Paper Chromatography of Unsaturated Fatty Acid Esters as Their Mercuric Acetate Addition Compounds

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As a microanalytical method for fatty acids and their derivatives, paper chromatography has recently become available to the lipid field. Various applications of paper chromatography to these fatty substances were shown by several workers (5, 7, 8, 9, 10, 11). For the separation of longer-chain fatty acids the reversed-phase paper chromatography was proved to be the most satisfactory method of general application. Using this reversed-phase technique, Boldingh (2) first separated fatty acid esters on rubber-treated filter paper. Inouye and Noda (6), Baker (1), Kaufmann and Nitsch (12), and Wegmann *et al.* (20) performed the separation of higher fatty acids on paper impregnated with hydrocarbons. Cellulose acetate paper was also used to separate the hydroxamates of C_5-C_{22} fatty acids by Micheel and Schweppe (14).

In the present investigation, paper chromatography of the methyl esters of unsaturated acids as their mercuric acetate addition compounds, which give a very sensitive color reaction with diphenylcarbazone (phenylazoformic acid phenylhydrazide), has been carried out in order to increase the sensibility and separability of this reversed-phase method. As for the addition of mercuric salts to ethylenic bonds, a large number of studies were made especially on the olefins (3). In the fat field the action of mercuric acetate on olein was first reported by Leys (13). Various addition compounds of unsaturated acid esters with mercuric salts were prepared by Ralston *et al.* (18), who stated that the addition of mercuric acetate in methanol solution yielded acetoxymercuri-methoxy derivatives as follows:

$$-CH{=}CH- \xrightarrow[CH_3OH]{Hg(OCOCH_3)_2} -CH(OCH_3)-CH(HgOCOCH_3)-$$

It was found that the acetylenic acids, except those containing a terminal acetylene group, reacted with mercuric acetate in acetic acid to form diacetoxymercuri-ketonic acids, which on subsequent decomposition with mineral acids gave the corresponding keto acids (15, 16, 17, 19). The following scheme was postulated for this addition reaction:

$$-C{\equiv}C- \xrightarrow[CH_3COOH]{Hg(OCOCH_3)_2} -C(OHgOCOCH_3){=}C(HgOCOCH_3)-$$

This paper also deals with the application of paper chromatography by the mercuration method to the analysis of the component acids of natural fats.

Experimental

Fatty Acid Esters. Oleic (m.p. 13°, I.V. 89.5), erucic (m.p. 34°, I.V. 74.9), β-eleostearic (m.p. 72°, I.V. 274.3), and ricinoleic (m.p. 5.5°, I.V. 84.8) acids were prepared from natural sources in the usual manner. Elaidic (m.p. 43°, I.V. 89.0) and stearolic (m.p. 48°, I.V. 89.4) acids were derived from oleic acid, and brassidic (m.p. 60°, I.V. 74.6) and behenolic (m.p. 57°, I. V. 75.0) acids from erucic acid by usual methods. 10-Undecenoic acid (m.p. 24°, I.V. 137.1) was prepared by the pyrolysis of ricinoleic acid. The 9-monoethenoid acids, decenoic to hexadecenoic, were isolated from cow milk fat by fractional distillation and crystallization while the 2-monoethenoid acids were synthesized by the dehydrobromination of the corresponding 2-bromo acid esters with diethylaniline (4). These fatty acids were all esterified with methanol and subjected to mercuration. Methyl linoleate (I.V. 172.0) and methyl linolenate (I.V. 259.9) were prepared by the debromination method.

TABLE I

Analysis and R_F Values of the Mercuric Acetate Addition Compounds of Methyl Esters of Unsaturated Fatty Acids

Mercuric compound of	Hg, %		R_F Value		
	Calcd.	Found	MAT[a]	DAT[a]	MAP[a]
9-Decenoic[b]	.....	.....	0.67	0.88	0.85
10-Undecenoic	41.0	40.6	0.59	0.71	0.84
9-Dodecenoic[b]	.....	.....	0.56	0.65	0.84
9-Tetradecenoic[b]	.....	.....	0.42	0.46	0.80
9-Hexadecenoic[b]	.....	.....	0.27	0.29	0.74
Oleic	34.2	34.2	0.17	0.20	0.62
Erucic	31.2	30.9	0.07	0.10	0.40
2-Dodecenoic	39.9	39.4	0.57	0.66	0.85
2-Tetradecenoic	37.8	37.2	0.43	0.47	0.81
2-Hexadecenoic	35.9	35.3	0.29	0.30	0.74
2-Octadecenoic	34.2	34.1	0.17	0.21	0.62
Elaidic	34.2	33.3	0.19	0.21	0.65
Brassidic	31.2	30.4	0.08	0.10	0.44
Linoleic	45.8	45.5	0.57	0.62	0.82
Linolenic	51.7	51.2	0.78	0.87	0.84
β-Eleostearic	.....	.....[c]	0.77	0.86	0.81
Stearolic	48.4[d]	47.8	0.75	0.84	0.82
Behenolic	45.3[d]	44.5	0.50	0.64	0.77
Ricinoleic	33.3	32.8	0.64	0.53	0.81

