COMPLETE STRUCTURAL ANALYSIS OF FATTY ACID MIXTURES BY THIN-LAYER CHROMATOGRAPHY

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INTRODUCTION

Gas–liquid, thin-layer and paper chromatography permit the separation of complex mixtures of fatty acids according to chain length, number of double bonds, configuration, etc. (for reviews, see refs. 1, 2, 3). As yet, however, no satisfactory method has been devised for separating structurally isomeric monoenoic acids containing the same number of carbon atoms, but having the double bonds in different positions. Hence one could not hitherto carry out a complete analysis of fatty acids by purely chromatographic means and the components of fatty acid mixtures regarded in many papers as "oleic", "palmitoleic", etc. acids actually consisted of mixtures of position isomers. In the relatively few cases in which an exhaustive structural analysis was carried out, the composition of the unsaturated acid fractions with equal numbers of C-atoms could be determined only from the results of oxidative cleavage[4–8]. This part of the work had to be done on a preparative scale, required considerable amounts of material for analysis and was very time-consuming[9,10]. In a recent modification the methyl esters of the unsaturated fatty acids are fractionated prior to oxidation by preparative thin-layer and gas–liquid chromatography[11]. The modified method is highly sensitive, but still does not discriminate between stereoisomeric acids and involves a large number of operations (twofold runs each by thin-layer chromatography and gas–liquid chromatography, ozonization and reduction of the ozonides).

The present paper describes a method for carrying out the complete structural analysis of fatty acids using only thin-layer chromatography*. It is based on two-dimensional thin-layer chromatography of fatty acids and allows separation of positional isomers of unsaturated fatty acids having double bonds in any position. The spots can be specifically identified with the aid of reference substances and if these are unavailable, then by oxidation of the components directly in the thin layer of the adsorbent. The method is simple, requires very little material (0.5 mg of a mixture of the methyl esters of the fatty acids) and greatly shortens the time required for the analysis.

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DISCUSSION

With the objective of separating the structural isomers of unsaturated acids we investigated the chromatographic behaviour of their methyl esters on plates covered with silica gel impregnated with silver nitrate. Such plates have been recently employed for separation of *cis-trans* isomers[12]. It was shown that the π-complexes formed by the reaction of silver nitrate with the ethylenic bond of the stereoisomeric unsaturated esters differ, much more than the esters alone, in polarity, and hence in their mobility on the plates. One could therefore expect that such plates would also efficiently separate structurally isomeric mono-unsaturated fatty acid esters.

Our results with the chromatography of the methyl esters of structurally isomeric mono-unsaturated fatty acids in various systems showed that they could in fact be separated on silver nitrate-impregnated silica gel (see Table I and Fig. 1). The best separation was obtained with systems I, II, IV and V (see Table I). The same conditions, however, did not lead to satisfactory separation of unsaturated esters of varying chain lengths. In order therefore to achieve complete separation according to chain length, structure and configuration, we made use of two-dimensional chromatography. The mixture was first subjected to thin-layer partition chromatography on silica gel impregnated with dodecane in an acetonitrile–acetone system[13]. This led to separation into groups according to the number of C atoms (each double

