SEPARATION OF ISOMERIC LONG-CHAIN POLYHYDROXY ACIDS BY THIN-LAYER CHROMATOGRAPHY

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The resolution of higher fatty acid isomers and vinylogues by thin-layer chromatography (TLC) on adsorbents impregnated with complexing agents has recently been reported[1]. It was demonstrated that *threo* and *erythro* isomers of vicinal dihydroxy acids could be separated by TLC on silicic acid impregnated with boric acid. Boric acid or borates are known to form complexes more readily with *threo* glycols than with *erythro* glycols[2,3] and it is assumed that some such interaction was operative, under the conditions of chromatography, to result in the clear resolutions of dihydroxy isomers obtained.

These results were similar to those obtained by FRAHN AND MILLS by paper electrophoresis[3] and suggested the possibility that TLC on adsorbents impregnated with complexing agents might be of more general application to the resolution of certain isomeric compounds, particularly where lack of solubility in suitable electrolytes precludes the use of the electrophoretic method. It was therefore considered worthwhile to study the migrations of other polyhydroxy acids on thin-layer plates impregnated with boric acid and with other known glycol-complexing agents.

Some remarkable separations of isomeric polyhydroxy fatty acid esters were achieved and are described in this communication. The chemical and stereochemical factors which gave rise to these separations are not discussed in detail, since they have not yet been fully elucidated. These aspects of this work are currently being studied and will be reported in detail in a subsequent publication.

EXPERIMENTAL

Materials

The *erythro* and *threo* isomers of 6,7- and 9,10-dihydroxystearic acids were prepared by oxidation of the *cis* and *trans* forms of the corresponding olefinic acids with dilute alkaline permanganate[4]. The *erythro*- and *threo*-12,13-dihydroxyoleic and -stearic acids were derived from *cis*-12:13-epoxyoleic acid, isolated from *Vernonia anthelmintica* seed oil[5]. The trihydroxy- and tetrahydroxystearic acids were prepared, as described previously, from ricinoleic acid[6], from 9-hydroxyoctadec-12-enoic acid isolated from *Strophanthus* seed oils[7], and from *cis*-12:13-epoxyoleic acid[5]. Each hydroxylation reaction produced a pair of isomeric tri- or tetrahydroxy acids which were separated as their methyl esters by preparative TLC on Silica Gel G, except for the *erythro*- and

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threo-9,12,13-trihydroxystearic acid pairs which were not resolved for this work. Methyl esters were prepared by adding excess diazomethane in diethyl ether to methanol solutions of the acids.

Procedures

Thin layers (*ca.* 275 μ) of Merck "Silica Gel G"* were applied to glass plates (20 × 20 cm or 10 × 20 cm) with the Desaga equipment**, as described by Mangold[8]. Impregnation of the layers was carried out by using aqueous solutions of the relevant compounds, instead of water, to prepare the adsorbent slurry for spreading on the plates. As a standard procedure, 2.8 g of impregnating agent was dissolved in 50 ml of water and mixed with 25 g of Silica Gel G, so that uniform impregnation of 10% (w/w) was achieved. Silica gel layers were impregnated with the following compounds: boric acid, sodium borate, sodium arsenite, basic lead acetate, sodium metavanadate and sodium molybdate.

Alternatively, impregnation was achieved by spraying silica gel plates with *ca.* 20% solutions of the inorganic compounds *cf.* ref. 1. However, this latter procedure gave less uniform impregnation which resulted, in some cases, in rather different migration patterns for some groups of isomers, so that layers impregnated during preparation were preferred for all comparative work. All plates, whether impregnated or not, were activated by heating in an oven at 110° for 30 min just before use.

Samples were applied as dilute solutions in chloroform and plates were developed, under conditions of "tank saturation", in closed jars lined with solvent-soaked filter paper. Chloroform–methanol mixtures were used as developing solvents. Visualisation of separated components was achieved generally by charring at 200° after spraying with 50% aqueous sulphuric acid, or by viewing under ultraviolet light after spraying with a 0.2% ethanolic solution of 2',7'-dichlorofluorescein.

