, but it lacked sufficient sensitivity. The same deficiency was found with thiourea which can react under certain conditions with saturated straight-chain compounds (13). Chromatograms of esters were sprayed with thiourea dissolved in methanol and then dipped into a solution of $AgNO_3$ in aqueous ammonia. The formation of Ag_2S proceeds at a slightly different rate in blank and spotted areas, showing light spots on a drab green background. The spots could be detected only when the paper was heavily overloaded with lipides.

Saturated acids can be spotted with I_2 by introducing unsaturation. Their allyl esters can be obtained in good yields, for example, from the methyl esters and allyl alcohol by alkaline transesterification. Allyl stearate and palmitate respond with only about half the intensity of the same amount of oleic acid. This method has the disadvantage of subjecting a mixture to chemical reactions before analysis.

Täufel and Vogel recently reported a method for detecting and assaying peroxides in lipides by means of a spot test on paper (14). Ferrous sulfate + ammonium thiocyanate is a very sensitive reagent for peroxides and permits a convenient test for such contaminants in a sample. The reagent can serve, of course, for detecting autoxidizable lipides after they have been chromatographed. Autoxidation of unsaturated lipides takes place only to a slight extent during the final processing of the developed chromatograms. Therefore exposure of the chromatograms to UV light is helpful in applying the reagent as an indicator. No advantage is offered over the iodine method for detecting unsaturated compounds since the reagent on the background darkens very rapidly to the same shade as the spots of autoxidized compounds, so masking them.

Identification of Spots, R_f Values. Comparative chromatography of unknown substances with authentic samples is as desirable here as in other fields where paper chromatography has found application (15a). The effects of solvent composition, temperature, and amount of sample on the R_f values have been emphasized already. Other factors are the size of the spot applied to the paper, the amount of silicone on the paper, the distance the solvent has traveled (3a), and the distance between the spot and the surface of the solvent at the beginning of the development (15b). When, in spite of this, R_f values are listed, it is rather for the purpose of pointing out the characteristics of homologous series and their variations with solvent systems than for encouraging identification solely on such a basis. The steady change of R_f values within vinylogous series is shown in Figure

3. On the basis of these regularities one can limit the number of alternatives for the identity of an unknown, providing some of the members of a particular series are available as authentic materials.

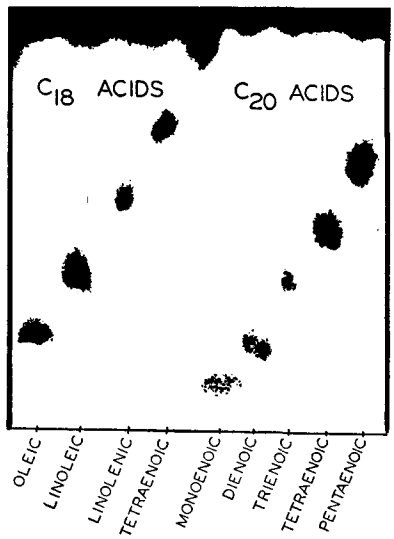


FIG. 3. Chromatograms of unsaturated C_{18} and C_{20} acids. Procedure: one sheet was used for each group; 42 HCOOH + 40.5 CH_3COOH + 17.5 H_2O, room temp., I_2 indicator. Photostats were made so that spots and front area appear black (courtesy of R. T. Holman).

The R_f values given in Table III are averages from several chromatograms of pure, individual substances run on different sheets. They represent the ratios of the distances, starting point—heaviest point of the spot:starting point—solvent front. Sample quantities were between 50 and 100 γ.

As with amino acids (15a), chromatograms of lipides can be calibrated by chromatographing suitable reference substances together with a sample. Migration values, when referred to such standard substances, turn out more consistently.

Superposition of Spots. With the more common acids the increment of R_f values found for one double bond is about equal to that found for shortening the chain length by two methylene groups. This rule (1) has proved valid experimentally for mixtures of palmitic + oleic, myristic + palmitoleic + linoleic, and palmitolinoleic + linolenic acids or their derivatives, which are not satisfactorily separable with any of the acidic solvents under the conditions so far described. Extrapolation from these examples fails since some separation was indicated for a mixture prepared from equal amounts of pure hexadecatrienoic, octadecatetraenoic, eicosapentaenoic, and docosahexaenoic acids when chromatographed in formic-acetic acid and water. The latter fatty acid had the lowest R_f value. It still must be expected that separation would fail completely with different amounts of the components in the mixture. Under the same conditions lauric acid, which does not belong in this series, migrated to the height of the octadecatetraenoic and eicosapentaenoic acids. The experiments show that, with high unsaturation, the influence of two methylene groups on the R_f values is not completely compensated for by that of one double bond and that new superpositions arise.

Iodine is specific for unsaturated lipides and is not interfered with by the presence of saturated ones, but there is no direct indicator which is specific for saturated compounds in the presence of unsaturated ones. A mixture of palmitic + oleic acids cannot be exhaustively interpreted chromatographically, to say nothing of more complex superpositions. In attempts to convert unsaturated compounds on the paper in order to change their chromatographic properties, bromination, epoxidation, and hydroxylation reactions have been carried out under various conditions. The subsequent chromatograms showed however that formation of isomers in these reactions complicates the picture. Furthermore, with compounds derived from higher unsaturated acids, the applicability of α-cyclo-

TABLE III

R_f Values of Monochain Lipides[a,b] at 30°C.