TABLE I

R_F VALUES OF ISOMERIC MONO-UNSATURATED METHYL ESTERS ON SILICA GEL IMPREGNATED WITH $AgNO_3$

Compounds	R_F					
	I	*II*	*III*	*IV*	*V*	*VI*
C_{16}						
$CH_3(CH_2)_5CH=CH(CH_2)_7COOCH_3$ (*cis*)	0.62	0.40	0.32	0.35	0.26	0.22
$CH_3(CH_2)_3CH=CH(CH_2)_9COOCH_3$ (*cis*)	0.68	0.46	0.38	0.44	0.34	0.28
C_{18}						
$CH_3(CH_2)_9CH=CH(CH_2)_5COOCH_3$ (*cis*)	0.56	0.34	0.29	0.28	0.23	0.18
$CH_3(CH_2)_7CH=CH(CH_2)_7COOCH_3$ (*cis*)	0.64	0.42	0.35	0.39	0.29	0.24
$CH_3(CH_2)_5CH=CH(CH_2)_9COOCH_3$ (*cis*)	0.73	0.49	0.42	0.49	0.41	0.34
$CH_3(CH_2)_7CH=CH(CH_2)_7COOCH_3$ (*trans*)	0.84	0.64	0.59	0.64	0.54	0.44
C_{20}						
$CH_3(CH_2)_7CH=CH(CH_2)_9COOCH_3$ (*cis*)	0.70	0.47	0.37	0.47	0.39	0.29
C_{22}						
$CH_3(CH_2)_{15}CH=CH(CH_2)_3COOCH_3$ (*cis*)	0.58	0.39	0.30	0.31	0.26	0.19
$CH_3(CH_2)_9CH=CH(CH_2)_9COOCH_3$ (*cis*)	0.72	0.51	0.40	0.50	0.41	0.32
C_{26}						
$CH_3(CH_2)_{15}CH=CH(CH_2)_7COOCH_3$ (*cis*)	0.65	0.46	0.34	0.42	0.30	0.23

Solvents: I = Diethyl ether–petroleum ether (b.p. 28–40°) (3:7)
II = Diethyl ether–petroleum ether (b.p. 28–40°) (9:41)
III = Diethyl ether–petroleum ether (b.p. 40–60°) (9:41)
IV = Dipropyl ether–petroleum ether (b.p. 28–40°) (2:3)
V = Dipropyl ether–petroleum ether (b.p. 28–40°) (1:3)
VI = Dipropyl ether–petroleum ether (b.p. 40–60°) (1:3)

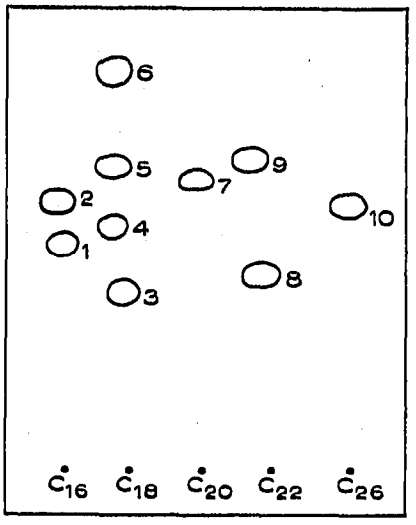

Fig. 1. Separation of isomeric mono-unsaturated methyl esters on $AgNO_3$-impregnated silica gel plates. Solvent: Dipropyl ether–hexane (2:3). Time for saturation of chamber: 40 min. Time of development: 90 min. Length of run: 14 cm. Detection: 50% H_2SO_4. Methyl esters of the acids (50 γ each): 1 = palmitoleic; 2 = palmitvaccenic; 3 = *cis*-octadecen-7-oic; 4 = oleic; 5 = *cis*-vaccenic; 6 = elaidic; 7 = *cis*-eicosen-11-oic; 8 = *cis*-docosen-5-oic; 9 = *cis*-docosen-11-oic; 10 = *cis*-hexacosen-9-oic.

bond being equivalent to shortening of the chain by two CH_2 units)[13,14]. The plates were then impregnated with a solution of silver nitrate and were developed in the second direction with a dipropyl ether–hexane (2:3) system.

