MULTI-DIMENSIONAL CHROMATOGRAPHY USING DIFFERENT DEVELOPING METHODS

IV. RATIONAL IDENTIFICATION OF FATTY ACID ESTERS BY MEANS OF PROGRAMMED DISTRIBUTION OF FRACTIONS IN TWO-DIMENSIONAL (GC–TLC) CHROMATOGRAMS*

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(Received July 23rd, 1965)

In preceding papers[1,2], we have pointed out the interesting possibilities offered by a new type of multi-dimensional chromatography in which a gas chromatogram is eluted on to the start of a travelling thin layer or chromatographic paper. Identification has been still further simplified by logarithmically programming the speed of travel of the starting line[3] which results in linear and additive spacing of neighbouring members of a homologous series. We have used this method to identify individual components of complex mixtures of tar constituents.

The present paper serves to illustrate the further possibilities offered by the above method for the identification of natural materials, *e.g.* fatty acids, as compared with the simple combination of gas chromatography and thin-layer chromatography, used by MANGOLD[4] for the identification of fatty acids, by NIGAM AND LEVI[5] for the identification of terpenes, and for the identification of phenols[6].

Gas chromatography on a non-polar stationary phase (first dimension) results in an excellent separation of fatty acids (as their methyl esters) according to the number of C-atoms in the molecule, and regardless of the number of double bonds or other bonds; thin-layer chromatography on silica gel impregnated with silver nitrate[7,8] (second dimension) separates the fatty acids according to their degree of unsaturation and regardless of the number of C-atoms. The logarithmic variation of the speed of travel of the starting line past the outlet of a GC column ensures that the identification of individual components in a developed two-dimensional chromatogram becomes a matter of linear geometric orientation. This method substantially simplifies comparison of various lipids, and of differences in their composition depending on their various biological sources and on the conditions of their exposure in the organism. The above method also allows one to look for the presence of possibly still unknown acids in natural and other products.

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* A part of the postdoctoral research work by N.R.A.

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PROCEDURE

The method used is as recently described[3]. Gas chromatography was carried out with a Chrom 3 instrument (Laboratory Instruments, Prague, Czechoslavakia). The column outlet was modified to permit the eluted fractions to be fed on to the thin layer[2]. The column employed was 240 cm long and 0.6 cm I.D. The column was packed with 25 % (w/w) Apiezon L on Chromosorb W (Johns Manville, London, Great Britain). Nitrogen was the carrier gas; detection was by a thermal conductivity cell. Column temperature: 180–220°. The injection block was maintained at 240°. The identification of esters extracted from silica gel after developing the chromatogram was carried out with 25 % (w/w) Reoplex 400 on Chromosorb W, using a flame ionization detector.

The thin-layer chromatography was carried out on silica gel PHH (Spolana, n.p., Neratovice, Czechoslovakia) wetted with saturated or dilute silver nitrate solution, 218 g $AgNO_3$ per 100 ml, and/or 200 ml, of water respectively (at 20°) and dried for 24 h at 180°. After this treatment, 1 g of silica gel contained 2.6 g or 1.3 g $AgNO_3$. Thin layers were prepared by coating glass plates, 180 × 90 × 1 mm or 180 × 190 × 1 mm, with loose dry powdered silica gel (metallic plates caused reduction). The chromatograms were developed within about 5 min in an S-chamber, in all cases, with approximately 25 ml of a 70:30 (v/v) petroleum ether–diethyl ether mixture. The carriage for shifting the plates past the outlet of the GC column was mounted at a distance of 1.5 to 2 mm from the outlet orifice.

Tests were carried out with a standard mixture of methyl esters of C_{14}–C_{18} fatty acids which contained unsaturated C_{18} acids or, eventually, with individual esters, synthesized[9] from pure free acids. The scope of our method is illustrated by examples chosen to match the most interesting papers in this field which have been published recently[10].

On thin layers, the spots were detected[11] by spraying the plates with an ethanolic solution of 2,7-dichlorofluorescein or Rhodamine B (0.2 % and 0.05 %, respectively); in all cases the plates were sprayed before complete evaporation of all the solvent (the dry loose thin layers are damaged by spraying in the dry state). When using 2,7-dichlorofluorescein, the spots became visible under ultraviolet light (360 mμ) as light green spots on a black-brown background. The spots are stabilized by this procedure after some time has elapsed; the detection threshold is 1–5 μg of ester. When using Rhodamine B, the spots are crimson on a pink background and become transiently visible for about 2 to 3 min even in daylight, as well as under an U.V. lamp. The detection limit is 1 μg.

