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SEPARATION AND IDENTIFICATION OF GEOMETRICAL ISOMERS OF 9,12-OCTADECADIENOIC AND 9,12,15-OCTADECATRIENOIC ACIDS*

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SUMMARY

Geometrical isomers of methyl 9,12-octadecadienoate and methyl 9,12,15-octadecatrienoate were fractionated by thin-layer chromatography on Silica Gel G modified with silver nitrate and ammonium hydroxide.

Dienoic esters were completely separated into three classes: (1) all *trans*; (2) *trans–cis* and *cis–trans*; and (3) all *cis*. Trienoic esters were similarly separated into four classes: (1) all *trans*; (2) di-*trans*, mono-*cis*; (3) di-*cis*, mono-*trans*; and (4) all *cis*.

Complete separation of the *cis*, *trans* isomers of methyl octadecadienoate and of the mono-*cis*, di-*trans* and mono-*trans*, di-*cis* isomers of methyl octadecatrienoate was achieved by gas–liquid chromatography on glass capillary columns (Apiezon L).

INTRODUCTION

The presence of geometrical isomers of mono-, and polyunsaturated fatty acids in lipids of animal orgin has been previously reported[1–6].

It is however generally believed that plant lipids contain, with a limited number of exceptions[7–10], only unsaturated acids with the double bonds in the *cis* configuration[7,11]. The formation of conjugated and non-conjugated isomers of polyunsaturated acids, during the refining of edible fats, has been reported[11], mainly on the basis of spectral data[12,13].

As the importance of polyunsaturated acids in metabolic and nutritional processes has been recognized, the need for adequate analytical techniques for their separation and identification has increased. The rapid development of thin layer and gas chromatographic techniques, during the last few years, has afforded a new and powerful means for their study.

In connection with a series of studies on the unsaturated fatty acids of natural fats and their modification during refining operations which are being carried out in this Institute, a number of techniques have been developed for the isolation and identification of the isomers of di- and tri-unsaturated octadecanoic acids.

In the present paper a method is presented for the separation of geometrical

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isomers of 9,12-octadecadienoic and 9,12,15-octadecatrienoic acids, as methyl esters, by thin layer chromatography on Silica Gel G, modified with ammonium hydroxide and silver nitrate. The identification of the separated isomers was carried out by GLC on capillary columns and I.R. spectrophotometry.

MATERIALS AND METHODS

Preparation of linoleic and linolenic acid isomers

About 1 g of linoleic acid* (Fluka: 89.1 % of 9-*cis*, 12-*cis* octadecadienoic acid, 10.9 % of geometrical isomers) and 1 g of linolenic acid* (Fluka: 86.1 % of 9-*cis*, 12-*cis*, 15-*cis* octadecatrienoic acid, 12.2 % of geometrical isomers, 0.58 % palmitoleic acid, 0.15 % oleic acid, 0.58 % linoleic acid) were isomerized by heating at 180° for 4 h with mechanical stirring under nitrogen with 2 % Se[14]. The percentage of *trans* double bonds formed, calculated from the mole percentage of geometrical isomers as determined by GLC on a BDS column by the procedure described later in this paper, was 46.9 % for linoleic and 44.4 % for linolenic acid.

Methyl esters of the isomerized acids were prepared with BF_3 and CH_3OH according to the procedure of METCALFE AND SCHMITZ[15].

TLC separations

28–30 % ammonium hydroxide solution was added to a solution of 12 g of $AgNO_3$ in 50 ml of distilled water until the precipitate initially formed was redissolved (about 9 ml). The solution was diluted to 100 ml with distilled water. Silica Gel G (50 g) was then slurried with the silver nitrate–ammonium hydroxide solution and the slurry used to prepare five 20 × 20 plates by spreading a uniform 0.30 mm layer. After the plates had air dried for 2 h, they were activated at 110° for 1 h and stored in a vacuum desiccator. In addition, chromatoplates with 8 g of $AgNO_3$ were prepared by the procedure described above. These plates were used for the separation of geometrical isomers of methyl linolenate. 600 μg per cm of methyl linoleate and 300 μg of methyl linolenate were applied in a continuous band approximately 3 cm from the lower edge of the plates.