	Acids			Methyl esters		Aldehydes	Alcohols
	85 CH_3COOH 15 H_2O	75 CH_3COOH 25 H_2O	42.0 HCOOH 40.5 CH_3COOH 17.5 H_2O[c]	85 CH_3COOH 15 H_2O	75 CH_3COOH 25 H_2O	85 CH_3COOH 15 H_2O	75 CH_3COOH 25 H_2O
Saturated							
Lauric	0.79			0.60		0.72	0.74
Myristic	0.73			0.44		0.59	0.62
Pentadecanoic	0.64			0.33			
Palmitic	0.59			0.26		0.49	0.46
Heptadecanoic	0.53			0.22			
Stearic	0.40			0.17		0.38	0.30
Arachidic				0.44[d]			
Behenic				0.43[e]			
Unsaturated							
Palmitoleic		0.40	0.36	0.55			
Palmitolinoleic		0.54	0.56	0.65			
Palmitolinolenic		0.63	0.71	0.74			
Hexadecatetraenoic		0.70	0.80				
Oleic	0.54	0.36	0.20	0.28	0.13	0.43	0.45
Elaidic	0.52						
Linoleic	0.66	0.40	0.35	0.38	0.20	0.54	0.58
Linolenic	0.73	0.55	0.52	0.50	0.26	0.64	0.69
				Other esters			
				90 CH_3COOH,10 H_2O			
Allyl palmitate				0.53			
Allyl stearate				0.37			

[a] R_f values of monoglycerides and of other glycerides are found in Table I of reference 1.
[b] The composition of all solvents is given by volume.
[c] 50 HCOOH, reagent grade, 88%, + 50 CH_3COOH, C. P., 85%, + 5 H_2O.
[d] 90 CH_3COOH + 10 H_2O.
[e] 95 CH_3COOH + 5 H_2O.

dextrin + I_2 is limited. The reagent responds to dibromostearic acid but is not reliable any more with polybromides. This implies that superimposed compounds could be analyzed for the presence of saturated and monounsaturated members after bromination but that higher unsaturated ones could not be detected. Experiments with methyl esters of C^{14} radioactive acids (16a) showed that palmitoleate, palmitolinoleate, oleate, and linoleate, brominated and chromatographed in aqueous acetic acid, produce spots in the region of caproate, laurate, and myristate. Radioactive spots were also found in the front line with all brominated samples (Table III), which might be explained by the presence of isomers.

More successful attempts to solve the problem of superposition are discussed in the following section and under Chromatography at Low Temperature.

Chain-Length Analysis. Paper chromatography is often applied to samples containing superimposing components. They are to be expected in crude mixtures of fatty acids and are encountered also with samples obtained from such mixtures by methods of separation other than paper chromatography. Unfortunately countercurrent distribution (17), displacement chromatography (18), and reversed phase column chromatography (19, 20) produce the same superpositions as paper chromatography. Therefore, to get the full value of paper chromatography as a monitoring device for other procedures, micro-scale, chain-length analysis becomes necessary. It is also necessary, of course, in analyzing the fractions of fatty esters obtained by distillation. To this end, samples of such fractions must be hydrogenated and then chromatographed, in addition to chromatographing them unchanged.

A few milligrams of the sample are hydrogenated with an equal amount or more of PtO_2 catalyst in 1 ml. of solvent. Acetic acid is used with fatty acids, methanol with methyl esters, and ethanol with ethyl esters. The standard Parr, low-pressure hydrogenation apparatus holds up to 10 samples in test tubes 15 x 125 mm. Hydrogenation is carried out at 40-50 lb. of pressure at room temperature for several hours. The product is chromatographed to test for uniformity of chain length. Once this is verified, homolog-vinylog superposition is ruled out. Complete hydrogenation should be ascertained in overloaded duplicate chromatograms, using iodine as an indicator.

Special Experiments

Chromatography at Low Temperature. The difficulty caused by superposition may be partly overcome by chromatography at low temperatures. The partition coefficients, and therewith the R_f values of the saturated acids, are much more affected by low temperatures than are those of unsaturated acids. The solvent, formic-acetic acid-water, at 31°C., cannot resolve palmitic and oleic acids, but slight resolution is indicated for the pair myristic and linoleic acids. At —5° the separation of the unsaturated acids has been well maintained, but both the saturated acids have been separated from them. The latter have hardly migrated and coincide under these conditions (Figure 4). It appears that shifting the pattern of superpositions by chromatographing at several temperatures can provide rather complete information on fairly complex samples which are too small for any of the present separation procedures other than paper chromatography. A similar problem of superposition arises with the chromatography of *cis* and *trans* isomers, where the pair oleic and elaidic acid has been studied. Figure 5 shows their superposition at normal temperature and the resolution at 0°.

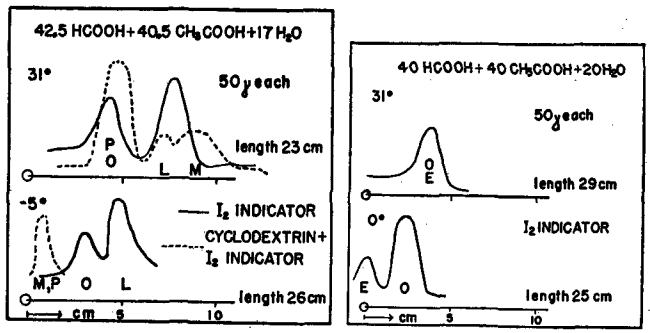


FIG. 4. Chromatography of myristic, palmitic, oleic, and linoleic acids (M, P, O, and L) at different temperatures. Procedure: chromatograms of the mixture and of the individual components were run at the specified temperature and stained with I_2 or α-cyclodextrin + I_2. The density and transmission curves of the chromatograms of the mixture are superimposed.

FIG. 5. Chromatography of oleic and elaidic acids (O and E). Procedure: the chromatograms were stained with I_2 and the density curves are plotted.

