CHROMATOGRAPHIC BEHAVIOUR OF ISOMERIC LONG-CHAIN ALIPHATIC COMPOUNDS

II. ARGENTATION THIN-LAYER CHROMATOGRAPHY OF ISOMERIC OCTADECENOATES

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(Received June 26th, 1967)

In the first papers describing chromatography of lipids on adsorbents impregnated with silver nitrate[1–3], two of the major attributes of this novel form of chromatography were well documented. These are that a given class of compounds can be separated firstly according to the number of isolated double bonds, more or less regardless of chain length, and secondly according to the geometry of such double bonds, whether *cis* or *trans*. More recently, a third major attribute of argentation chromatography has been recognised, namely the possibility of separating suitable positionally isomeric unsaturated compounds. (For a recent review of argentation chromatography see ref. 4). DE VRIES AND JURRIENS[5] showed that with dienoic esters on TLC the effect of silver ion complexing increased with increasing separation of the two double bonds so that 9,11-, 9,12- and 9,15-octadecadienoates could be readily separated. These authors also described the separation of 6-, 9- and 12-*cis*-octadecenoates by argentation-TLC with benzene–light petroleum (8:2) as solvent[5]. Similar separations of the 7-, 9- and 11-*cis*-octadecenoates and of the 9- and 11-isomers were reported by BERGELSON *et al.*[6] and by LEES AND KORN[7], respectively, using mixtures of various ethers and light petroleum fractions as solvents, and VERESHCHAGIN separated the 6- and 9-isomers by a reversed-phase partition system on paper[8].

We had also, independently, devised procedures based on multiple development of silver nitrate impregnated plates at low temperature which consistently gave excellent separations of positionally isomeric *cis*- and also *trans*-monoenoic esters. These separation conditions, described in this paper, have been used routinely in our laboratory for some three years to effect separations of mixtures of positionally isomeric monoenoates, both natural and synthetic. In our hands at least, these procedures are far superior in reproducibility and in resolution to the TLC systems described earlier[5–7].

EXPERIMENTAL

Materials

The *cis*-isomers of methyl 6-, 11- and 12-octadecenoates were very kindly donated

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by O. S. PRIVETT* and the *trans*-isomers of these and of methyl oleate (9-octadecenoate), which is readily available, were conveniently prepared by elaidinisation with catalytic amounts of nitric acid plus sodium nitrite in dimethylcellosolve (diglyme) solution at 65° and were isolated by preparative argentation-TLC. *cis*-8- and -10-Octadecenoates were generously provided by H. J. J. PABON**. The *trans*-3-monoenoate was isolated from the mixed esters of *Aster alpinus* seed oil[9] and elaidinisation of this compound gave approximately 10% yield of *cis*-3-octadecenoate and 50% yield of the rearrangement product *trans*-2-octadecenoate, which were isolated by argentation-TLC. 17-Octadecenoate was prepared by anodic coupling of 10-undecenoic acid with the half ester of nonanedioic acid. *cis*-15-Octadecenoate was isolated by our conditions of argentation-TLC from the product of partial reduction with hydrazine of 9,15-octadecadienoate, this latter compound having been isolated from the products of partial reduction of linolenate with hydrazine (*cf.* ref. 10).

Procedures

Silver nitrate impregnated thin layers (*ca.* 250 μ thick) were prepared conventionally using aqueous silver nitrate solutions of suitable concentrations for mixing with the Silica Gel G (Merck). Various levels of impregnation were investigated and are described as percentages of the total mixed adsorbent; thus, for example, 10% silver nitrate impregnated plates were obtained by mixing 22.5 g of silica gel with a solution of 2.5 g of $AgNO_3$ in 45–50 ml of water. Plates impregnated with ammoniacal silver nitrate, described by WOOD AND SNYDER[11], were also investigated and were prepared in the same way but using solutions of $AgNO_3$ in 0.88 ammonia instead of in water to mix with the silica gel. Initially, the layers were spread on glass plates (20 × 20 cm) using the Desaga equipment but this resulted in heavy corrosion of the spreader and more recently we have turned to the equipment of Quickfit & Quartz Ltd., Stone, Staffordshire, Great Britain, which is completely impervious to the corrosive effects of silver nitrate. Prepared plates were allowed to dry at room temperature, were activated for 1 h at 110° and were then stored in sealed glass tanks over saturated calcium chloride solution, *i.e.* at a relative humidity of *ca.* 30%. This not only protected the plates from the laboratory atmosphere but ensured that they were of a suitable and reproducible activity at any time that they were removed for use.