Development of the chromatoplates was carried out with the system benzene–petroleum ether (b.p. 40–70°) 30:70 (v/v) for the methyl linoleate isomers and benzene–petroleum ether 40:60 (v/v) for the methyl linolenate isomers. The bands were then visualized by spraying with a 0.2 % solution of 2′,7′-dichlorofluorescein in absolute ethanol and viewing under U.V. light (λ = 254 mμ).

GLC separation

GLC separation of the geometrical isomers of linoleic and linolenic acids was carried out on the corresponding methyl esters using a Carlo Erba gas chromatograph Model C equipped with a 60 m glass capillary column coated with Apiezon L, and a flame ionization detector. The column temperature was 180°; the injector and detector temperatures were kept at 260°. The carrier gas was nitrogen, with a flow rate of 1 ml/min; (the flow rate through the stream splitter was 100 ml/min). The number of

* The purity of methyl linoleate and methyl linolenate was determined by GLC on a butanediol succinate (BDS) packed column and a capillary column coated with Apiezon L.

theoretical plates of the column, calculated for methyl 9-*cis*, 12-*cis*-linoleate was about 30,000.

In order to determine the extent of isomerization, linoleic and linolenic acids were esterified with a methanol–BF_3 solution and the isomers separated by TLC. An internal standard (methyl elaidate) was added to the recovered fractions and their relative amounts determined by GLC on a column packed with 15 % BDS on silylated Chromosorb W, 80–100 mesh. A dual column Perkin Elmer Model 800 gas chromatograph, equipped with differential flame ionization detector was used. The column temperature was 200°; detector temperature: 210°; injector temperature: 260°; carrier gas: nitrogen, 30 ml/min.

Infrared analysis

Different geometrical isomers were identified by I.R. analysis of the material recovered from the various TLC bands, by the procedure reported elsewhere[16].

RESULTS AND DISCUSSION

The isomers which can be formed on isomerization of 9,12-octadecadienoic and 9,12,15-octadecatrienoic acids with Se are listed in Table I.

TABLE I

GEOMETRICAL ISOMERS OF 9,12-OCTADECADIENOIC AND 9,12,15-OCTADECATRIENOIC ACIDS

9-cis, 12-cis	*9-cis, 12-cis, 15-cis*
9-*cis*, 12-*cis*	9-*cis*, 12-*cis*, 15-*cis*
9-*cis*, 12-*trans*	9-*cis*, 12-*cis*, 15-*trans*
9-*trans*, 12-*cis*	9-*cis*, 12-*trans*, 15-*cis*
9-*trans*, 12-*trans*	9-*trans*, 12-*cis*, 15-*cis*
	9-*cis*, 12-*trans*, 15-*trans*
	9-*trans*, 12-*cis*, 15-*trans*
	9-*trans*, 12-*trans*, 15-*cis*
	9-*trans*, 12-*trans*, 15-*trans*

Migration of double bonds on isomerization, with formation of conjugated isomers occurred to a very limited extent, as demonstrated by GLC, U.V. and I.R. analysis of the Se isomerized products[16,17]. These results are in agreement with the data reported by SCHOLFIELD *et al.*[14]. The position of the double bonds of the different isomers has been investigated and the work will be the subject of a separate note[17].

Thus four isomers are to be expected as a result of Se isomerization of 9,12-octadecadienoic acid and eight isomers can be formed from 9,12,15-octadecatrienoic acid. Separation of the 9,12-octadecenoic acid isomers was achieved by SCHOLFIELD *et al.*[14] by means of "Argentation Countercurrent Distribution" (CCD) and GLC on capillary column. Complete separation of the geometrical isomers of methyl linolenate was not obtained[18]. Geometrical isomers of methyl linoleate and methyl linolenate were separated on silver nitrate impregnated Silica Gel G plates by DE VRIES[19], but no details concerning the source and the purity of these compounds were given.

Figs. 1 and 2 show the TLC separation of the geometrical isomers of the methyl esters of 9,12-octadecadienoic and 9,12,15-octadecatrienoic acids, respectively, obtained on Silica Gel G modified with ammonium hydroxide and silver nitrate. Three main bands are obtained in the first case (Fig. 1), and four bands in the second case (Fig. 2).