The experiments were carried out in standard chromatographic jars, which had been immersed in a triple wall chamber insulated with Vermiculite. Two lead discs, fitted into the jars, could be lifted in and out with screw-in handles. The solvent was placed in a dish on top of these weights, and the jar was surrounded with an ice-salt mixture. After thermoequilibration for 1 to 2 hrs. the chromatograms, with two samples per strip, were hung in the jar and developed for 20 hrs. There is a time-temperature gradient with this improvised device: beginning at —10°C., the temperature rises to about 0° at the end of the period. The essential feature certainly is the low temperature rather than its gradient. Chromatograms developed at from —18° to —10° showed streaking of oleic acid when the same amount was used as in the experiment between —10° and 0°.

Numerous experiments have been carried out in a variety of solvents and at several temperatures between —20° and —78°, using the acids C_{12}, C_{14}, C_{16} (0 to 3 double bonds), and C_{18} (0 to 3 double bonds) in different combinations. The results obtained so far can be summarized as follows. Chromatography at temperatures below —20° offers mainly separation of saturated from unsaturated acids. Extremes (in these mixtures, lauric acids for the saturated and oleic acid for the unsaturated group) exhibit ambivalent properties, that is, have a tendency to streak between the two groups.

Autoxidation. Once the samples are placed on the paper, precautions against oxygen attack have not been taken in the chromatographic procedures. The question arises whether autoxidation occurs and, if so, to what extent it may alter or make the results subject to uncontrollable factors, particularly in quantitative work. Siliconized papers spotted with peroxide-negative linoleic acid (14) were exposed to air for 24 hrs. The spots which had not been chromatographed then showed a weak peroxide reaction.

Similarly, slight autoxidation was found after developing and processing a chromatogram of the same fresh acid. Samples badly autoxidized during careless storage leave a spot of polymerized material at the starting point and show heavy staining in the front line, indicating oxidation products of low molecular weight. The latter are marked by a very dark spot in the solvent front at the end of the track, after exposure to I_2. The front area however is always more darkly stained than the other part of the chromatogram. The use of radioactive fatty acids affords a more conclusive test for autoxidation during the chromatographic procedures. Methyl esters of C_{18} acids, labeled randomly with C^{14} and having 0 to 3 double bonds (16a), were applied to the paper and chromatographed after 1 hr. and after 2, 7, and 21 days. Radioautographs of the chromatograms were taken on x-ray films and measured densitometrically. The curves obtained are given in Figure 6. The

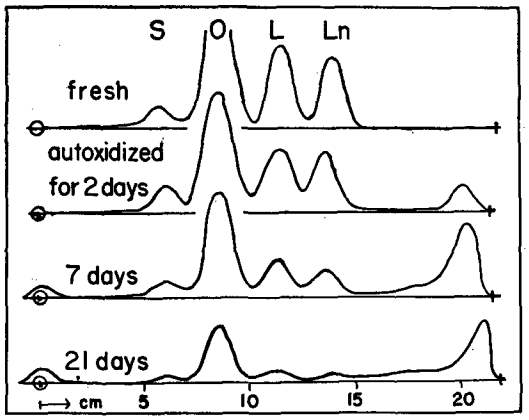


FIG. 6. Chromatography of methyl esters of radioactive C_{18} acids, fresh and autoxidized. The initial composition is 5.7% stearate, 50.8% oleate, 22.9% linoleate, and 20.6% linolenate (S, O, L, and Ln). The sample amounts are not equal so that only relative comparison can be made. Procedure: 85 CH_3COOH + 15 H_2O, room temp. The density of radioautographs is plotted.

chromatograms of fresh samples proved to be "clean" whereas those exposed to air for 2 days showed 2% of the total radioactivity in the front line and none at the starting point. The corresponding values found with the chromatograms exposed for 7 and 21 days were 27.5 and 47.5%, respectively, of the total radioactivity in the front area and 3.7% and 6.6% at the starting point. Streaks of radioactive material which one would expect from autoxidation during the development could not be detected with the 1-hr. samples. Obviously autoxidation is negligible when such common lipides are chromatographed. In addition, the experiments outline a simple technique to follow the progress of autoxidation.

Quantitative Paper Chromatography

Quantitative paper chromatography of fatty acids has been reported by Wagner, Abisch, and Bernhard (21) and by Seher (22), who used reversed partition on hydrocarbon-treated paper (5, 6) and determined the acids on the paper in form of Cu salts (6). Perilä (23) proceeded similarly but worked with silver salts of the fatty acids. The quantitative use of mercuric acetate complexes with unsaturated acid methyl esters (8) has been reported by Kaufmann (24) together with a survey of other suggestions.

Indicator methods 1 and 2 (Table I) have been used for the quantitative determination of acids, esters, and glycerides (2). The spots were measured in a densitometer (Photovolt Corporation, 52-C and 521A) with a stage attached to it for semiautomatic plotting of curves. The density of yellow spots was recorded, using a blue filter (445 mμ), and the transmission of white spots on purple background was measured by using a green filter (525 mμ). The slit size of the densitometer was 1 x 25 mm., and readings were taken at 2-mm. intervals. The transparency of the paper is increased by the impregnation with silicone, which is of advantage, particularly with method 1. The background of papers stained directly with I_2 often is not quite constant over the length of the chromatogram. This difficulty can be minimized, but not completely eliminated, by washing the papers (see General Procedures). The density gradient of the background is nearly linear in the useful region and has little effect on the accuracy of the measurements. With both staining methods the front area is darker than the background and measurements are acceptable only when base-line values are obtained between the highest R_f spot and the front line. The scanning guide for the papers requires strips about 3.4 cm. wide so that a sheet of 11.5-cm. width provides tracks for three chromatograms. Samples meant for comparison were applied in equal volumes, which is a general rule for quantitative paper chromatography (3b, c). Reference can be made only within the same paper strip which is not cut to size for the densitometer until the final processing is finished.