In this way, complete separation of the groups into individual components was achieved, the components appearing as clearly defined spots on spraying with an alkaline or ammoniacal solution of bromothymol blue. The R_F values of the methyl

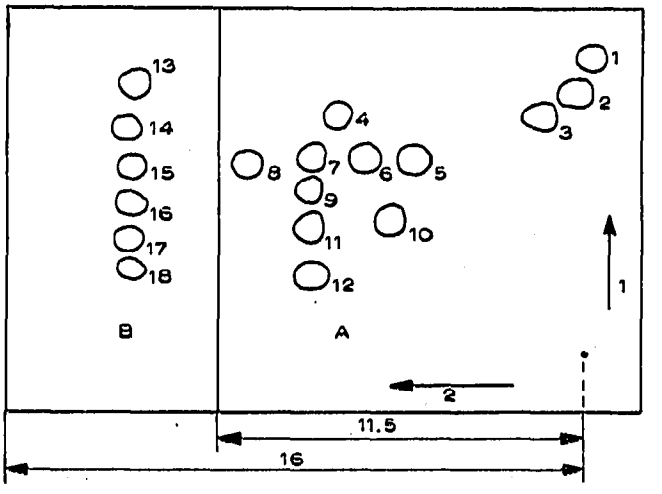

Fig. 2. Two-dimensional chromatography of a mixture of methyl esters of saturated and unsaturated fatty acids (dimensions in cm). Adsorbent: silica gel. First direction: impregnation with a 10% solution of dodecane in hexane. Solvent: acetonitrile–acetone (1:1). Developing time: 90 min. Second direction: impregnation with a 20% $AgNO_3$ solution (zone A only). Solvent: dipropyl ether–hexane (2:3). Developing time: 60 min. Methyl esters of acids (50 γ each): 1 = arachidonic; 2 = linolenic; 3 = linoleic; 4 = palmitvaccenic; 5 = *cis*-octadecen-7-oic; 6 = oleic; 7 = *cis*-vaccenic; 8 = elaidic, 9 = *cis*-eicosen-11-oic; 10 = *cis*-docosen-5-oic; 11 = *cis*-docosen-11-oic; 12 = *cis*-hexacosen-9-oic; 13 = lauric; 14 = myristic; 15 = palmitic; 16 = stearic; 17 = arachidic; 18 = behenic.

esters are given in Table II. Fig. 2 shows the separation of an artificial mixture of the methyl esters of six saturated, nine monoethylenic and three polyenic esters.

It is particularly noteworthy that under the above conditions esters of different chain length, but with the same distance of the double bond from the carboxyl group possess R_F values that are close together, which values increase as the double bond is further removed from the carboxyl group; *trans* isomers move faster than *cis* isomers (see Table II). This circumstance allows the method to be used, not only for complete separation of the mixtures, but also for identification and study of the structure of the components.

TABLE II

R_F VALUES IN TWO-DIMENSIONAL CHROMATOGRAPHY OF METHYL ESTERS OF SATURATED AND UNSATURATED FATTY ACIDS ON SILICA GEL

Compounds	*1st Direction*	*2nd Direction*
Methyl esters of saturated acids		
Dodecanoic	0.78	0.80
Tetradecanoic	0.67	0.80
Hexadecanoic	0.56	0.80
Octadecanoic	0.46	0.80
Eicosanoic	0.36	0.80
Docosanoic	0.28	0.80
Methyl esters of unsaturated acids:		
C_{16}		
cis-Hexadecen-9-oic	0.71	0.39
cis-Hexadecen-11-oic	0.71	0.46
C_{18}		
cis-Octadecen-7-oic	0.60	0.34
cis-Octadecen-9-oic	0.60	0.42
cis-Octadecen-11-oic	0.60	0.51
trans-Octadecen-9-oic	0.60	0.64
C_{20}		
cis-Eicosen-11-oic	0.50	0.52
C_{22}		
cis-Docosen-5-oic	0.40	0.39
cis-Docosen-11-oic	0.40	0.52
C_{26}		
cis- Hexacosen-9-oic	0.25	0.47
Octadeca-9,12-dienoic	0.71	0.08
Octadeca-9,12,15-trienoic	0.80	0.03
Eicosa-5,8,11,14-tetraenoic	0.89	0.00