[a] Solvent systems: MAT, methanol-acetic acid-tetralin; DAT, diethylene glycol-acetic acid-tetralin; MAP, methanol-acetic acid-petroleum hydrocarbon.
[b] Contaminated with small amounts of saturated acids which have no effect on the R_F values.
[c] The percentage of mercury was considerably lower than theoretical.
[d] Calculated as diacetoxymercuri-ketonic acid methyl ester according to the formula given by Myddleton *et al.* (16, 17).

Mercuration. A mixture of the methyl ester of unsaturated fatty acid and 20% excess of mercuric acetate in absolute methanol(in the ratio of 1-2 ml. of methanol per 1 g. of mercuric acetate) was heated at 80°C. for about 30 min. until the mercuric acetate almost dissolved. To the reaction mixture a large quantity of distilled water was added, and the lower oily layer was washed several times with distilled water to remove the excess of mercuric acetate and the very small amount of the unreacted ester. The oily layer was then extracted with ether, and ether extract washed with distilled water and dried with anhydrous sodium sulfate. After evaporating the ether under reduced pressure, the residue was dried in a vacuum desiccator placed in a dark chamber. The addition compound was obtained as a colorless viscous oil; the yield was nearly theoretical. The mercury contents of the addition compounds are shown in Table I.

The mercuric addition derivatives from ethenoid acid esters liberated the original esters by treating with hydrochloric acid while those from the ethynoic acid gave the corresponding keto acid esters by the same treatment.

TABLE II

Separation and Identification of the Component Unsaturated Acids of Natural Fats by Paper Chromatography [a]

Fat [b]	Methyl esters			R_F values of mercuric derivatives of component acids identified							
	B.p. range of distillates		No. of fractions [c]	Decenoic	Dodecenoic	Tetradecenoic	Hexadecenoic	Oleic	Linoleic	Linolenic	C_{20}-C_{22} unsatd.
	°C.	mm.									
Olive oil	136-180	3	4	.....	.....	0.40	0.28	0.17 [d]	0.57	.....	.....
Soybean oil	129-184	2	5	.....	.....	.....	0.28	0.18 [d]	0.56 [d]	0.76	0.08
Linseed oil	131-169	2	4	.....	.....	.....	0.27	0.17	0.57 [d]	0.78 [d]	.....
Cow milk fat	31- 50	24	8	0.67	0.56	0.41	0.27	0.17 [d]	0.58 [e]	0.76 [e]	0.07
	43-173	2									

[a] Descending chromatography at 20°C. Solvent system: Methanol-acetic acid-tetralin.
[b] Olive oil: A.V., 3.7; Sap. V., 195.2; I.V., 81.3. Soybean oil: A.V., 1.8; Sap. V., 194.6; I.V., 134.0. Linseed oil: A.V., 1.1; Sap. V., 193.9; I.V., 178.6. Cow milk fat: A.V., 0.4; Sap. V., 225.1; I.V., 39.3.
[c] The residue is included among the number and also analyzed by paper chromatography.
[d] Major component acid detected in large amounts on the chromatograms.
[e] Identified as octadecadienoic and octadecatrienoic acids (traces in the latter), respectively, because their structures have not been definitely proved.

For only paper-chromatographic purposes, a more simplified procedure of mercuration was available. In this case the reaction mixture was diluted with ether or benzene, and then a large quantity of water was added with shaking. The upper organic layer was directly used as the sample solution for paper chromatography.