The areas under the densitometer curves were measured with a planimeter. Triplicates of pure samples run on the same sheet showed very good reproducibility. Moreover these areas are directly proportional to the amount of sample used. This is demonstrated in Figure 7, where three different amounts of the same ester have been chromatographed on one sheet. When mixtures are chromatographed however, the linearity is not well maintained. The same phenomenon has been elaborated upon, in particular by Fischer in quantitative paper chromatography of amino acids (3d). Therefore reference model mix-

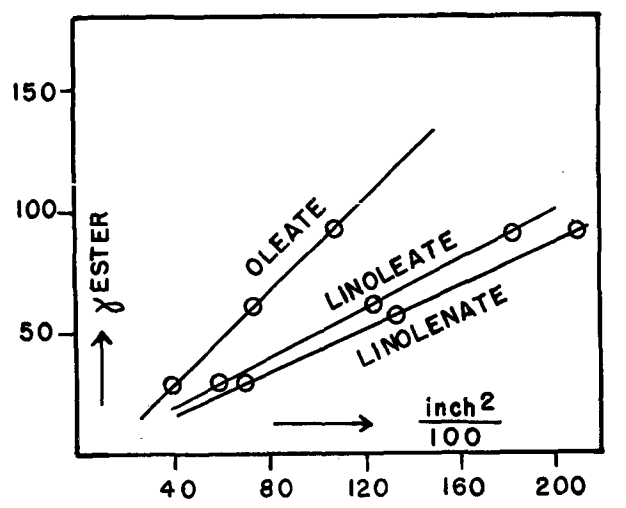


FIG. 7. The relation of areas under the density curves to the amounts of unsaturated esters chromatographed (I_2 indicator).

tures were taken, the composition of which was adjusted so that the difference from the unknown did not exceed 10%, which often is an accepted margin of error in paper chromatographic analyses. Linear interpolation appears permissible within that limit. The average obtained from at least three chromatograms, run together with the model mixture, must be taken. When irregularities in the background cannot be overcome, a more accurate background is obtained from a blank track on the same sheet. For the quantitative determination of saturated and unsaturated components of a mixture, separate chromatograms must be run. With some experience the initial chromatograms can be evaluated visually, and the optical measurements may be restricted to the second approximation, which often is the final one. The procedures were tested with mixtures of compositions unknown to the analyst (J.L.G.). All analyses carried out so far are listed in Table IV, numbered in the order in which they were performed.

TABLE IV

Quantitative Analysis of Lipides[a]

Analysis number	Stearic % Found (Given) Rel. error	Oleic % Found (Given) Rel. error	Linoleic % Found (Given) Rel. error	Linolenic % Found (Given) Rel. error
	Acids			
1	Not determined (10)	38.0 (32.0) *19.0*	36.0 (35.0) *3.0*	20.0 (23.0) *13.0*
2	Not given	30.8 (29.3) *5.1*	23.2 (24.0) *3.4*	46.5 (46.8) *0.6*
5	5.5 (6.95) *20.8*	36.8 (34.6) *6.4*	8.2 (8.3) *1.2*	49.6 (50.0) *0.8*
6	20.4 (16.8) *21.4*	17.1 (17.3) *1.2*	37.4 (42.1) *11.1*	25.0 (23.4) *6.8*
	Methyl esters			
3	9.3 (10.0) *7.0*	27.5 (26.2) *5.2*	33.4 (31.5) *6.0*	29.8 (32.3) *7.7*
7	9.9 (10.3) *4.0*	51.5 (51.6) *0.2*	25.8 (24.8) *4.0*	13.0 (13.3) *2.0*
8	41.3 (42.0) *1.7*	31.6 (30.1) *4.8*	20.8 (21.4) *2.9*	6.17 (6.97) *5.0*
	Triglycerides			
4	Not given	20.3 (19.2) *5.7*	46.5 (47.0) *1.1*	35.4 (33.9) *4.4*

[a] Most papers had been washed with acidic solvent, including those for glyceride analysis. Developing times were between 15 and 20 hrs. Conditions:
No. 1: 88 HCOOH + 12 H_2O, 30°.
No. 2: Same as in No. 1.
No. 3: 44.0 HCOOH + 42.5 CH_3COOH + 13.5 H_2O, 30°.
No. 4: 25 $CHCl_3$ + 75 CH_3OH + 5 H_2O, approx. 20°.
No. 5, 6: Stearic acid: 80 CH_3COOH + 20 H_2O, 40°, one determination only; unsaturated acids: 42 HCOOH + 40.5 CH_3COOH + 17.5 H_2O, 30°; chromatographed 200 γ for major, 400 γ for minor components.
No. 7: Stearate: 85 CH_3COOH + 15 H_2O, 30°; unsaturated esters: 42 HCOOH + 40.5 CH_3COOH + 17.5 H_2O, 30°; chromatographed 200 γ and 300 γ.
No. 8: Stearate: 80 CH_3COOH + 20 H_2O, 40°, and 85 CH_3COOH + 15 H_2O, 30°; unsaturated esters: same conditions as above, and 42 HCOOH + 40.5 CH_3COOH + 17.5 H_2O, 30°; chromatographed 500 γ for determination of linolenate.