If reference substances are available, the method permits a complete structural analysis of fatty acids to be carried out without any supplementary procedure. If they are not available, the unsaturated acids can be identified by oxidative cleavage directly in the thin layer of the adsorbent. For this purpose the individual substances were scraped off together with the adsorbent and were applied in the form of an ethereal extract to plates covered with a thin layer of cellulose impregnated with a benzene

solution of dimethylformamide. Oxidative fission of the compounds by the periodate–permanganate reagent[15] was carried out directly on the plates. Development of the resultant mixture of the monocarboxylic acid and the monomethyl ester of the dicarboxylic acid was carried out in the system hexane–diethyl ether–dimethylformamide (40:20:1), the compounds being detected with the aid of a sensitive acid–base indicator[16].

Higher polyenic (*e.g.* arachidonic) acids are difficult to elute from silica gel impregnated with silver nitrate. They were therefore oxidized directly on the silica gel taken from the plate. The mixture was then extracted with ether and the extract applied to the plate covered with dimethylsulphoxide or dimethylformamide-impregnated cellulose (Fig. 4).

The spots obtained for the *n*-alkanoic acid and the monomethyl ester of the dicarboxylic acid were identified with the aid of reference substances (malonic acid derived from divinylmethane patterned esters does not give spots). The results of the oxidation of ten monoethylenic and polyenic acids are shown in Figs. 3 and 4.