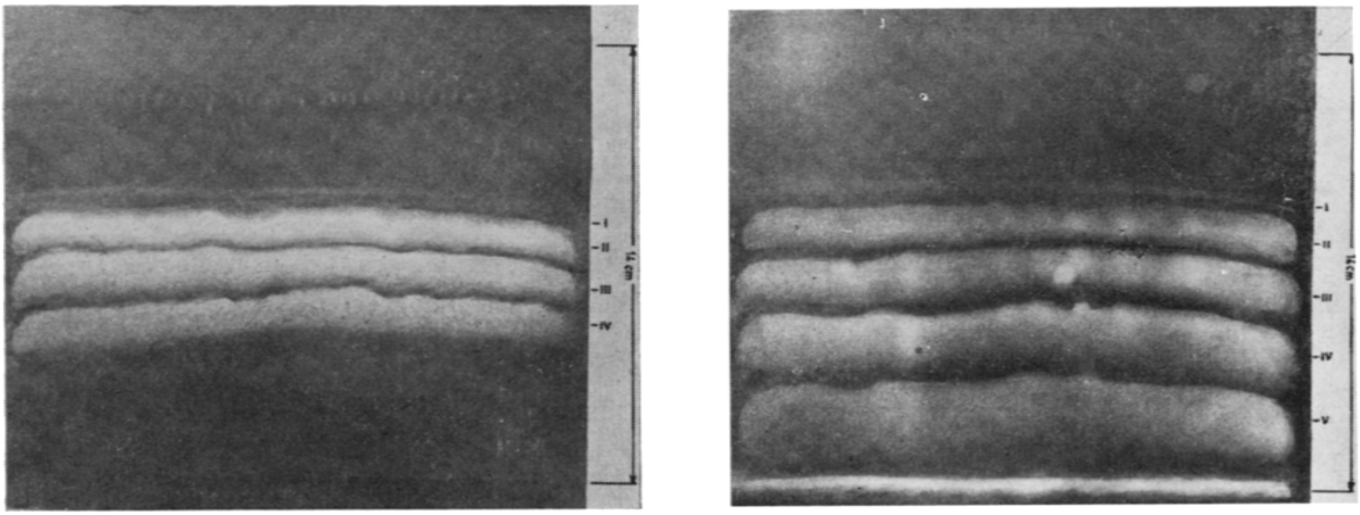

Fig. 1. TLC separation of non-conjugated geometrical isomers of methyl linoleate (methyl 9,12 octadecadienoate). (I) Traces of conjugated isomers; (II) methyl (*trans–trans*) linoleate; (III) methyl (mono-*trans*, mono-*cis*) linoleate; (IV) methyl (*cis–cis*) linoleate.

Fig. 2. TLC separation of non-conjugated geometrical isomers of methyl linolenate (methyl 9,12,15 octadecatrienoate). (I) Traces of conjugated isomers; (II) methyl (*trans–trans–trans*) linolenate; (III) methyl (di-*trans*, mono-*cis*) linolenate; (IV) methyl (di-*cis*, mono-*trans*) linolenate; (V) methyl (*cis–cis–cis*) linolenate.

A weak band (Band I: Figs. 1 and 2) is visible immediately above the main bands, which contains the small amount of conjugated isomers formed[17,20]. In Fig. 2 a band which remains at the start is also present and is very likely due to polymerization products of 9,12-15-octadecatrienoate.

The separated bands were scraped off the plates and extracted with anhydrous ethyl ether. The nature of the recovered substances was then studied by I.R. spectrophotometry and by GLC on a BDS packed column, and an Apiezon L capillary column.

In the case of the methyl 9,12-octadecadienoate isomers, bands II and III show a strong absorption at 965 cm^{-1}, characteristic of isolated *trans* double bonds; no absorption is shown by band IV. It can therefore be assumed that band II contains the *trans-trans*-isomer, band III the two possible *cis,trans* isomers, and band IV the *cis,cis* isomer.

If this assumption is correct, and the absorption at 965 cm^{-1} also behaves additively[16] when several isolated double bonds are present in the same molecule, the ratio (1) given below must be equal to 0.5.

$$\frac{a_{ct}}{a_{tt}} = \frac{a_{III}}{a_{II}} = \frac{A_{III}/C_{III}}{A_{II}/C_{II}} = \frac{A_{III}C_{II}}{A_{II}C_{III}} = 0.5 \qquad (1)$$

(where a_{ct} = absorptivity of the *cis,trans* isomers; a_{tt} = absorptivity of the *trans, trans* isomer; a_{II} = absorptivity of substances recovered from band II; a_{III} = absorptivity of substances recovered from band III; A_{II} = absorbance measured for band II; A_{III} = absorbance measured for band III; C_{II} = concentration of band II (*trans,trans* isomer); C_{III} = concentration of band III (*cis,trans* isomers)).