Samples were applied as dilute (*ca.* 1%) solutions in light petroleum by Hamilton microsyringe or, for preparative separations, with the Desaga streak applicator and development was carried out in closed tanks lined with solvent soaked filter paper. When multiple development was involved the evaporation of the solvent from the layer between developments was assisted by a current of air from a hair drier. Development at low temperatures was carried out in tanks kept in cold rooms at + 4° and —8° and in freezers at —15° and —25°. The plates, however, were not equilibrated to these low temperatures either before or between developments. After development, spots were located by thoroughly spraying with 30% chlorosulphonic acid in acetic acid followed by heating at 200° to effect charring. The results were documented by photographing with a Polaroid Land camera or by photocopying on blue-line diazo paper.

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RESULTS AND DISCUSSION

When, during our early work on argentation-TLC of lipids, we occasionally noted significant differences in mobilities of some positionally isomeric *cis*-octadecenoates we realised the potential value that a system to effect such separations would have in the chemical and biochemical investigations of our group. A wide variety of solvents of similar eluting power were therefore investigated, notably benzene, carbon tetrachloride and mixtures of each of these with various proportions of light petroleum and also binary mixtures of light petroleum with diethyl ether and other volatile ethers. These initial studies were carried out on plates having a 10 % level of silver nitrate impregnation which were activated for 30 min at 110° immediately before use. The results were that our trial mixture of equal amounts of the 6-, 9- and 11-*cis*-octadecenoates was only partially resolved with one development with diethyl ether–light petroleum (10:90) or with benzene–light petroleum (80:20) and that this resolution was improved by multiple development (two or three times) with solvents having a higher proportion of light petroleum. Other volatile ethers were, in general, less good than diethyl ether and solvents based on carbon tetrachloride effected little or no resolution. The solvents based on benzene gave consistently better results than those containing diethyl ether in that the spots remained more symmetrical and were less prone to tailing.

Because the stability of silver ion–olefin complexes decreases with increasing temperature, we considered that chromatography at low temperatures might enhance complex formation and improve separations of these isomers. As benzene (m.p. 4°) is not suitable for low temperature work, we developed a series of plates with toluene at room temperature, +4°, —8°, —15° and —25°. It was found that the mobilities of all the test compounds, including stearate, were decreased with decreasing temperature such that although only one development could be carried out at room temperature and +4°, two developments could be accommodated at —8° and —15° and three developments were possible at —25°, without causing the *cis*-monoenes to migrate into the upper quarter of the plate. The resolution of the 6-, 9- and 11-*cis*-isomers progressively improved with decrease in temperature and at —25° was much better than any previously obtained. A parallel series of experiments using xylene as solvent gave very similar results.

During the course of these various experiments, it was found that the mobilities of our various trial samples frequently varied considerably from day to day, even though the impregnated plates used were made each day and each one was activated just before use. This variability in the activity of plates was considered to be due to variations in the relative humidity of the laboratory atmosphere (*cf.* ref. 12). By storing plates, after they have been activated, in sealed glass tanks over saturated calcium chloride solutions for some time (*i.e.* at a relative humidity of *ca.* 30 %) these variations in activity were drastically reduced. Although better separations could sometimes be achieved on freshly activated plates, those stored in this way gave consistently good separations and, furthermore, retained their resolving power during storage for a week or more.

