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Note

Isolation of *trans*-hexadecenoic and *trans*-octadecenoic fatty acid methyl esters from lipid extracts by means of argentation and reversed-phase thin-layer chromatography

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The use of glass capillary gas–liquid chromatography (GLC) on highly polar cyanosiloxane phases, studied in detail previously[1–4], has become common practice for the qualitative and quantitative analysis of *trans* fatty acids from complex fatty acid mixtures.

Biological and industrial hydrogenation does not only induce geometrical isomerization and thereby the formation of *trans* fatty acids whose prototype is *trans*-octadecenoic acid, but also leads to positional isomerization so that widespread shifts in the positions of the double bonds occur. In rats the pattern of incorporation of individual isomeric *trans*- (and *cis*-)octadecenoic acids has been found to be specific for different tissues[5]. For humans no comparable data are yet available.

The location of double bonds is determined by ozonolysis followed by GLC separation of the fragments. This method is well established, commonly used and free from technical problems. In contrast to ozonolysis, the isolation of individual pure fatty acid methyl esters (FAMEs) for studies on the position of double bonds is laborious, time-consuming and cumbersome, so that in general no series of analyses have been described. Usually, argentation chromatography on thin-layer plates (Ag^+/TLC)[6,7], column chromatography[8] or argentation based on counter-current distribution[9,10] are applied. Unfortunately, extensive overlapping of different FAMEs often occurs. Therefore preparative Ag^+/TLC of FAMEs has mostly been combined with preparative GLC[5]. Formerly, other methods were used, such as crystallization and repeated fractional distillation of FAMEs followed by column chromatography of previously formed mercuric acetate adducts[11]. Recently, Özcimder and Hammers[12] described the usefulness of reversed-phase and argentation high-performance liquid chromatography as a pre-fractionation method.

In order to continue our studies on the occurrence and the possible biological mechanisms of *trans* monoenoic fatty acids in different human tissues[3,13], we have searched for a reliable method for the isolation of pure *trans*-hexa- and -octadecenoic fatty acids that would be rapid, specific and sensitive and would not require expensive apparatus. This paper describes such a method, combining preparative argentation and reversed-phase high-performance TLC. The method can be extended to the isolation of other pure fatty acids after some minor modifications. In terms of simplicity, it is preferable to earlier methods.

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MATERIALS AND METHODS

All FAME standards used were purchased from Nu-Check-Prep (Elysian, MN, U.S.A.). Their purity was at least 99% when analyzed by capillary column GLC. Investigations were also carried out with FAME mixtures obtained from crude lipid extracts of different human tissues[3].

Argentation TLC (Ag^+/TLC)

Analytical silica gel H (Kieselgel 60) glass plates (E. Merck, Darmstadt, G.F.R.) were used after impregnation with $AgNO_3$. The $AgNO_3$ (10 g) was dissolved in distilled water (10 ml), and methanol was added to 100 ml. The plates were dipped in this solution, the excess of solution was allowed to run off and the plates were dried for 15 min. They were then activated by heating at 40°C for 30 min, and after cooling to room temperature were kept in a dry dark storage box.

trans-Monoenes were separated from the crude FAME samples by preparative Ag^+/TLC with dichloroethane as developing solvent. A methyl elaidate standard was cochromatographed. The spots were located by spraying with 2′,7′-dichlorofluorescein and viewing under ultraviolet light (254 nm). The marked spots were scraped off and eluted with chloroform–methanol (2:1). The eluates were evaporated, the FAME redissolved in a small volume of chloroform and rechromatographed. The isolated *trans*-monoene band was then used for reversed-phase HPTLC.