The ratio (1) was determined by GLC by the following procedure. The substances recovered from band II and band III were dissolved in equal volumes of hexane (solution 1 and 2). One ml of each of the two solutions was added to 1 ml of a 0.2 % solution of methyl elaidate in hexane and the resulting mixtures were separately analyzed by GLC on the BDS column.

The ratio C_{II}/C_{III} could then be calculated from the following expression:

$$\frac{C_{II}}{C_{III}} = \frac{q_{II}}{q_{III}} = \frac{S_{II}/S_{sII}}{S_{III}/S_{sIII}} = \frac{S_{II} \cdot S_{sIII}}{S_{sII} \cdot S_{III}}$$

(where q_{II} = absolute amount of component of band II; q_{III} = absolute amount of component of band III; S_{II} = area of the peak of component of band II; S_{sII} = area of the peak of the standard added to band II; S_{III} = area of the peak of component of band III; S_{sIII} = area of the peak of the standard added to band III).

Solutions 1 and 2 were taken to dryness; the residues dissolved in 1 ml of CS_2, and their absorbance (A_{II} and A_{III}) determined. The values of the ratio C_{II}/C_{III} and A_{III}/A_{II}, determined as described, substituted in equation 1 give a value of a_{ct}/a_{tt} = 0.51 in good agreement with the expected value.

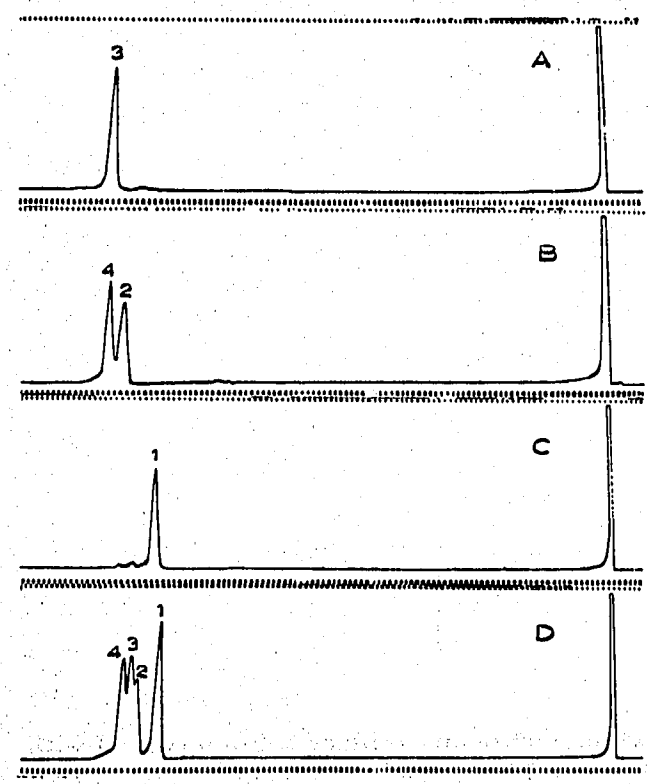

Fig. 3. GLC separation of geometrical isomers of methyl linoleate on capillary column coated with Apiezon L. (A) Methyl (9-*trans*, 12-*trans*) linoleate (peak 3); (B) methyl (9-*cis*, 12-*trans*) linoleate (peak 2) + methyl (9-*trans*, 12-*cis*) linoleate (peak 4); (C) methyl (9-*cis*, 12-*cis*) linoleate (peak 1); (D) Se isomerized methyl linoleate.

Each TLC fraction was subsequently analyzed by GLC on an Apiezon L coated capillary column (Fig. 3). As expected, bands II and IV contain only one component, which can be identified as methyl 9-*trans*, 12-*trans* octadecadienoate (gas chromatogram A: peak 3) and methyl 9-*cis*,12-*cis* octadecadienoate (gas chromatogram C: peak 1) respectively; two components are present in band III, which correspond to the two possible *cis–trans* isomers (gas chromatogram B: peaks 2 and 4).