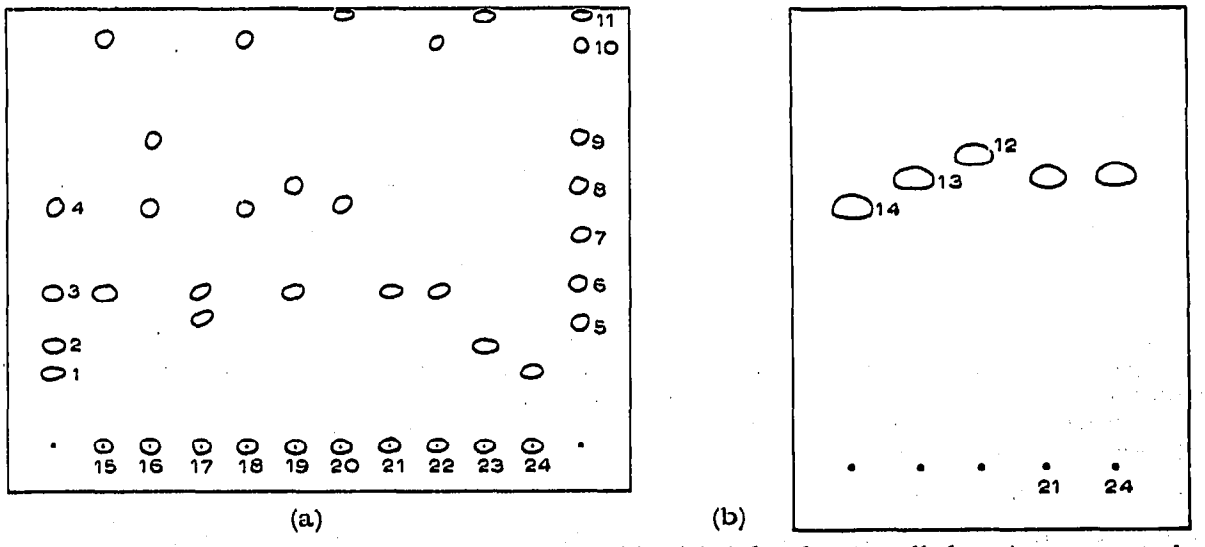

Fig. 3. Oxidative cleavage of unsaturated fatty acids. (a) Adsorbent: cellulose impregnated with a 25% solution of dimethylformamide in benzene. Solvent: hexane–diethyl ether–dimethylformamide (40:20:1). Developing time: 20 min. (b) Adsorbent: silica gel impregnated with a 10% solution of dodecane in hexane. Solvent: acetic acid–acetonitrile (1:4) (saturated with dodecane). Developing time: 60 min. Reference substances: (I) Monomethyl esters of the acids: 1 = glutaric; 2 = pimelic; 3 = azelaic; 4 = nonanedicarboxylic-1,9. (II) Acids: 5 = propionic; 6 = butyric; 7 = valeric; 8 = capronic; 9 = enanthic; 10 = pelargonic; 11 = hendecanoic; 12 = palmitic; 13 = margaric; 14 = stearic. Methyl esters of unsaturated acids subjected to oxidation: 15 = oleic; 16 = *cis*-vaccenic; 17 = linolenic; 18 = *cis*-eicosen-11-oic; 19 = linoleic; 20 = *cis*-docosen-11-oic; 21 = *cis*-hexacosen-9-oic; 22 = elaidic; 23 = *cis*-octadecen-7-oic; 24 = *cis*-docosen-5-oic.

In this system, *n*-alkanoic acids with more than 11 C atoms move along with the front. Oxidation of the higher unsaturated fatty acids yielding such alkanoic acids must therefore be carried out on two plates. In order to develop and identify the monomethyl ester of the dicarboxylic acid, oxidation is carried out on cellulose plates as described above. To develop and detect the second fraction, *i.e.* the monocarboxylic acid with more than 11 carbon atoms, oxidation is carried out on a thin-layer of silica gel impregnated with dodecane and developed with dodecane-saturated acetonitrile–acetic acid (4:1) (Fig. 3b).

It can be seen from Table III that the R_F values of some monocarboxylic acids and monomethyl esters of dicarboxylic acids coincide. However, the combined data of two-dimensional chromatography and oxidative fission always allow accurate determination to be made of the structure of the original unsaturated acids together with a complete structural analysis of any straight chain fatty acid mixture. Quan-

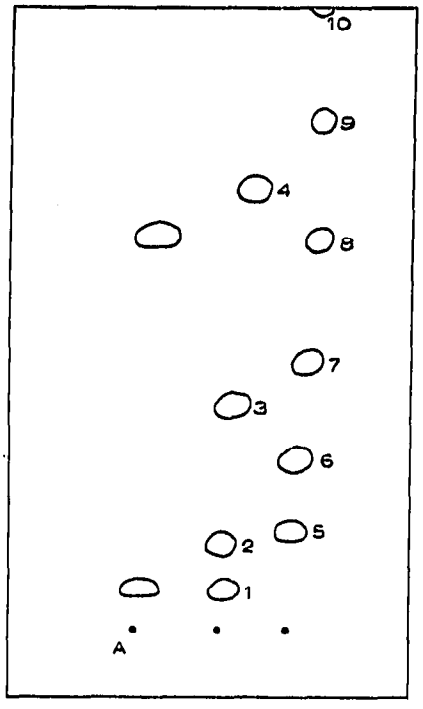

Fig. 4. Chromatography of the oxidation products of methyl arachidonate. Adsorbent: cellulose impregnated with a 25 % solution of dimethylsulfoxide in toluene. Solvent: diethyl ether–hexane (1:2) (chamber saturated with dimethylsulfoxide vapor). Developing time: 35 min. A: Oxidation products of methyl arachidonate. For enumeration of reference substances, see Fig. 3.

titative determination of the components can be achieved either by one of the methods usually employed in thin-layer chromatography[17,18] or by a combination of thin-layer and gas–liquid chromatography[19].

The proposed method thus makes possible the complete structural analysis of mixtures of fatty acids differing not only in the number of C atoms and in the degree of unsaturation, but also in the position of the double bonds, and may therefore become a convenient tool in lipid chemistry.