Paper Chromatography. Tetralin and petroleum hydrocarbon (b.p. 140-170°C.) were used as the stationary solvents, and the developing solvent used against the former was 90% (v/v) aqueous methanol-acetic acid-tetralin (30:1:3 by volume) or diethylene glycol-acetic acid-tetralin (60:20:11), and that against the latter was methanol-acetic acid--petroleum hydrocarbon (b.p. 140-170°C.) (30:1:7). Before developing, 2.5 × 60 or 4 × 60 cm. strips of Toyo No. 2 filter paper were dipped in the tetralin or petroleum hydrocarbon, depending upon the developing solvent to be used, and the excess liquid was removed by slight pressing between dry filter paper. From 0.5 to 3 μl. of the sample solution in benzene or ether, containing 10-300 μg. of each mercuric addition compound, was then immediately placed on the starting line of the impregnated paper, which was developed at once by the descending technique in the glass cylinder similar to that described by Wender and Gage (21). The temperature was maintained at 20°C. during the course of the development. A 40-cm. flow from a starting line took 8-10 hrs. with the solvent system of methanol-acetic acid-tetralin (MAT) or methanol-acetic acid-petroleum hydrocarbon (MAP), and 70 hrs. with diethylene glycol-acetic acid-tetralin (DAT). When development was complete, the paper was removed from the cylinder, dried overnight at room temperature (or at 80°C. for 30 min. without exposure to light), and sprayed with a 0.2% solution of diphenylcarbazone in alcohol. In the case of the solvent system DAT, the paper should be washed with water before drying. The mercuric addition compounds were revealed by spraying as purple or bluish-purple spots, which became more distinct by re-spraying with a 0.05 *N* solution of nitric acid in alcohol. The R_F values of the addition compounds measured with their pure samples are given in Table I. Several paper chromatograms illustrating the separability of the compounds are shown in Figure 1 as examples.

Analysis of Natural Mixed Fatty Acids. In order to examine the practical utility of this method, it was applied to the separation and identification of the component unsaturated acids in some natural fats, such as olive oil, soybean oil, linseed oil, and cow milk fat. The mixed fatty acids obtained after hydrolysis of 10 g. of each fat were converted into methyl esters, which were fractionally distilled under a reduced pressure through an electrically heated fractionating column. Each of several fractions thus obtained was treated with mercuric acetate and separately subjected to paper chromatography, using the systems of MAT, DAT, and MAP. A number of unsaturated acids were identified on the chromatograms by comparing their spots with those of standard compounds and referring to the physical and chemical constants of the original ester fractions. Among these acids, tetradecenoic acid in olive oil and hexadecenoic acid in linseed oil, the presence of which was not hitherto reported in the literature, were first identified by this analysis. The results are summarized in Table II.

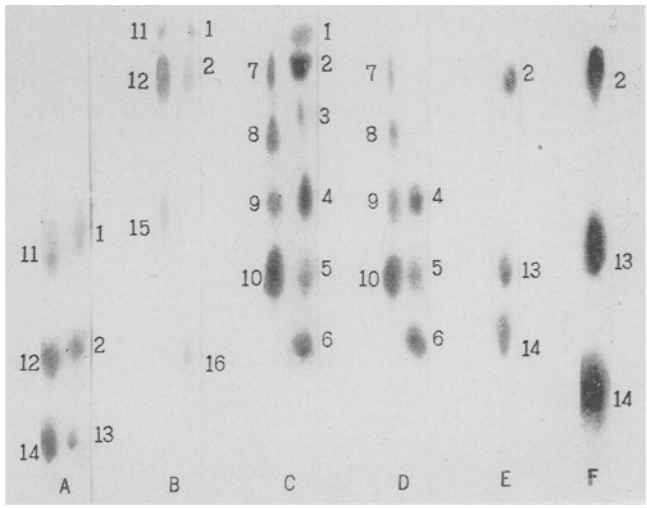

FIG. 1. Paper chromatograms of the mercuric addition compounds of methyl esters of unsaturated acids: 1, erucic; 2, oleic; 3, 9-hexadecenoic; 4, 9-tetradecenoic; 5, 9-dodecenoic; 6, 9-decenoic; 7, 2-octadecenoic; 8, 2-hexadecenoic; 9, 2-tetradecenoic; 10, 2-dodecenoic; 11, brassidic; 12, elaidic; 13, linoleic; 14, linolenic; 15, behenolic; 16, stearolic. Solvent systems: A, MAP; B-E, MAT; F, DAT. The spots obtained from a lower boiling point fraction of the natural mixed component acids of cow milk fat in comparison with those from 2-monoethenoid acids are shown in chromatogram D.