The extraction of esters from silica gel was carried out (*cf.* ref. 2) with ethanol and the first two drops were injected into the chromatograph.

RESULTS AND DISCUSSION

Both the amount of silver nitrate on silica gel and the polar component of the solvent system substantially influence the R_F values as it is shown in Tables I and II. The degree of impregnation of the silica gel must be considerable lest the silica gel should influence the development of the chromatograms (causing tailing) and the R_F values of acids with several double bonds should be too high (decrease of resolution).

TABLE I

INFLUENCE OF THE AMOUNT OF $AgNO_3$ ON SILICA GEL ON R_F VALUES OF THE METHYL ESTERS OF STEARIC, LINOLEIC, AND LINOLENIC ACIDS (DEVELOPED WITH PETROLEUM ETHER–DIETHYL ETHER (70:30, v/v))

g $AgNO_3$/1 g silica gel	R_F C_{18}	$C_{18}^{=}$	$C_{18}^{==}$
2.62	0.84	0.73	0.32
1.31	0.81	0.65	0.40
0.52	0.81	0.70	0.58
0.24	0.76	0.70	0.62

TABLE II

INFLUENCE OF THE CONTENT OF DIETHYL ETHER ON R_F VALUES OF METHYL ESTERS OF STEARIC, LINOLEIC, AND LINOLENIC ACIDS (2.6 g $AgNO_3$/1 g SILICA GEL)

Petroleum ether–diethyl ether v/v	R_F C_{18}	$C_{18}^{=}$	$C_{18}^{==}$
100:0	0.12	0.09	0.05
90:10	0.67	0.55	0.09
80:20	0.79	0.70	0.32
70:30	0.84	0.73	0.31
60:40	0.93	0.67	0.25

When these conditions are satisfied, the silver nitrate content ceases to be critical. In our experiments these conditions were fulfilled when the minimum content of $AgNO_3$ was 1 g per g of silica gel. Similarly, an increase in the content of the polar component in the solvent accelerates migration of the unsaturated acids up to a certain limit, whereas a further increase of the former causes tailing of the zones. That was the reason why an apparently good resolution at a ratio of 60:40 had no practical value. The optimum solvent system was 70:30 (v/v) petroleum–diethyl ether. The reproducibility of R_F's has been checked by repeating the experiments twenty times with methyl esters of acids which had a different number of C-atoms but were of the same structure, and it has been shown that the R_F values are characteristic for the number of double bonds, being at the same time independent of the length of the carbon chain (Table III). It also seems to be possible to separate some individual stereoisomers (see refs. 7 and 12).

TABLE III

R_F VALUES AND THE MEAN ERROR OF MEASURING R_F'S OF METHYL ESTERS OF ACIDS WITH VARIOUS NUMBERS OF C-ATOMS AND DOUBLE BONDS

20 experiments	$C_{14:0}$	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{20:1}$	$C_{24:1}$	$C_{18:2}$
Ø	0.84	0.83	0.84	0.73	0.74	0.74	0.32
% rel.*	0.8	1.1	1.0	1.0	0.9	1.2	1.1
% rel.**	4.0	4.5	4.0	3.5	4.8	5.0	5.0

* Experiments carried out with single batches of silica gel and solvent system, respectively.
** Experiments carried out with freshly prepared silica gel and solvent system for each run.

ANALYTICAL APPLICATION

The application of the above method for analyses is obvious from the excellent resolution of individual components of a very complex mixture of saturated and unsaturated fatty acids on two-dimensional chromatograms. For the sake of comparison, the gas chromatograms of the mixture are illustrated (*cf.* ref. 10). Fig. 1A shows a gas chromatogram developed on Apiezon L (as a non-polar phase).

The non-selective grouping of acids according to the number of C-atoms is obvious. This chromatogram was eluted on to the start of a thin layer, travelling at a logarithmically programmed speed. Fig. 2 shows the distribution of individual chromatographed components after developing the second dimension. Good separation of all components is obvious.

Fig. 1B shows a gas chromatogram of the same mixture, developed on the polar Reoplex 400. In this case the well-known distinction between saturated and unsaturated acids takes place but with the simultaneous occurrence of overlapping resulting from the regrouping.