One further practical aspect remained to be investigated, namely the effect of differing levels of silver nitrate impregnation on resolution of positional isomers. Following the general directions of DE VRIES[1], most workers have used 20–30 %

levels of impregnation (*cf.* refs. 2, 5–7). However, we had concluded that, for separations on the basis of number and geometry of double bonds, little if any improvement in separation occurred on increasing the level of impregnation above about 2.5 %[13], and we had routinely used 5 % impregnated plates for such separations. Our conclusion has been supported by KLEIN *et al.*[14] and by STAHL AND VOLLMAN[15] who, working with steroids and with terpenoid alcohols respectively, demonstrated that about 3 % silver nitrate was the optimum and most economical level of impregnation. However, when we began the present work we compromised and worked with 10 % levels of impregnation. When a series of plates impregnated with silver nitrate at levels of 5 %, 10 %, 20 % and 30 % were developed three times with toluene at —25°, however, there was an increase in the separations of stearate, oleate and linoleate but a rather greater improvement, relatively, in the separation of the 6-, 9- and 11-positional isomers, as summarised in Fig. 1. Exactly the same effect was obtained when xylene was used as the developer at —25°. The reason for this effect is unknown but it may be that disilver–olefin complexes assume some importance at higher concentrations of silver nitrate (*cf.* ref. 16) and that the effect of these is more pronounced on the monoenes than on linoleate. While we still retain our preference for 5 % or 10 % impregnated plates for separations according to degree and geometry of unsaturation, we now routinely use 30 % impregnated plates for separation of positional isomers of monoenes.

We have also investigated plates impregnated with ammoniacal silver nitrate as described by WOOD AND SNYDER[11]. We found that these provided rather more cohesive layers and, compared to the normal silver nitrate layers, gave slightly improved resolution of positional isomers but we considered this improvement to be too small to justify the inconvenience and discomfort involved in the preparation of such plates.

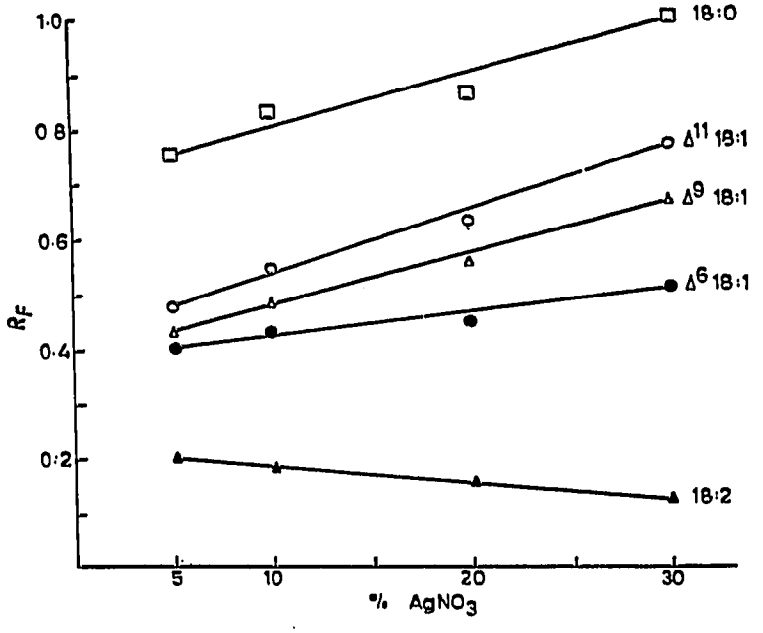

Fig. 1. Graphical representation of variation in R_F values of methyl stearate (18:0), methyl linoleate (18:2), and the *cis*-isomers of methyl 6-, 9-, and 11-octadecenoates (18:1) on thin-layer plates with differing levels of silver nitrate impregnation. Each plate was developed three times with toluene at —25°.