EXPERIMENTAL

Chromatography was carried out on plates coated with a thin layer of silica gel as follows: A paste of 6.5 g KSK silica gel (150–200 mesh), 0.4 g gypsum and 17 ml 12 % $AgNO_3$ solution is applied to a 9 × 24 cm glass plate and after drying at room temperature for 5–8 h is dried in an oven, the temperature of which is slowly (40–60 min) raised to 104–106° at which temperature the plate is kept for another hour. The plates gradually darken on prolonged storage, but this does not affect their separating efficiency. The grey background disappears when detecting the substances

TABLE III

R_F VALUES OF MONOCARBOXYLIC ACIDS AND MONOESTERS OF DICARBOXYLIC ACIDS ON DIMETHYLFORMAMIDE-IMPREGNATED CELLULOSE

Compounds	R_F
Monocarboxylic acid	
Propionic	0.20
Butyric	0.31
Valeric	0.43
Caproic	0.54
Enantic	0.65
Pelargonic	0.84
Hendecanoic	0.94
Monomethyl esters of dicarboxylic acids	
Glutaric	0.11
Pimelic	0.17
Azelaic	0.31
Nonanedicarboxylic	0.54

by spraying with 50 % H_2SO_4 followed by heating for 5–10 min with a 500 W I.R.-lamp. The substances are revealed as brown, rapidly vanishing spots. It should be mentioned that the separation of the substances and the reproducibility of the results depend greatly upon the degree of saturation of the chamber. Best results were obtained by preliminary saturation for 40–50 min [for the system I and II (see note to Table I) 5–10 min are sufficient]. According to reported data[20] supersaturation of the chamber by attaching filter paper to the walls cuts the developing time by one third. Our observations have shown that despite this, poorer separation of the substances is obtained.

Two-dimensional chromatography was carried out on plates (18 × 18 cm) covered with a paste of 10 g silica gel, 0.6 g gypsum and 25 ml water. After the usual drying procedure, the plates were impregnated by gradual immersion in 10 % (v/v) of dodecane solution in hexane. The extent of impregnation (weight ratio of impregnating substance to adsorbent) can vary within the limits of 0.1–0.2 without any noticeable effect on the separating power. For lower degrees of impregnation the capacity of the layer becomes inadequate and part of the substance moves with the front; for higher degrees, a second frontal line appears.

In the first direction, the mobile phase consisted of a 1:1 acetonitrile–acetone mixture, of which 90 % had been previously saturated with dodecane (6–6.4 ml dodecane per 90 ml mixture at 18–20°). Development took 70–90 min. Mean R_F values for the runs in the first direction are given in Table II.

After development in the first direction the plates were dried for 30 min at room temperature and for 30–40 min at 90–95°, and were then left overnight in air. Part of the plate (zone A, Fig. 2) was impregnated with 20 % $AgNO_3$ solution and the plate was activated by slow heating (1 h) up to 100° and holding at 100–102° for one h*.

* The reagent is much less sensitive for the detection of saturated acids on $AgNO_3$-impregnated silica gel than on the non-impregnated adsorbent. The part of the plate where the saturated methyl esters should be located after the run in the second direction (zone B, Fig. 2) was therefore not sprayed with $AgNO_3$ solution.

A dipropyl ether–hexane mixture (2:3) served as mobile phase for the second direction. The mean R_F values for this direction are also shown in Table II.

After developing in the second direction the plates were dried at 70–80° for 20 min, zone B was sprayed with an alkaline solution of bromothymol blue* (40 mg indicator in 100 ml 0.01 *N* NaOH solution) and the plates were again heated for 10–15 min at the same temperature. The saturated esters were detected as yellow spots on a blue background. The sensitivity was 15–20 γ.

After detection of the saturated esters the plates were sprayed with an ammoniacal solution of bromothymol blue (40 ml of bromothymol blue in 100 ml 20 % NH_4OH)**. The methyl esters of unsaturated acids were detected as quickly fading, light or dark blue spots on a gray background. The sensitivity was 40–50 γ.