By comparison with the product obtained from ricinoleic acid by dehydration with $NaHSO_4$[20] the first of these two peaks was identified as methyl 9-*cis*, 12-*trans* octadecadienoate (gas chromatogram B; peak 2).

The same procedure was used in order to characterize the isomerization products of methyl 9,12,15-octadecatrienoate. Infrared measurements showed that the isomers recovered from bands II, III and IV contain three, two and one *trans* double bonds, respectively, whereas band V does not show the characteristic absorption band of an isolated *trans* double bond at 965 cm^{-1}.

Fig. 4 shows the chromatograms of the four fractions obtained with the Apiezon

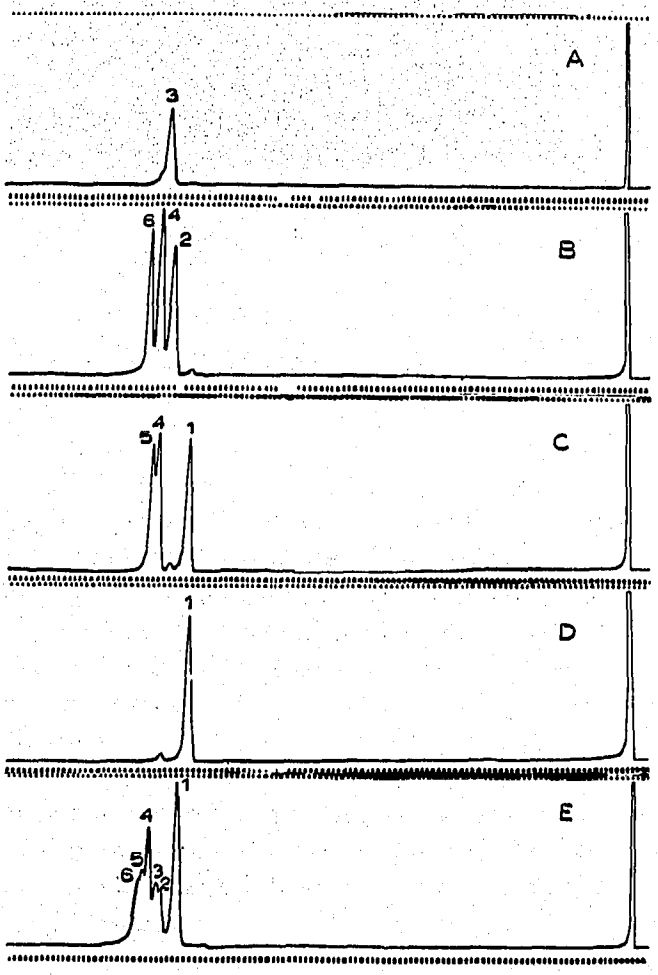

Fig. 4. GLC separation of geometrical isomers of methyl linolenate on capillary column coated with Apiezon L. (A) methyl (9-*trans*, 12-*trans*, 15-*trans*) linolenate (peak 3); (B) methyl (9-*trans*, 12-*trans*, 15-*cis*) + (9-*trans*, 12-*cis*, 15-*trans*) + (9-*cis*, 12-*trans*, 15-*trans*) linolenates (peaks 6,2,4); (C) methyl (9-*cis*, 12-*cis*, 15-*trans*) + (9-*cis*, 12-*trans*, 15-*cis*) + (9-*trans*, 12-*cis*, 15-*cis*) linolenates (peaks 1,4,5); (D) methyl (9-*cis*, 12-*cis*, 15-*cis*) linolenate (peak 1); (E) Se isomerized methyl linolenate.

L column. The first fraction (band II) contains only one product, which on the basis of the infrared data, can be identified as methyl 9-*trans*, 12-*trans*, 15-*trans* octadecatrienoate (gas chromatogram A: peak 3).

The second and third fraction (band III and IV) both contain three components which correspond to the possible di-*trans*, mono-*cis* (gas chromatogram B: peaks 2,4,6) and di-*cis*, mono-*trans* isomers (gas chromatogram C: peaks 1,4,5). The assignment of the three compounds present in each fraction will be the subject of a separate note[21]. The last fraction (band V) again contains only one component, the all-*cis* isomer (Fig. 4–gas chromatogram D: peak 1).