In the two-dimensional chromatograms of Fig. 3 all the components are well separated again, but obey a principle other than that in the former case (see Fig. 2). Individual zones, extracted and reinjected into the gas chromatograph, gave single peaks.

A small zone of an impurity was detected in the examined mixture. The location of this zone in the chromatogram according to Fig. 2 unambiguously corresponds to a C_{18} acid, and with respect to its R_F, to a higher number of double bonds than three. The correlation shown in Fig. 4, which was compiled from the relative locations of

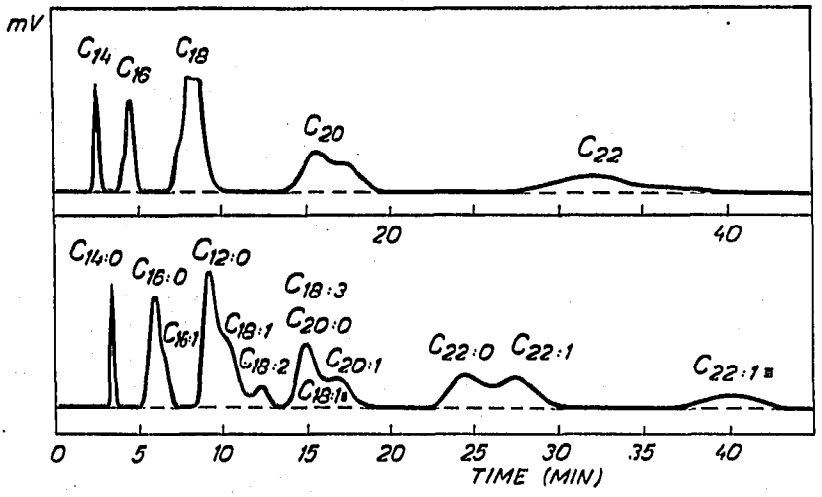

Fig. 1. Chromatograms of a mixture of C_{14}–C_{22} fatty acid methyl esters. (A) Separation on Apiezon L at 220°. (B) Below: Separation on Reoplex 400 at 200°.

saturated and unsaturated C_{18} acids, as obtained in the two-dimensional chromatogram shown in Fig. 3, confirms that the impurity is a C_{18} acid, and unambiguously establishes the presence of four double bonds in the molecule.

The scope and simplicity of our method for identification of fatty acids of most diverse structures can be demonstrated by studying the locations shown by the printed