The detailed procedure for analysis No. 7 of Table IV is described in the following. A sample of the mixture (approximately 0.22 ml.) was pipetted into a 10-ml. volumetric flask, weighed (0.1958 g.), and made up to volume with methanol. A model mixture, consisting of 100 ± 5 mg. of each of the components, was prepared in 10 ml. of the same solvent. The requirement of equal amounts in equal volumes is not mandatory for the initial estimation. One sample of the unknown (15 λ, or about 300 γ) and two samples of the model mixture (between 2 and 15 λ or 80 and 600 γ) were chromatographed on three tracks of one sheet. Six such sheets were prepared, three of which were developed in formic + acetic acid + water and stained with I_2 to estimate the unsaturated components and three of which were developed in acetic acid + water and stained with α-cyclodextrin + I_2 for stearate. Visual assay of the composition of the unknown showed stearate, 40 γ; oleate, 150 γ; linoleate, 75 γ; and linolenate, 40 γ (total 305 γ), or 12, 50, 25, and 13%, respectively. Aiming for such composition at a concentration close to 2%, a new model mixture was prepared, containing 0.0201 g. stearate, 0.0918 g. oleate, 0.0503 g. linoleate, and 0.0297 g. linolenate. In a series of chromatograms 10 λ of this and of the unknown mixture were developed, stained, and measured optically on the same day. The base lines showed a slight but constant gradient so that all chromatograms could be evaluated. Areas under the curves were measured with a planimeter. The amounts were calculated for each ester according to the equation

$$\gamma\ (\text{unknown}) = \frac{\gamma}{\text{in}^2}\ (\text{known}) \times \text{in}^2\ (\text{unknown})$$

TABLE V

Paper Chromatographic Determination of C_{18} Methyl Esters (Details of analysis No. 7 of Table IV. Stearate determined on 3, unsaturated esters determined on 5 chromatograms.)

Stearate	Oleate	Linoleate	Linolenate
%	%	%	%
9.4	55.8	25.6	13.0
	44.5	23.5	11.5
8.8	58.5	31.8	14.6
	50.1	26.1	13.2
12.3	54.8	25.3	14.0
Av.: 10.2	52.8	26.5	13.3
	Total 102.8%		

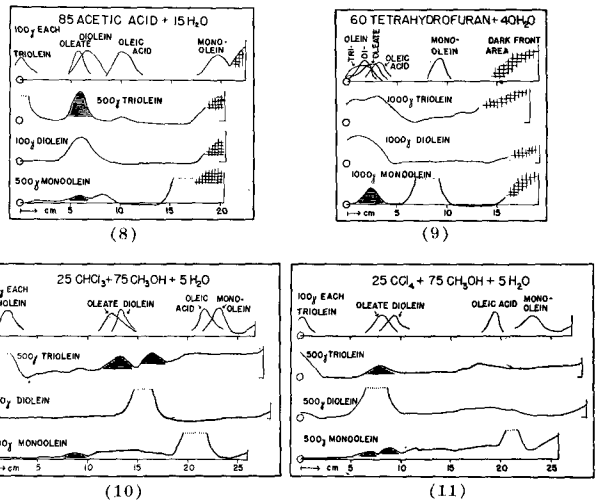
The results of the individual chromatograms are listed in Table V. The average values (total 102.8%) were corrected proportionately to 100%. These final values are found in Table IV, No. 7.

The direct determination of oleic and stearic acids or their esters, which is afforded by paper chromatography, is often desirable. Higher unsaturated acids are commonly determined by alkaline isomerization (25), but there are advantages in procedures based on separation. Assay of glycerides so far has not been reported with any other paper chromatographic method.

Pure authentic samples must be on hand for determining the constituents of a mixture. The areas under the optical curves of chromatograms of equal amounts of oleic, linoleic, and linolenic acids show a ratio close to 1:1.5:2.2, *i.e.*, with unsaturation the sensitivity increases. Use of the same proportions for the unsaturated members of other series does not appear warranted as long as experimental verification is lacking. The quantitative determination of radioactive lipides however is independent of the comparison with authentic samples, provided the radioactivity is distributed randomly. It is possible to obtain this advantage with nonradioactive lipides, for example, by preparing quantitatively methyl-C^{14} esters from "cold" acids with radioactive diazomethane. The mole ratios of the component esters can be calculated from the radiation intensities of the different spots since the distribution of the radioactivity is virtually equimolar. Nonacidic impurities, in this case, must be detected chemically.

Paper Chromatography of Glycerides

An analysis of monoacid triglycerides is listed in Table IV. The separation, though merely academic, encourages future attempts to chromatograph natural fats. Although this goal has not been achieved yet, paper chromatography is already of great value in research on synthetic glycerides (16b). The solvent systems suitable for separation of mono-, di-, and triglycerides from each other are listed in Table II and Figures 8–11. Chromatograms developed in these solvents afford an evaluation of the purity of a preparation. The curves obtained from chromatograms of preparations of mono-, di- and triolein are presented in Figures 8–11. Guiding schemes for the most common contaminants help to interpret the curves. These are drawn for each solvent system in the upper part of the figures. The purity of the acid moiety is taken for granted. According to Figure 8, the sample of triolein is contaminated with methyl oleate and/or diolein, and possibly traces of oleic acid; Figure 9 should indicate monoolein, which is hard to detect in other solvent systems; Figure 10 indicates the presence of methyl oleate and diolein; Figure 11 shows methyl oleate and/or diolein, and possibly traces of oleic acid. The chromatograms of diolein do not reveal any contamination. Methyl oleate and/or diolein are indicated as contaminants in monoolein. The chromatograms were greatly overloaded to detect minor components. For diolein however this was not possible in all solvent systems because of streaking.