Oxidative cleavage of the methyl esters of unsaturated acids

(*a*) *Preparation of the plates.* A plate (18 × 18 cm) covered by a thin layer of cellulose as described earlier[21] (9 g cellulose powder, 0.7 g gypsum, 25 ml water) was impregnated by immersion for 10–15 sec in a 25 % solution of dimethylformamide in benzene. The plate was then dried for 20 min at room temperature and 2–3 min at 60–70°, following which it was immediately covered by glass (to prevent further evaporation of the impregnated composition). A 1.5–2 cm band was left uncovered for application of the oxidation products.

(*b*) *Oxidation and chromatographic analysis of the oxidation products.* After detecting the methyl esters on silica gel the spots were scraped off together with the adsorbent and the substance extracted with ether (4 × 10 ml). The combined ether extracts were evaporated *in vacuo* to 0.3 ml and were spotted on a cellulose plate.

Each methyl ester spot was oxidized by applying by means of a capillary tube a small amount of oxidizing reagent (0.001 mole K_2CO_3 and 0.001 mole $KMnO_4$ dissolved in 10 ml of water and mixed with a solution of 0.001 mole $NaIO_4$ in 10 ml of water) and heating the plate in a drying oven at 55–60° until the pink potassium permanganate color disappeared (3–5 min). This operation was repeated 2 or 3 times (over-all oxidation time 30–40 min), after which the brown spots formed were moistened with 2 *N* HCl solution. After applying the reference substances the chromatogram was developed in the system hexane–diethyl ether–dimethylformamide (40:20:1). The developing time was 20–30 min. The plate was then placed in an ammonia-saturated glass chamber and sprayed with indicator (200 mg methyl red, 200 mg bromothymol blue, 100 ml formalin, 400 ml ethanol, 3 ml 1 *N* NaOH)[16]. The monocarboxylic acids and monomethyl esters of dicarboxylic acids formed as the result of the oxidation process were detected as yellow spots on a green background (Fig. 3a). At the starting line, a pink 1.5–2 cm band that passes into yellow is observed, whose appearance is apparently due to the presence of hydrochloric acid and of dimethylformamide degradation products. On this band the monomethyl ester of glutaric acid can be detected as an orange colored spot. The sensitivity is 5 γ.

Methyl arachidonate was oxidized directly on silica gel, the reaction mixture then being extracted with ether (3 × 10 ml). The ether extract was then evaporated

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** The reagent should be kept from zone A in order to avoid hydrolysis of the unsaturated esters.

*** A more sensitivitive reagent (10–15 γ) is a solution of 40 mg bromothymol blue in 100 ml water and 2 ml 2 *N* HCl. However, it gives poorer results on further oxidation.

to 0.1 ml and the mixture applied on a cellulose-coated plate, impregnated with dimethylformamide or a 25 % solution of dimethylsulfoxide in toluene (Fig. 4).

In order to identify the compounds which on oxidation yield an *n*-alkanoic acid with more than 11 carbon atoms, the resultant monomethyl ester of the dicarboxylic acid is detected on cellulose plates as described above. To identify the monocarboxylic acid fraction the oxidation is carried out on a thin silica gel layer, impregnated with dodecane*, by applying to the methyl ester spot the oxidizing reagent (0.001 mole $KMnO_4$, 0.001 mole $NaIO_4$, 20 ml water) as described above. The chromatogram is developed in the system acetonitrile–acetic acid (4:1), saturated with dodecane. The developing time was 50–60 min. The plates are then dried at 110–120° for 1 h, sprayed with 10 % solution of phosphomolybdic acid in ethanol and heated by an I.R. lamp (Fig. 3b).

SUMMARY

A method for the complete structural analysis of complex mixtures of fatty acids has been developed based on two-dimensional thin-layer chromatography of their methyl esters on silica gel and identification of the unsaturated acids by oxidative cleavage directly in the adsorbent layer. The method permits the determination both of positional isomers and of stereoisomers of unsaturated fatty acids.

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* Optimal results were obtained with silica gel prepared from liquid glass[22].