Reversed-phase HPTLC

The doubly developed reversed-phase HPTLC plates contained a hydrocarbon phase (C_8 or C_{18}) chemically bonded to silica gel (HPTLC-Fertigplatten RF-8 and RF-18; E. Merck). For the RF-8 plates, formic acid–acetonitrile–acetone (2:2:1) gave excellent separations; acetonitrile was likewise used for the RF-18 plates. The separation characteristics of FAMEs were studied on plates containing only 75% or 50%, respectively, of *n*-octadecylsilyl reversed phase (Nano-SIL C_{18}-75 and Nano-SIL C_{18}-50 Fertigplatten für die Nano-DC; Machery, Nagel & Co., Düren, G.F.R.).

GLC

All analytical steps were followed by GLC analyses[1,2]. The conditions are summarized in Table I. In order to determine the accuracy of the method, some

TABLE I

ANALYTICAL CONDITIONS FOR GLC SEPARATION OF FAMEs

Column	Wall-coated open tubular, glass, 50 m × 0.30 mm I.D.; SP 2340 (Chrompack, Berlin, G.F.R.)
Gas chromatograph	Carlo Erba Fractovap 2900
Carrier gas	Nitrogen
Inlet pressure	0.9 bar
Sample size	0.1–0.3 μl chloroform
Splitting ratio	1:6
Oven temperature:	125–175°C, at 2°C/min; 175–230°C, at 3°C/min
Detector	flame ionization
Chart speed	0.5 cm/min

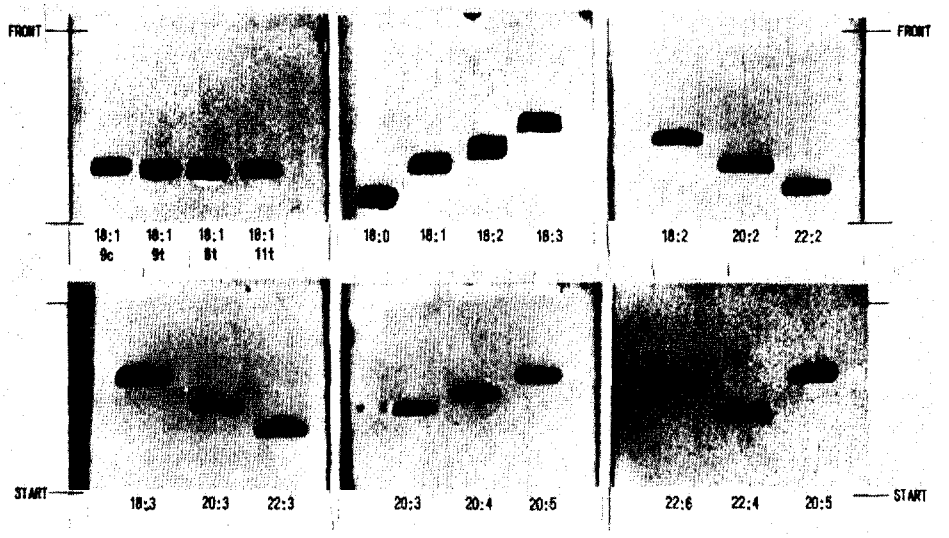

Fig. 1. Chromatographic separation of fatty acid methyl esters on reversed-phase HPTLC plates containing 100% *n*-octylsilyl functional groups bonded to the silica surface (HPTLC-Fertigplatten RF-8; E. Merck). The plates were developed twice in formic acid–acetonitrile–acetone (2:2:1) at room temperature. The spots were detected under UV light and then blackened for photographic purposes.

samples of FAME mixtures were re-analyzed after admixture of a known amount of *trans*-octadecenoic acid methyl esters. Prior to all GLC analyses, methyl palmitate was added as internal standard.