FIGS. 8–11. Chromatography of oleic acid glycerides in different solvent systems. Procedure: I_2 indicator, the density is plotted. The top line in each figure was obtained by superposition of curves from individual chromatograms. It indicates the approximate positions of the contaminants to be expected. Spots of contaminants detected visually are shaded horizontally; dark-front areas are shaded horizontally and vertically.

Acetic acid solvent is needed for detecting free acids and will also show other contaminants. Tetrahydrofuran serves mainly for detecting and separating monoglycerides. The deeply stained front area is rather large on papers developed with this solvent. Chloroform is better for chromatographing different triglycerides than is carbon tetrachloride, which in turn gives a wider spread between the contaminating components. The complete series of curves is presented here although some of them do not contribute

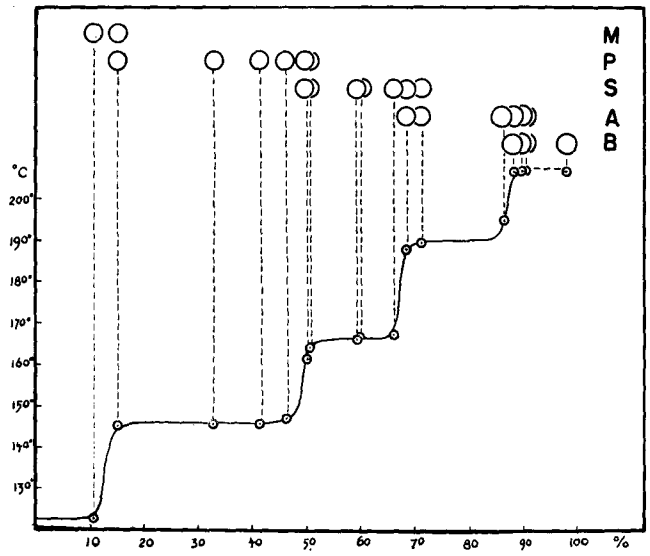


FIG. 12. Distillation of hydrogenated methyl esters of menhaden oil fatty acids at 1.6–1.8-mm. pressure, and paper chromatographic analysis of the fractions. (M = myristate, P = palmitate, S = stearate, A = arachidate, and B = behenate; esters not identified are omitted).

to the conclusions drawn for these particular samples. Each glyceride presents a somewhat different problem, which makes this choice of slovent systems very desirable. In addition, the R_f values can be regulated by the same measures as for acetic acid + water so that the conditions of development can be adjusted to the requirements of glycerides derived from quite different acids.

It was possible now to ascertain the purity of mono- and dipalmitin which had been synthesized and labeled with C^{14} in the glycerol as well as in the fatty acid moiety (26, 27). The small amounts prepared in concentrated radioactive form did not warrant direct chemical determination of their purity. The identity and purity had been indicated so far by their melting points and by chemical analyses of "cold" samples.

Application of Paper Chromatography to Fatty Acid Analysis of Menhaden Oil

Several portions of menhaden oil were hydrogenated and saponified. After removal of the unsaponifiable material the acids were esterified with methanol. Distillation of 490 g. of esters was carried out in a 25-mm. x 48-in. heligrid column (Podbielniak Inc.) during a period of 24 hrs. Pure methyl behenate could be obtained only after changing to a 25-cm. Vigreux column. Each fraction was analyzed several times by paper chromatography. The distillation curve is given in Figure 12, where the circles indicate the esters identified in the preceding fraction. Chromatographically pure fractions were recrystallized once from petroleum ether. Free acids obtained from these fractions did not show any impurity when chromatographed. The melting points of esters and acids agreed with those given in the literature.

The pure fractions from the distillation constituted 81% of the starting material. The composition of the mixed fractions, amounting to 17%, could have been arrived at in several conventional ways, such as determining the ester values, or from the weight-temperature curve, all based on the assumption that not

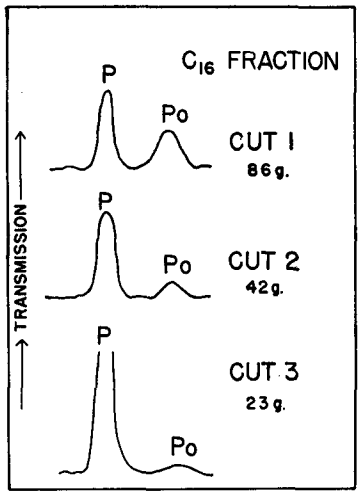


FIG. 13. The enrichment of palmitate towards the end of the C_{16} fraction in distilling menhaden fatty esters. Procedure: different-size samples chromatographed on one sheet, α-cyclodextrin + I_2 indicator; the transmission curve is plotted (P = palmitate, Po = palmitoleate).

more than two components are present in any one fraction. Chromatographic assays were made instead. The impure fractions were chromatographed on the same sheet with 1:1 mixtures of the expected components. It could be decided visually whether to adjust the ratios of the model mixtures to 1:3 or 3:1 in the first approximation. After comparison with these compositions the amounts of the unknown components were determined within limits of 25%, and a further approximation to the limit of 13% could have been achieved visually. After the first step however the error can be estimated as less than 1% for the over-all analysis, which is narrower than could be claimed for the preceding preparative procedures. The chain length composition of the major components in this sample of menhaden oil fatty acids is C_{14} 13.8%, C_{16} 33.6%, C_{18} 20.6%, C_{20} 19.4%, C_{22} 10.6%, and residue ($>C_{22}$?) 2%.