The standard procedure, therefore, that we now use for separation of positionally isomeric monoenoic esters, whether for analytical or preparative purposes, is to use plates with a 30 % level of impregnation of silver nitrate, prepared as described in the experimental section, and to develop these three times with toluene at —25°. When only *cis*-monoenoates are involved these developments are each allowed to run almost to the top of the plate. When *trans*-isomers are also present, as in Figs. 2 and 3, we restrict the first two developments, respectively, to approximately half and three-quarters of the height of the plate and allow only the last development to reach the top. Having thus optimised the conditions for separation of isomers using toluene as solvent we then re-investigated some of the other types of solvent but found that the separations were considerably inferior to those obtained with toluene or xylene.

The migration behaviour of a fairly wide range of positionally isomeric *cis*- and *trans*-octadecenoates using this procedure is illustrated in Fig. 2(a). The marked differences in mobilities of isomers in both the *cis* and the *trans* series is clearly evident and the pattern obtained by running the isomers side by side in this way is very similar to that obtained on normal silica gel plates with the series of positionally isomeric hydroxystearates[17], and also keto- and acetoxystearates, hydroxystearyl alcohols and diacetates[18]. All these series of oxygenated stearate derivatives assume a sinusoidal type of curve, when chromatographed in this way, with decreasing mobilities from the 2- to the 5- or 6-substituted isomers, progressively increasing mobilities to the 12- and 13-substituted compounds and then decreasing mobilities again to the terminally substituted isomer. We believe that the same factors, whether electronic or conformational, which cause these regular variations in the mobilities of the individual isomers of all these series of oxygenated aliphatic compounds, are operative in the series of isomeric monoenes, in this case enhancing or diminishing the ability of the π-electrons of the double bonds to form ligands with the silver ions in the adsorbent. We therefore predict that if all the isomeric members of the *cis*- and *trans*-octadecenoate series were available and were chromatographed side by side by our procedure the result would be approximately as represented in Fig. 2(b). We obviously cannot be certain that the minima of these curves will be represented by the Δ^5 isomers but, by analogy with the various series of oxygenated compounds[17,18], we consider this most likely. Also, the position indicated for the *cis*-Δ^2-isomer is highly speculative and it may well be coincident with the *trans*-Δ^2-isomer, because the steric effect and/or the delocalisation of the π-electrons of the double bond by the adjacent carbomethoxy group may entirely suppress complex formation by both these isomers.

What the exact nature of the factors giving rise to such a pattern of chromatographic behaviour may be we have as yet no clear idea. We believe to be significant the considerably greater increase in mobilities from the 6- or 5-isomer to the 2-isomer, relative to the rest of the pattern, for the isomeric monoenes as compared to the isomeric hydroxy esters and other oxygenated derivatives[17,18]. This may indicate that over this range of positions an inductive effect of the carboxylic ester group is predominant, becoming more pronounced as the double bond or substituent more closely approaches this group. Partial withdrawal or delocalisation of the electrons of a double bond by such an inductive effect would impair its ability to complex with silver ions to a far greater extent relatively than a similar degree of electron withdrawal from an oxygenated substituent would affect its affinity for the adsorbent. We are less certain as to the factors influencing the mobilities of the remaining isomers of these