RESULTS AND DISCUSSION

As demonstrated in Figs. 1 and 2, FAME standards can be separated in the reversed-phase systems according to chain length and the number of double bonds. The R_F values decrease with increasing chain length and increase with a increasing number of double bonds. As previously reported by Kaufmann and co-workers[14,15] and by Bergelson *et al.*[16], each double bond is nearly equivalent to a chain shortening by two CH_2 units. Neither of the two TLC systems tested proved superior to the other with regard to the resolution of fatty acids. In comparison, plates coated with 75% or 50% *n*-octadecylsilyl reversed phase exhibited higher R_F values for individual standards, but resulted in a progressively lower resolving capacity. Geometrical and positional isomers cannot be separated by this reversed-phase system. Since Ag^+/TLC basically separates according to the degree and *cis-trans* type of unsaturation with little separation of differing chain lengths[17], it is particularly suitable for combination with the reversed-phase TLC method described above[16].

Following argentation, chromatography of *trans*-monoene fractions of FAMEs of biological origin[3] showed contamination with some very long-chain monounsaturated *cis* FAME such as 22:1 (ω 9) and 24:1 (ω 9). After rechromatography in the reversed-phase TLC system, complete discrimination of *trans*-hexadeconoic, *trans*-octadecenoic, *cis*-docosenoic and *cis*-tetracosenoic methyl esters was

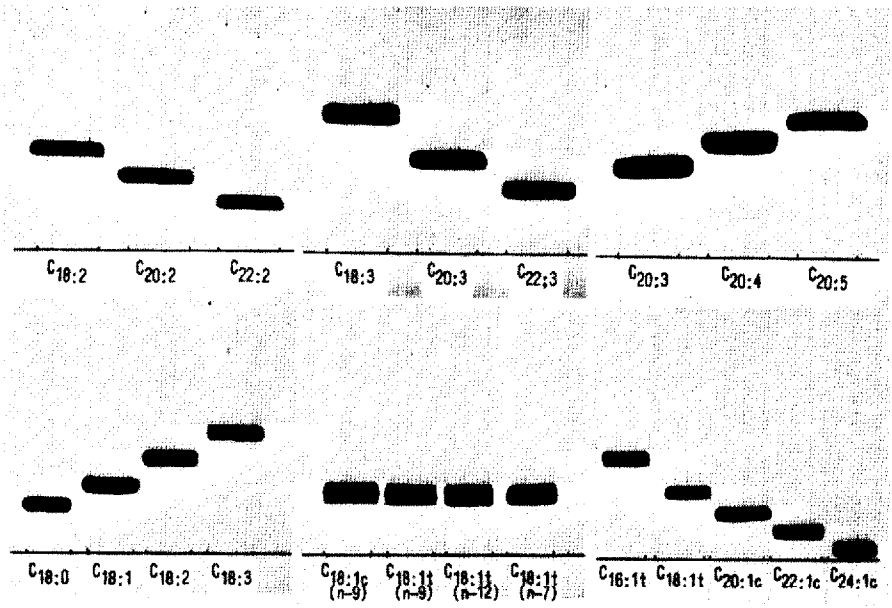

Fig. 2. Chromatographic separation of fatty acid methyl esters on reversed-phase HPTLC plates containing 100% *n*-octadecylsilyl functional groups bonded to the silica surface (HPTLC-Fertigplatten RF-18; E. Merck). Acetonitrile was used as the solvent system. For further details see Fig. 1.

possible after GLC control of the re-extracted individual spots. The pure individual *trans* monoenoic methyl esters could then be subjected to ozonolysis and further analysis to determine the position of the double bonds.

Extensive experiments to separate complex FAME mixtures on only one RF-18 plate were ineffective. In these experiments, a small border strip of the plate was dipped in a methanol–silver nitrate solution for silver nitrate impregnation and developed in the first direction in two-dimensional TLC, whereas for the second direction the remaining non-impregnated RF plate was used.

After admixture of a known quantity of *trans*-octadecenoic methyl ester to six FAME samples derived from human tissues, repeated analysis yielded a 98% recovery of this *trans* monoenoic acid. The whole analytical procedure takes 2.5 h.

The described method is very simple, rapid and highly specific and does not require expensive apparatus in comparison to other methods, *e.g.*, combined TLC–GLC[5].

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