RESULTS

The chromatograms of the methyl esters of long-chain di-, tri- and tetrahydroxy acids on untreated Silica Gel G (A), boric acid-impregnated Silica Gel G (B), sodium borate-impregnated Silica Gel G (C), and sodium arsenite-impregnated Silica Gel G (D) are reproduced in Figs. 1, 2 and 3.

It was found that Silica Gel G layers impregnated with basic lead acetate, sodium metavanadate, or sodium molybdate resulted in migrations of all components almost identical to those obtained on untreated Silica Gel G.

Dihydroxy esters

On untreated Silica Gel G, there is a slight gradation in mobility of dihydroxy esters according to the position of the glycol group in the chain, the polarity sequence being 6,7- > 9,10- > 12,13-dihydroxystearate > 12,13-dihydroxyoleate (Fig. 1A). There is, however, no difference in mobility according to *erythro*- or *threo*-configuration of the glycol group.

* E. Merck, A.-G., Darmstadt, Germany; British distributor: Anderman and Co. Ltd., Tooley Street, London, S.E. 1.

** Desaga, G.m.b.H., Heidelberg, Germany; British distributor: Camlab (Glass) Ltd., Cambridge.

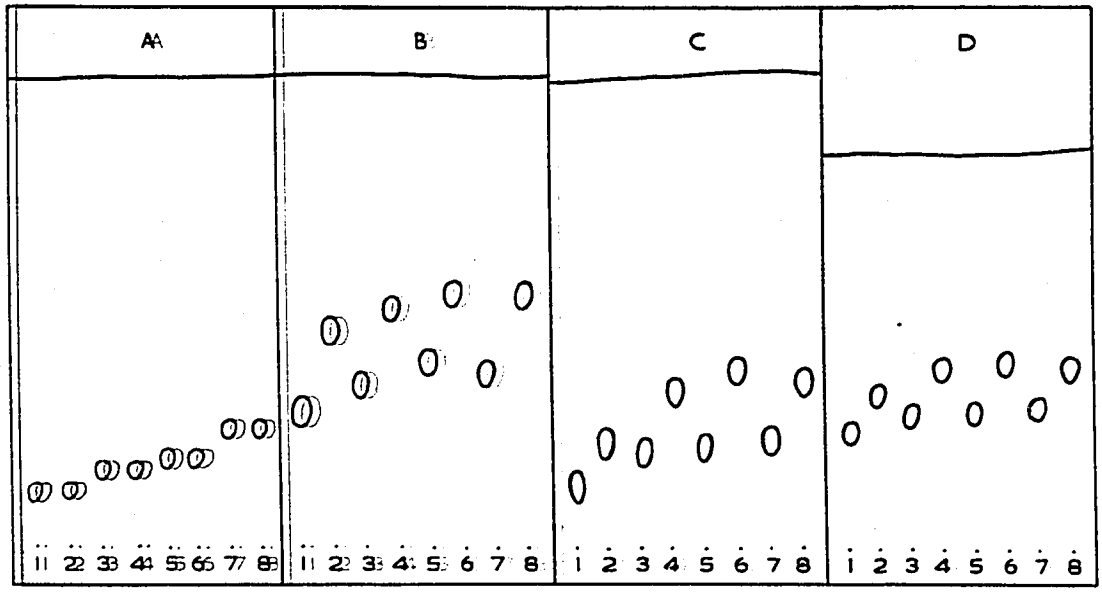

Fig. 1. Thin-layer chromatograms on Silica Gel G (A), boric acid-impregnated Silica Gel G (B), sodium borate-impregnated Silica Gel G (C), and sodium arsenite-impregnated Silica Gel G (D), of methyl esters of