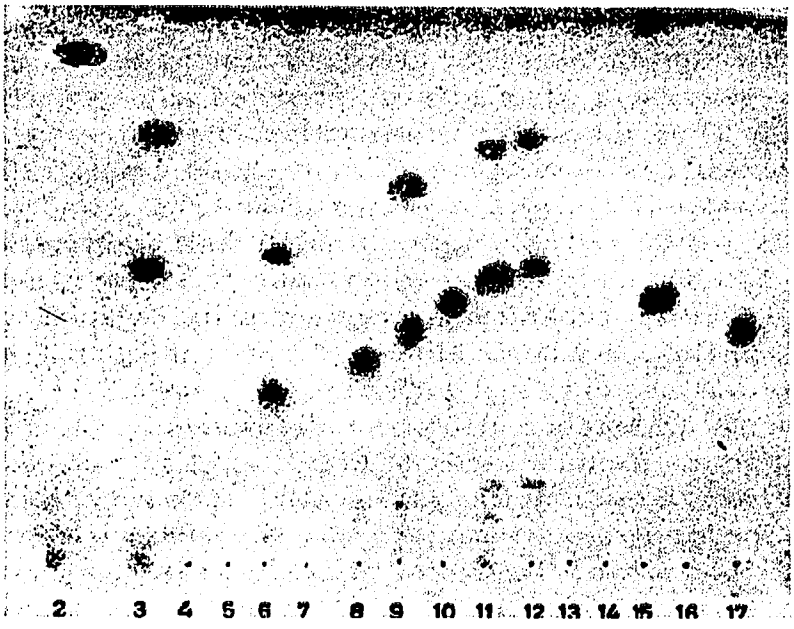

Fig. 2(a). Thin-layer chromatogram of isomeric methyl octadecenoates on silver nitrate–Silica Gel G (30:70). The position of the double bond is indicated by the sample number, the samples being the 2-, 3-, 6-, 9-, 11- and 12-*trans*-octadecenoates, the 3-, 6-, 8-, 9-, 10-, 11-, 12- and 15-*cis*-octadecenoates and the vinyl compound, 17-octadecenoate. The plate was developed, at —25°, three times with toluene (to one half, three-quarters and the full height of the plate respectively) and spots were located by spraying with chlorosulphonic acid–acetic acid (1:2) and charring.

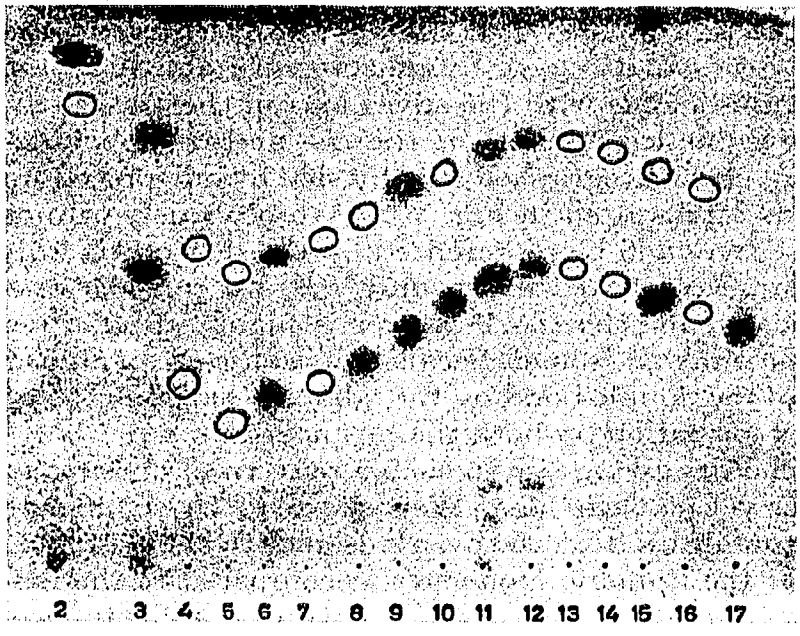

Fig. 2(b). As (a) but with the predicted positions of the 4-, 5-, 7-, 8-, 10-, 13-, 14-, 15- and 16-*trans*-octadecenoates and the 2-, 4-, 5-, 7-, 13-, 14- and 16-*cis*-octadecenoates drawn in.

various series, namely those with unsaturation or substitution in the 6- to 17- or 18-positions but we hope that further results from chromatography and from infrared and NMR spectra of such isomers may enable us to propose a reasoned hypothesis in a subsequent paper.