Absorption Spectra. The absorption spectra of the diphenylcarbazone complexes of the mercuric acetate addition compounds were examined as a preliminary step towards the spectrophotometric determination adapted to the quantitative use of this paper chromatography. Color was developed in the benzene solu-

tion by adding diphenylcarbazone to each pure mercuric addition compound in the ratio of 2 moles of the former to 1 g. atom of mercury in the latter, and the mixture was diluted with benzene to a $2 \times 10^{-5} M$ concentration of the mercuric addition compound. After 10 min. the spectrophotometric measurements were carried out on the solution, using the Beckman DU spectrophotometer with cells of 1-cm. path length. The spectra of the diphenylcarbazone complexes obtained from the mercuric acetate addition compounds of methyl oleate, linoleate, linolenate, elaidate, and ricinoleate in benzene showed absorption maxima at 560, 535, 582, 560, and 560 mμ, respectively, as presented in Figure 2. The influence of the excess of diphenylcarbazone upon the absorbances of its mercuric complexes was practically negligible at the wavelengths of absorption maxima of the complexes for diphenylcarbazone in benzene displayed much lower absorption maximum at 465 mμ. It was found that the absorbance values of the complexes from methyl linoleate and linolenate at the wavelengths of their absorption maxima were approximately twice and thrice, respectively, those from the methyl esters of monoethenoid acids except oleic acid in the same concentration ($2 \times 10^{-5} M$).

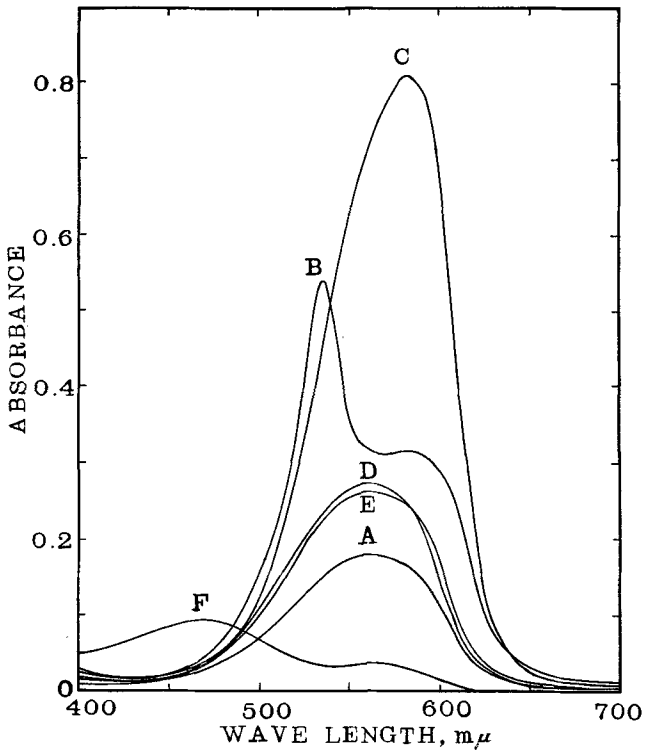

FIG. 2. Absorption spectra of diphenylcarbazone (10^{-3} M) and its complex salts derived from the mercuric acetate addition compounds of methyl esters (2×10^{-5} M), in benzene: A, oleate; B, linoleate; C, linolenate; D, elaidate; E, ricinoleate; F, diphenylcarbazone.

Discussion

The fact that the keto acid esters were formed by decomposing the mercuric addition compounds of ethynoic acid esters with hydrochloric acid seems to indicate that the addition in methanol solution also yields the same diacetoxymercuri-ketonic acid esters as those obtained in acetic acid solution (16, 17).

R_F values vary considerably with temperature, and it is therefore necessary to maintain a constant temperature. Among the solvent systems employed, the system MAT is most suitable for the general separation of the methyl esters of unsaturated acids, particularly of monoethenoid acids, while the system MAP is effective for the separation of higher monoethenoid acid esters than oleate, and the system DAT for that of di- and triethenoid acid esters. The increase of acetic acid content in the systems gives increasing clearness of the spots, contrarily decreasing the separability owing to shifting towards higher R_F values. About the effect of the structures of original unsaturated esters on the R_F values, certain relationships may be found in conclusion. Generally, the longer the aliphatic chain of an ester, the lower was the R_F value. With esters of equal chain length the following relative order of the R_F values is shown, for example with the system MAT: *cis*-monoethenoid < *trans*-monoethenoid < diethenoid < hydroxymonoethenoid < monoethynoic < triethenoid (conj.) < triethenoid acid esters. Similar relations are also found with other systems. Differences in R_F values between 9- and 2-monoethenoid, conjugated and nonconjugated, or *cis*- and *trans*-isomers are slight.

The qualitative application of this technique has proved useful in analyzing the component unsaturated acids of natural fats, particularly superior in detecting minor component acids. The esters of coexisting saturated acids give no color reaction and do not interfere in the R_F values of the mercuric derivatives from unsaturated esters.