Tables II and III report the relative retention volumes of the isomers of methyl 9,12-octadecadienoate and methyl 9,12,15-octadecatrienoate, as determined on the Apiezon L capillary column, and the relative abundance of each isomer.

It is interesting to note that, contrary to previously published work[14,22–24], the 9-*trans*, 12-*cis* (Fig. 3 – gas chromatogram B: peak 4) and 9-*trans*, 12-*trans* (Fig. 3 – gas chromatogram A: peak 3) isomers of methyl octadecadienoate and the 9-*trans*,12-*trans*,15-*trans* (Fig. 4 – gas chromatogram A: peak 3) and one of the di-*trans*, mono-*cis* (Fig. 4 – gas chromatogram B: peak 2) isomers show different retention volumes. On the other hand, methyl 9-*cis*, 12-*cis* octadecadienoate (Fig. 3 – gas chromatogram C: peak 1), methyl 9-*cis*,12-*cis*, 15-*cis* octadecatrienoate (Fig. 4 – gas chromatogram D: peak 1) and one of the di-*cis*, mono-*trans* isomers (Fig. 4 – gas chromatogram C: peak 1) are not separated under the same experimental conditions. Also one of the mono-*trans*, di-*cis* (Fig. 4 – gas chromatogram C: peak 4) and one of the mono-*cis*, di-*trans* (Fig. 4 – gas chromatogram B: peak 4) isomers of methyl octadecatrienoate show the same retention volumes.

It may be possible, however, to separate all eight possible geometrical isomers by means of a more efficient capillary column.

TABLE II

ANALYSES OF FRACTIONS (BY TLC) FROM SELENIUM ISOMERIZED METHYL LINOLEATE

TLC fractions (Fig. 1)	*Geometrical isomers (Fig. 3)*	*GLC analyses*				*I.R. analyses absorption at 965 cm^{-1}*
		BDS column (mol %)*	*Trans double bonds formed (%)*	*Apiezon L capillary column*		
				(mol %)	*V_r** relative to 9-cis, 12-cis octadecadienoate*	
II	9-*trans*, 12-*trans* (peak 3)	25.10	25.10	—	1.058	++
III	9-*cis*, 12-*trans* (peak 2)	43.76	21.88	19.48	1.045	+
	9-*trans*, 12-*cis* (peak 4)			24.28	1.073	
IV	9-*cis*, 12-*cis* (peak 1)	31.14	—	—	1.000	—

* Internal Standard: methyl elaidate.
** Time calculated from the solvent $(C_2H_5)_2O$.
+ Absorption at 965 cm^{-1}.
— No absorption at 965 cm^{-1}.

TABLE III

ANALYSES OF FRACTIONS (BY TLC) FROM SELENIUM ISOMERIZED METHYL LINOLENATE

TLC fractions (Fig. 2)	*Geometrical isomers (Fig. 4)*	*GLC analyses*				*I.R. analyses absorption at 965 cm^{-1}*
		BDS column (mol %)*	*Trans double bonds formed (%)*	*Apiezon L capillary column*		
				(mol %)	*V_r** relative to 9-cis, 12-cis octadecadienoate*	
II	*9-trans, 12-trans, 15-trans* (peak 3)	11.95	11.95	—	1.046	+++
III	three isomers:					
	(peak 2)	26.92	17.95	9.05	1.035	++
	(peak 4)			10.09	1.065	
	(peak 6)			7.77	1.086	
IV	three isomers:					
	(peak 1)	43.37	14.46	14.30	1.000	+
	(peak 4)			15.38	1.065	
	(peak 5)			13.68	1.077	
V	9-*cis*, 12-*cis*, 15-*cis* (peak 1)	17.76	—	—	1.000	—

* Internal Standard: methyl elaidate.
** Time calculated from the solvent $(C_2H_5)_2O$.
\+ Absorption at 965 cm^{-1}.
— No absorption at 965 cm^{-1}.

The relative abundance of the different isomers was determined by GLC on a BDS packed column, by adding methyl elaidate as an internal standard to the fractions recovered from the plates. It should be noted that on the BDS column the two *cis*, *trans* isomers of octadecadienoic acid methyl ester and the three mono-*cis*, di-*trans* isomers of octadecatrienoic acid methyl ester are not separated, while the three di-*cis*, mono-*trans* isomers show only partial separation. They are therefore determined as a single component; the relative quantities of each isomer are then determined from the data obtained with the Apiezon L capillary column.