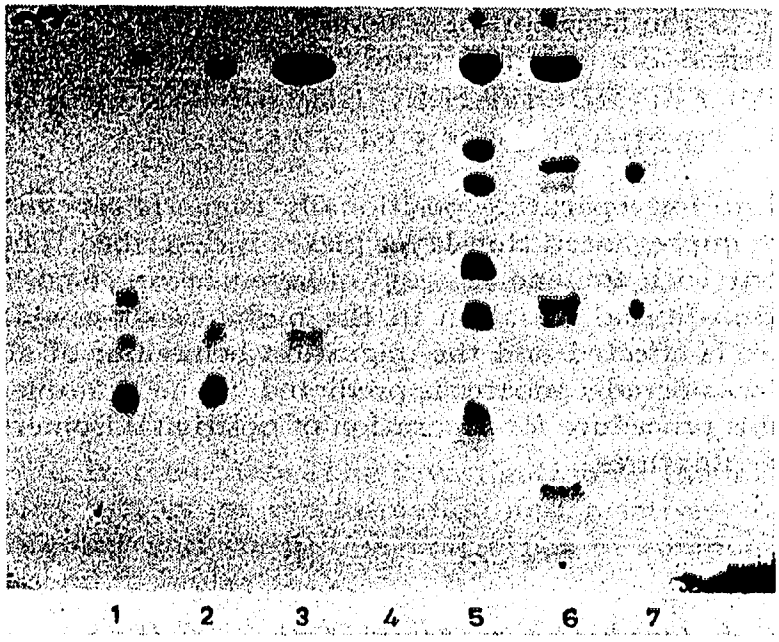

Fig. 3. Thin-layer chromatogram of some natural and synthetic methyl ester mixtures and standards on silver nitrate–Silica Gel G (30:70), developed as described in Fig. 2. The samples (the components of which are listed in order of increasing mobility) are: (1) *cis*-6-, *cis*-9- and *cis*-11-octadecenoates and trace of stearate; (2) mixed esters from parsley seed oil, showing petroselinate (*cis*-6-18:1) and oleate (*cis*-9-18:1) and saturated esters (16:0 and 18:0); (3) mixed esters from the blue-green alga, *Anacystis nidulans*, showing *cis*-9-hexadecenoate + *cis*-9-octadecenoate, a small amount of *cis*-11-octadecenoate (not clearly visible on this illustration), and saturated esters (16:0 and 18:0); (4) 9-octadecynoate (stearolate); (5) product of partial reduction with deuterated hydrazine of *cis*-9,*trans*-12- plus *trans*-9,*cis*-12-octadecadienoates, showing unreduced dienoates, *threo*-12,13-diD-*cis*-9-octadecenoate, *threo*-9,10-diD-*cis*-12-18:1, *erythro*-12,13-diD-*trans*-9-18:1, *erythro*-9,10-diD-*trans*-12-18:1, and tetradeuterostearates, the product of total reduction; (6) product of partial reduction with deuterohydrazine of *cis*-9,*trans*-15- plus *trans*-9,*cis*-15-octadecadienoates, showing unreduced dienoate, *threo*-15,16-diD-*cis*-9-18:1, *threo*-9,10-diD-*cis*-15-18:1, *erythro*-15,16-diD-*trans*-9-18:1, *erythro*-9,10-diD-*trans*-15-18:1, and tetradeuterostearates, the product of total reduction; (7) oleate (*cis*-9-18:1) and elaidate (*trans*-9-18:1).

Fig. 3 illustrates the separation of our trial mixture of 6-, 9- and 11-*cis*-octadecenoates along with a few mixtures, both natural and synthetic, to demonstrate some of the uses to which this chromatographic procedure has been put. For example, the ability to separate, intact, the two *cis*-octadecenoate isomers occurring together in parsley seeds, petroselinate (Δ^6) and oleate (Δ^9), is of obvious value in distinguishing between their biosynthetic pathways in the seed[19]. On the other hand, the possibility of separating the positional isomers of the monoenes generated by partial reduction of dienes with hydrazine has enabled us to prepare *erythro*-12, 13- ditritio-oleic acid, and the *erythro*- and *threo*-isomers of 12,13-dideutero-oleic acid and 15,16-dideutero-oleic acid. (The mixtures from which were isolated the *threo*-12,13- and -15,16-dideutero-oleates are shown in Fig. 3.) These specifically labelled compounds have now been used to elucidate the mechanism of ricinoleic acid biosynthesis in the castor bean and the stereochemistry of fatty acid desaturation in photosynthetic tissues[20,21].