From the spectrophotometric experiments it is apparent that no detaching of mercury from the addition compounds occurs when diphenylcarbazone complexes are formed because they exhibit characteristic absorption spectra, respectively. These results also seem to suggest the possibility of the spectrophotometric determination in conjunction with this paper-chromatographic separation. However care should be taken to control change of color intensity after color formation, especially to prevent noticeable fading caused by exposing to light for many hours.

Summary

A method is described by which unsaturated fatty acid esters can be separated and identified by reversed-phase paper chromatography. The procedure is based upon the formation of the mercuric acetate addition compounds of the esters and the detection of the compounds on the chromatograms, using the sensitive color reaction with diphenylcarbazone. The application of this technique to the analysis of the component unsaturated acids of natural fats has been examined, and tetradecenoic acid in olive oil and hexadecenoic acid in linseed oil both formerly unidentified have been detected as the minor component acids by means of the method. The preliminary investigation on the absorption spectra of diphenylcarbazone complexes derived from the addition compounds has been made to bring the method into quantitative use.

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Reactions of Fatty Materials with Oxygen. XVII.[1] Some Observations on the Secondary Products of Autoxidation of Methyl Oleate[2]

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THE PRIMARY PRODUCTS of autoxidation of methyl oleate, as well as other fatty materials, are the subject of intensive study both in this laboratory and elsewhere (2,8,9,10,14,15,17,19). Even in the earliest stages of autoxidation however it is evident (14) that secondary products are being formed concurrently although to only a limited extent. Secondary products of autoxidation have received considerable attention but largely from the standpoint of their separation (4,5,6,11,16,18). At best, this is an intricate, tedious, and often inefficient process in view of the variety of products formed.

The most obvious method for ascertaining the nature and quantity of secondary products and at the same time for eliminating product isolation is determination of the composition of autoxidation mixtures analytically. Although this sounds as though it would be a simple procedure, it has only been within the past few years that reliable chemical, spectroscopic and other physical methods have become available. In an earlier investigation (9) we applied to methyl oleate autoxidized at 35°, 70°, and 100° analytical methods whose reliability had been checked with known mixtures the composition of which simulated that of autoxidized methyl oleate (13). In this initial analytical study (9) much useful and new information was collected, but it was concluded that a more productive approach would involve fractionation of the autoxidation products followed by an analytical investigation of the fractions.

This paper describes some of the results obtained in elaborating the composition of autoxidized methyl oleate by examining fractions obtained by urea complex separations. The results of this study have permitted us a) to reevaluate and reinterpret the analytical results on unfractionated methyl oleate reported earlier (9), b) to obtain a more reliable concept of the composition of autoxidized methyl oleate, c) to understand better why methyl oleate cannot be directly autoxidized to a peroxide content in excess of about 35-40%, and d) to develop a procedure for converting autoxidized methyl oleate (or oleic acid) to a relatively simple system having potential commercial value. Details of this last point are the subject of the following paper in this series (3).

[1] Paper XVI is reference 14.
[2] Presented at the Fall Meeting of the American Oil Chemists' Society, Minneapolis, Minn., Oct. 11-13, 1954.
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Experimental

Starting Material. The preparation of pure methyl oleate has been described (12). It contained 97-99% methyl oleate, less than 0.2% polyunsaturates, and was free of *trans* isomers.

Oxidation Procedure. Oxidations at or above 70° were conducted in the dark in Pyrex flasks. A vigorous, finely dispersed stream of pure oxygen was passed through the methyl oleate, and samples were withdrawn at intervals for analysis or fractionation.

Analytical Methods. These have already been described (13).

Urea Complex Separations. The procedure of an earlier paper was employed (2).

Typical Autoxidation and Fractionation Procedure. Methyl oleate was autoxidized in the dark at 80° until the peak in peroxide content (38%) had been passed and the material contained about 33% peroxide (peroxide oxygen content 1.60%). This required about 96 hours. One hundred and fifty-five grams were added to a hot solution of 728 g. of urea in 2,080 ml. of methanol. The solution was cooled to room temperature and filtered. From the filtrate, 90 g. of pale-yellow oil were recovered; from the precipitate, 62 g. (Table I).

This autoxidation-fractionation procedure was applied to many of the samples described in our previous paper (9). Those particularly studied contained from 5% peroxide to peak peroxide values (35-40%) and from the peak down to 10%. Analytical data similar to that in Table I were obtained but are not given here because of their large number and complexity. The data in Table I are most noteworthy, and the other data are briefly discussed in "Results and Discussion."

Results and Discussion

The motivation for a more detailed study of the secondary products of autoxidation of methyl oleate stemmed from a desire on our part to autoxidize methyl oleate directly to a peroxide content in excess of 35-40%, the maximum usually obtained (9). Various hypotheses to account for this levelling off had