The data reported in Table II and Table III, and the results of the GLC analysis of the fractions separated by TLC, show that the ammonium hydroxide–silver nitrate modified layers permit very satisfactory separations of the geometrical isomers of di- and tri-unsaturated fatty acids.

The use of ammoniacal silver nitrate was proposed by WOOD AND SNYDER[25] for the separation of saturated and *cis*-unsaturated fatty acids, using a large excess of ammonium hydroxide (10:1). Very recently DUTTA AND BARNA[26] reported a similarly modified support for the separation of *cis* and *trans* isomers of α,β-unsaturated acids. These authors suggest that a possible explanation for the better separation obtained with ammoniacal silver nitrate plates could be found in the strong coordination complexes formed by the silver-diammino ion $[Ag(NH_3)_2]^+$ with the π-bonds of the olefinic compounds.

We have noticed, however, that, in order to obtain the same R_F on both $AgNO_3$ impregnated plates and ammonium hydroxide–silver nitrate plates for a given isomer,

a benzene–petroleum ether 8:2 (v/v) solvent system was necessary in the first case while in the second case the 3:8 v/v mixture of benzene–petroleum ether was adequate. This seems to indicate that the interaction of the olefinic compounds with the substrate is lower when the silver nitrate silica gel adsorbent is modified with ammonium hydroxide. This is not unexpected when the generally accepted type of interaction metal–olefin is taken into consideration[27–31]. The bonding in metal olefin complexes is schematically represented in Fig. 5. A primary σ bond is formed in which the π-electrons of the olefin are donated to a vacant atomic orbital of the metal (5s and 5p orbital in the case of silver); an additional bond is formed by interaction of d electrons of the metal with the π^* antibonding molecular orbital of the olefin. In the $[Ag(NH_3)_2]^+$ ion two sp orbitals are used to coordinate the ammonia molecules and it seems unlikely that this ion could form stronger complexes with the olefins than with the Ag^+ ion.

Fig. 5. Representation of the bonding in metal olefin complexes: (a) the σ bond in which a π MO on the olefin overlaps with a metal orbital; (b) the σ bond in which the π^* antibonding MO of the olefin overlaps with a metal d orbital.

Although the complexity of the system ammonium hydroxide–silver nitrate–silicic acid does not allow us to define with certainty the active species which are present after activation of the plates, it is likely that the silver is only in part present as the silver diammino ion. By extracting the plates with water after activation at 110° for 1 h only 47 % of the added silver was recovered and the determined ratio $(NH_3)/Ag^+$ was: 1.6 as compared to the theoretical value: 2.0.

The evidence available at present therefore seems to indicate that the better separation obtained when the silver–silica gel substrate is modified with ammonium hydroxide may be explained as due to the lower and more specific interaction of the silver diammino ion with the π electrons of the olefin. The physical and chemical absorption of NH_3 on the silica gel surface[32–37] may, however, play an important role in partially deactivating the substrate.

Silicic acid, in fact, mainly interacts with the unsaturated fatty esters by hydrogen bond formation with the carbomethoxyl group. This kind of interaction is the same for all the isomers and tends to level out their behaviour. When hydrogen bond formation is reduced by addition of ammonium hydroxide, it seems reasonable to think that the formation of coordination complexes of the olefins with Ag^+ and/or $[Ag(NH_3)_2]^+$ ions becomes the determining factor in the separation process.

CONCLUSIONS

Geometrical isomers of di- and tri-unsaturated acids methyl esters can be conveniently separated on ammonium hydroxide–silver nitrate modified silica gel plates.

The coordinated use of thin layer and gas chromatographic techniques allows the separation and the characterization of the various geometrical isomers of 9,12-octadecadienoic and 9,12,15-octadecatrienoic acids. The possibility of determining, as a function of time, the isomer content of the mixture permits the study of the kinetics of the isomerization of these acids by Se.

The results obtained in this work are valid when the double bonds are in the 9,12 and 9,12,15 position. The possibility that a different position of the double bonds on the aliphatic chain may cause a different chromatographic behaviour of the molecule cannot be excluded.

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