One further attribute of this procedure is that the acetylenic ester, 9-octadecynoate or stearolate, is readily resolved from oleate as shown. This has already enabled us to detect stearolic acid as a natural constituent of some seed oils[22]. This

chromatographic procedure also led to the isolation of a component of *Exocarpus cupressiformis* oil which was characterised as a unique furanoid fatty acid[23].

SUMMARY

A novel and improved procedure for separating positionally isomeric *cis*- and *trans*-octadecenoates on silver nitrate impregnated thin-layer plates is described. The method consists of triple development with toluene at —25° of layers having a 30% level of silver nitrate impregnation. Substantial variation in the mobilities of a wide range of *cis*- and *trans*-octadecenoates is effected and the migration behaviour of all of the positionally isomeric *cis*- and *trans*-octadecenoates is predicted. Some examples are shown of the practical utility of this procedure for separation of positional isomers in natural and synthetic methyl ester mixtures.

REFERENCES

1 B. DE VRIES, *Chem. Ind. (London)*, (1962) 1049.
2 C. B. BARRETT, M. S. J. DALLAS AND F. B. PADLEY, *Chem. Ind. (London)*, (1962) 1050.
3 L. J. MORRIS, *Chem. Ind. (London)*, (1962) 1238.
4 L. J. MORRIS, *J. Lipid Res.*, 7 (1966) 717.
5 B. DE VRIES AND G. JURRIENS, *Fette, Seifen, Anstrichmittel*, 65 (1963) 725.
6 L. D. BERGELSON, E. V. DYATLOVITSKAYA AND V. V. VORONKOVA, *J. Chromatog.*, 15 (1964) 191.
7 A. M. LEES AND E. D. KORN, *Biochim. Biophys. Acta*, 116 (1966) 403.
8 A. G. VERESHCHAGIN, *J. Chromatog.*, 17 (1965) 382.
9 L. J. MORRIS, M. O. MARSHALL AND E. W. HAMMOND, *Lipids*, in press.
10 C. R. SCHOLFIELD, E. P. JONES, J. NOWAKOWSKA, E. SELKE AND H. J. DUTTON, *J. Am. Oil Chemists' Soc.*, 38 (1961) 208.
11 R. WOOD AND F. SNYDER, *J. Am. Oil Chemists' Soc.*, 43 (1966) 53.
12 M. S. J. DALLAS, *J. Chromatog.*, 17 (1965) 267.
13 L. J. MORRIS, *Lab. Pract.*, 13 (1964) 284.
14 P. D. KLEIN, J. C. KNIGHT AND P. A. SZCZEPANIK, *J. Am. Oil Chemists' Soc.*, 43 (1966) 275.
15 E. STAHL AND H. VOLLMAN, *Talanta*, 12 (1965) 525.
16 W. FEATHERSTONE AND A. J. S. SORRIE, *J. Chem. Soc.*, (1964) 5235.
17 L. J. MORRIS AND D. M. WHARRY, *J. Chromatog.*, 20 (1965) 27.
18 L. J. MORRIS AND D. M. WHARRY, to be published.
19 P. HARRIS AND A. T. JAMES, unpublished results.
20 L. J. MORRIS, to be published.
21 L. J. MORRIS, R. V. HARRIS, W. KELLY AND A. T. JAMES, *Biochem. Biophys. Res. Commun.*, in press.
22 L. J. MORRIS AND M. O. MARSHALL, *Chem. Ind. (London)*, (1966) 460.
23 L. J. MORRIS, M. O. MARSHALL AND W. KELLY, *Tetrahedron Letters*, (1966) 4249.