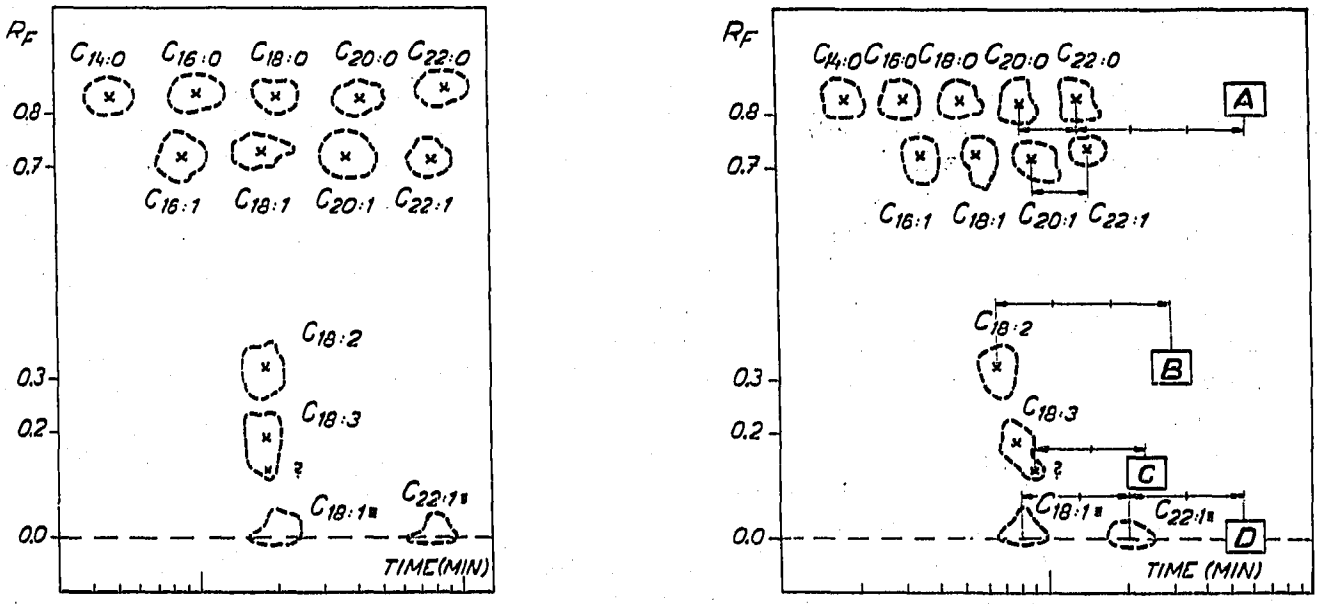

Fig. 2. Programmed distribution of individual C_{14}–C_{22} fatty acid methyl esters in the two-dimensional chromatogram. First dimension (GLC): Apiezon L at 220° (isothermal). Second dimension (TLC): Silica gel + $AgNO_3$ (logarithmical shifting); petroleum ether–diethyl ether (70:30).

Fig. 3. Distribution and band correlation of known and unknown fatty acid methyl esters in the two-dimensional chromatogram. First dimension (GLC): Apiezon L at 220° (isothermal). Second dimension (TLC): Silica gel + $AgNO_3$ (logarithmical shifting); petroleum ether–diethyl ether (70:30).

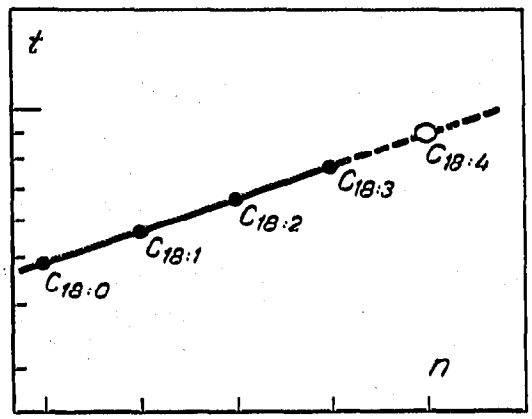

Fig. 4. Identification of an unknown fatty acid methyl ester from band position of known members of the homologous series.

symbols, which indicate where the presence of acids of certain structures can be expected. The position of these symbols was determined by measuring the distance corresponding to the sum of increments for an arbitrary member of a homologous series of an arbitrary structure. Area A corresponds therefore to the location of the methyl ester of a saturated C_{28} acid, area B to an ester of a C_{24} acid with two double bonds, area C to a C_{22} ester with four double bonds, and area D to the location of a C_{20} ester with one triple bond, etc. This procedure is extremely advantageous when studying differences among unknown lipids of natural and other origins and their transformations.

CONCLUSIONS

A two-dimensional chromatography of fatty acids is described, using elution of a gas chromatogram (first dimension) on to the start of a thin layer, travelling at a logarithmically programmed speed. The use of a non-polar stationary phase in gas chromatography causes the fatty acid esters to be separated according to their number of C-atoms, and regardless of their structure. The logarithmic speed of travel of the thin layer secures a linear spacing of individual members of a homologous series along the starting line. Development of the thin layer chromatogram of silica gel, impregnated with silver nitrate (second dimension) results in a separation of the fatty acid esters according to their degree of unsaturation and regardless of the number of C-atoms.

Thus, the determination of the location of any chromatographic zone in the two-dimensional chromatogram becomes a matter of rational geometric orientation, based on the knowledge of the location of a few (*e.g.* two to four) arbitrarily chosen acids. Individual esters, which were studied in complex mixtures of fatty acids, gave separate zones that did not overlap. We are, therefore, entitled to predict precise locations of fatty acids of arbitrary structures, and check for the presence of these acids in the chromatogram.

Our method can be used with advantage for investigating miscellaneous lipids, as well as differences in their composition when they originate from different biological materials and after having been exposed to various conditions in the organism. The method is also well suited for the detection of some possibly unknown acids in natural products.

SUMMARY

An identification method for complex fatty acid mixtures has been developed by using a new two-dimensional chromatographic technique. The first dimension is represented by a gas chromatogram eluted on to the logarithmically travelling start line of a thin layer, the second dimension by the thin-layer chromatogram (silica gel coated with $AgNO_3$).

The location pattern of the chromatographic bands is then a matter of rational geometric orientation due to the simple and characteristic distances of components equivalent to their molecular structure (C-number, number of double bonds). This pattern can be derived from the location of 2–4 known standard substances.

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