In the course of these paper chromatographic determinations it was found that each of the fractions between methyl myristate, palmitate, stearate, and arachidate contains a third component besides the two expected ones. Their sequence, according to R_f values in decreasing order, is: myristate—substance A—palmitate—substance B—stearate—substance C—arachidate. Chromatography of the free acids gave analogous results. The R_f values of synthetic pentadecanoic (28) and heptadecanoic acids (29) and of their methyl esters correspond well to those of substances A and B. Further work is mandatory to make definite structural assignments. Morice and Shorland reported the isolation of odd-numbered fatty acids from shark liver oil (30), and their occurrence in menhaden oil appears quite possible. Their presence was considered in adjusting the model mixtures for the mixed fractions, but the amounts were too low to have a significant role in the chain-length analysis of the major components.

Methyl esters of nonhydrogenated fatty acids were prepared from the same lot of menhaden oil in the conventional manner; unsaponifiables were removed. Distillation in the heligrid column used previously had to be terminated before a pure C_{20} fraction was obtained, because of extensive polymerization. All fractions were subjected to chain-length analysis. The amounts of C_{14}, C_{16}, and C_{18} esters compared well with those obtained from the hydrogenated esters. Substances A, B, and C were found in the intermediate fractions after they had been hydrogenated.

Two fractions had been collected in the course of distilling pure C_{18} esters. According to chromatographic analyses, they consisted of 3% and 25% stearate, 60% and 50% oleate, 16% and 13% linoleate, 8% and 6% linolenate, and about 12% and 8% octadecatetraenoate. The amount of stearate was greatly increased in the second fraction. Similarly palmitate is greatly enriched at the end of the distillation of C_{16} esters, where three fractions proved to be pure in regard to chain length. As the boiling points of saturated and unsaturated C_{18} methyl esters reportedly are 171° and 166–168° at 2-mm. pressure (31), segregation is to be expected and has been borne out experimentally, in particular by Norris and Terry (32). Such enrichment is shown in Fig. 13, where the ratios of palmitate:palmitoleate are strikingly different in the three cuts.

As in the group of C_{18} acids, unsaturated C_{16} acids having up to four double bonds are represented in menhaden oil. Hexadecatetraenoic acid amounts to about 3% of the whole fraction. The presence of this component has been verified by countercurrent separation. Myristic acid and an insignificant amount of myristoleic acid are the only components found in the C_{14} group.

Paper chromatographic, chain-length analysis is not restricted to mixtures containing two or three compounds as they occurred in the above example. It was also applied, without distillation, to rape seed oil fatty acids which contain all even-numbered members between C_{16} and C_{22}. Another example for chain-length analysis of a multicomponent mixture is presented in Figure 14. Palmitoleic acid (Bios Laboratories Inc.) was hydrogenated and chromatographed. The lot was calculated to contain approximately 20% C_{14}, 50% C_{16}, 16% C_{18}, 5% C_{20}, and 2% C_{22} acids, and 7% of higher fatty acids or polymerized material.

Paper chromatography of lipides is being used at the Hormel Institute: in checking the completeness of routine reactions (esterification, interesterification, saponification, etc.); in evaluating synthesizing procedures (aldehydes [33], glycerides [16b]); in the analysis of the highly unsaturated components of fish oils (16c); for monitoring the results of distillations, of countercurrent distributions (16a), and of displacement chromatography (fatty acids and esters

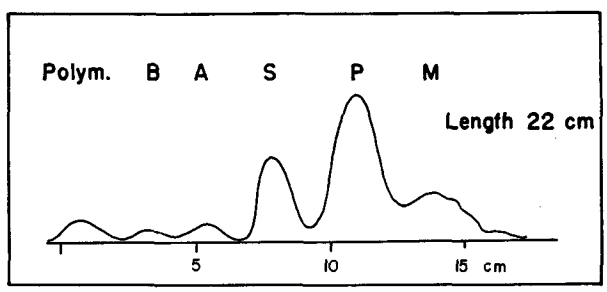


FIG. 14. Chain length analysis of commercial palmitoleic acid. Procedure: 200 γ of a hydrogenated sample, 85 CH_3COOH + 15 H_2O, 30°, α-cyclodextrin + I_2 indicator; the transmission curve is plotted (M = myristic, P = palmitic acid, etc.).

[16d]); and is being adapted in work under the Hormel Foundation fatty acid project. The experiences of our colleagues have contributed to the progress reported here.

Acknowledgments

It is our pleasant duty to thank D. M. Sand, who was in charge of the preparations of α-cyclodextrin. W. O. Lundberg, R. T. Holman, J. R. Chipault, and O. S. Privett provided numerous samples of lipides. Generous gifts of glycerides were obtained from Miss Audrey T. Gros, Southern Regional Research Laboratories, and F. H. Mattson, Procter and Gamble Company. The substances were of great value to us, and many of them have been listed here.

Summary

Procedures for qualitative and quantitative paper chromatography of lipides have been described in detail, together with some practical applications.

With the monochain lipides it was found that one developing system serves universally. The long aliphatic chains essentially determine the chromatographic properties so that suitable conditions for chromatographing any lipide or lipide-like entity are predictable. For the same reason total analysis of natural mixtures cannot be done on one paper chromatogram due to superpositions.

For complete analysis of natural mixtures of fatty acids it is advisable at the present time to combine paper chromatography with some other separation method. Distillation supplements best but requires gram quantities of material while paper chromatography is applicable on gamma or milligram scale. Chain-length analysis by paper chromatography and low-temperature chromatography have been introduced and are promising methods to approach a completely micro method for analyzing complex mixtures.

REFERENCES

1. Mangold, H. K., Lamp, B. G., and Schlenk, H., J. Am. Chem. Soc., *77*, 6070 (1955).
2. Gellerman, J. L., and Schlenk, H., Experientia, *12*, 342 (1956).
3a. Block, R. J., Durrum, E. L., and Zweig, G., "Paper Chromatography and Paper Electrophoresis," p. 11, 47, and 107, New York, Academic Press, 1955.
3b. *Ibid.*, p. 51.
3c. Fischer, F. G., and Dörfel, H., Biochem. Zeitschr., *324*, 544 (1954).
3d. *Ibid.*, p. 550.
4. Inouye, Y., and Noda, M., J. Agr. Chem. Soc., Japan, *27*, 50 (1953).
5. Inouye, Y., and Noda, M., J. Agr. Chem. Soc., Japan, *26*, 634 (1952).
6. Kaufmann, H. P., and Nitsch, W. H., Fette Seifen Anstrichmittel, *56*, 154 (1954).
7. Ashley, B. D., and Westphal, U., Arch. Biochem. and Biophys., *56*, 1 (1956).
8. Inouye, Y., Noda, M., and Hirayama, O., J. Am. Oil Chemists' Soc., *32*, 132 (1955).
9. Freudenberg, K., Plankenhorn, E., and Knauber, H., Lieb. Ann., *558*, 1 (1947).
10. French, D., Levine, M. L., Pazur, J. H., and Norberg, E., J. Am. Chem. Soc., *71*, 353 (1949).
11. Reviews on inclusion compounds: Schlenk, W., Fortschr. chem. Forsch., *2*, 92 (1951); Cramer, F., "Einschlussverbindungen," Berlin, Springer, 1954.
12. Schoch, T. J., J. Am. Chem. Soc., *64*, 2954 (1942).
13. McLaughlin, R. L., and McClenahan, W. S., J. Am. Chem. Soc., *74*, 5804 (1952).
14. Täufel, K., and Vogel, R., Fette Seifen Anstrichmittel, *57*, 393 (1955).
15a. Cassidy, H. G., "Technique of Organic Chemistry," vol. 5, p. 309, New York, Interscience Publishers Inc. (1951).
15b. *Ibid.*, p. 312.
16. Annual Report of the Hormel Institute, 1955–56: a) Schlenk, H., and Mangold, H. K., p. 49; b) Privett, O. S., p. 13; c) Privett, O. S., p. 59; and d) Holman, R. T., p. 32.
17. Ahrens, E. H., and Craig, L. C., J. Biol. Chem., *195*, 299 (1952).
18. Holman, R. T., J. Am. Chem. Soc., *73*, 5289 (1951).
19. Howard, G. A., and Martin, A. J. P., Biochem. J., *46*, 532 (1950).
20. Crombie, W. M. L., Comber, R., and Boatman, S. G., Biochem. J., *59*, 309 (1955).
21. Wagner, H., Abisch, L., and Bernhard, K., Helv. Chim. Acta, *38*, 1536 (1955).
22. Seher, A., Fette, Seifen, Anstrichmittel, *58*, 498 (1956).
23. Perilä, O., Acta Chem. Scand., *10*, 143 (1956).
24. Kaufmann, H. P., Fette, Seifen, Anstrichmittel, *58*, 492 (1956).
25. Herb, S. F., J. Am. Oil Chemists' Soc., *32*, 153 (1955); Holman, R. T., in "Methods of Biochemical Analysis," vol. 4. New York, Interscience Publishers Inc., p. 99.
26. Schlenk, H., Lamp, B. G., and DeHaas, B. W., J. Am. Chem. Soc., *74*, 2550 (1952).
27. Reiser, R., and Williams, M. C., J. Biol. Chem., *202*, 815 (1953).
28. Links, J., and deGroot, M. S., Rec. trav. chim., *72*, 57 (1953)
29. Dauben, W. G., J. Am. Chem. Soc., *70*, 1376 (1948).
30. Morice, I., and Shorland, F. B., Biochem. J., *61*, 453 (1955).
31. Murray, K. E., in "Progress in the Chemistry of Fats and Other Lipids," vol. 3, p. 265, London, Pergamon Press, 1955.
32. Norris, F. A., and Terry, D. E., Oil and Soap, *22*, 41 (1945).
33. Mangold, H. K., and Schlenk, H., manuscript in preparation.

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The Viscosities of Nonaqueous Solutions of Magnesium Caprate and Laurate

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VISCOSITIES of nonaqueous solutions of sodium and potassium soaps were first studied by Mata Prasad (1). Ostwald and Riedel (2), Cawley, Carlie, King, and Kingman (3), and Wood, Nissan, and Garner (4) determined the viscosities of solutions of aluminum soaps in nonaqueous solvents. Alexander and Grey (5) made a critical study of the viscosity of aluminum soaps in benzene and studied the effect of the peptizer, cresol. They also tested the validity of an empirical equation given by Schulze and Blaschke (6) and later derived theoretically by Huggins (7).

In the present study the viscosities of magnesium caprate and magnesium laurate in aromatic hydrocarbons and aliphatic alcohols were determined at different temperatures and concentrations in an endeavor to determine whether the temperature affects the micelle size.

Materials Used. Capric and lauric acids (B.D.H.) were distilled under reduced pressure. Magnesium chloride (Merck Puriss) was used for the preparation of soaps.

Hydrocarbons were kept over calcium chloride for 15 days and then distilled and stored over sodium wire. They were again distilled at the time of preparation of solutions. The solvents had the following boiling points at atmospheric pressure:

	B.P.
Benzene (B.D.H.) (Analar)	79.5°C.
o-Xylene (Merck Puriss)	143–144°C.
p-Xylene (Kodak)	138.5°C.
Toluene (Thomas Tyrer)	110.5°C.
m-Xylene (Merck Puriss)	138°C.

Alcohols were kept over potassium hydroxide for a week, then distilled and refluxed over 1% calcium for