the following fatty acids: 1 = *erythro*-6,7-dihydroxystearic; 2 = *threo*-6,7-dihydroxystearic; 3 = *erythro*-9,10-dihydroxystearic; 4 = *threo*-9,10-dihydroxystearic; 5 = *erythro*-12,13-dihydroxystearic; 6 = *threo*-12,13-dihydroxystearic; 7 = *erythro*-12,13-dihydroxyoleic; 8 = *threo*-12,13-dihydroxyoleic. Developing solvent: methanol–chloroform (2:98). Spots were located by spraying with aqueous sulphuric acid (1:1) and charring, and were reproduced by tracing.

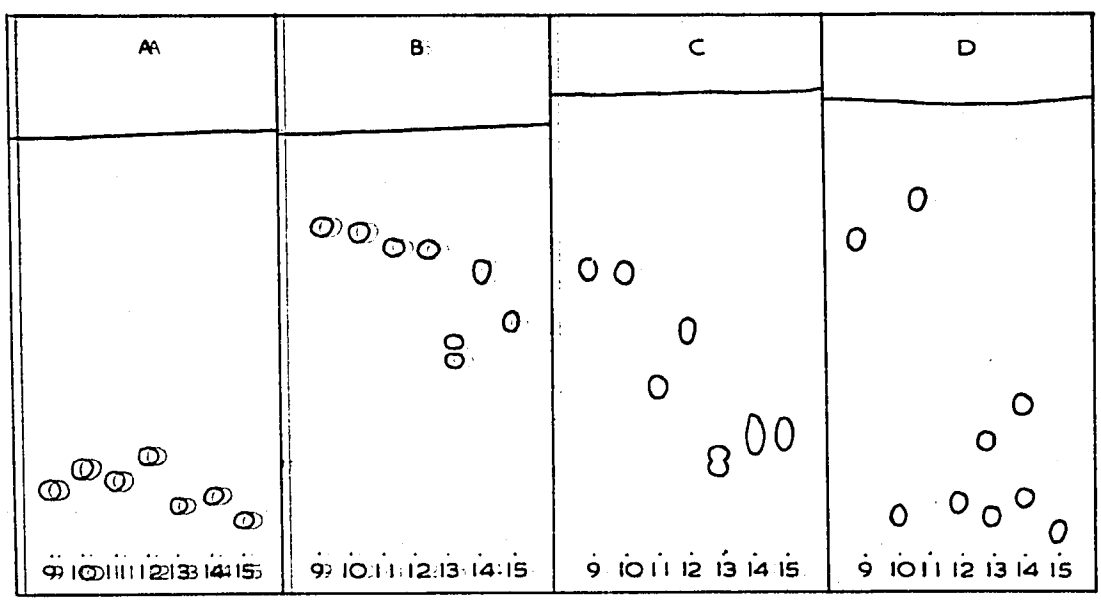

Fig. 2. Thin-layer chromatograms on Silica Gel G (A), boric acid-impregnated Silica Gel G (B), sodium borate-impregnated Silica Gel G (C), and sodium arsenite-impregnated Silica Gel G (D), of methyl esters of the following fatty acids (literature melting points of acids in parentheses): 9 = *erythro*-9,10,12-trihydroxystearic (138°); 10 = *erythro*-9,10,12-trihydroxystearic (112°); 11 = *threo*-9,10,12-trihydroxystearic (110°); 12 = *threo*-9,10,12-trihydroxystearic (87°); 13 = *erythro*-9,12,13-trihydroxystearic isomers (148° and 102°); 14 = *threo*-9,12,13-trihydroxystearic isomers (89° and ?)); 15 = *threo*-9,10,16-trihydroxypalmitic. Developing solvents: A, B and C, methanol–chloroform (5:95); D, methanol–chloroform (1:99). Spots were located by spraying with aqueous sulphuric acid (1:1) and charring, and were reproduced by tracing.

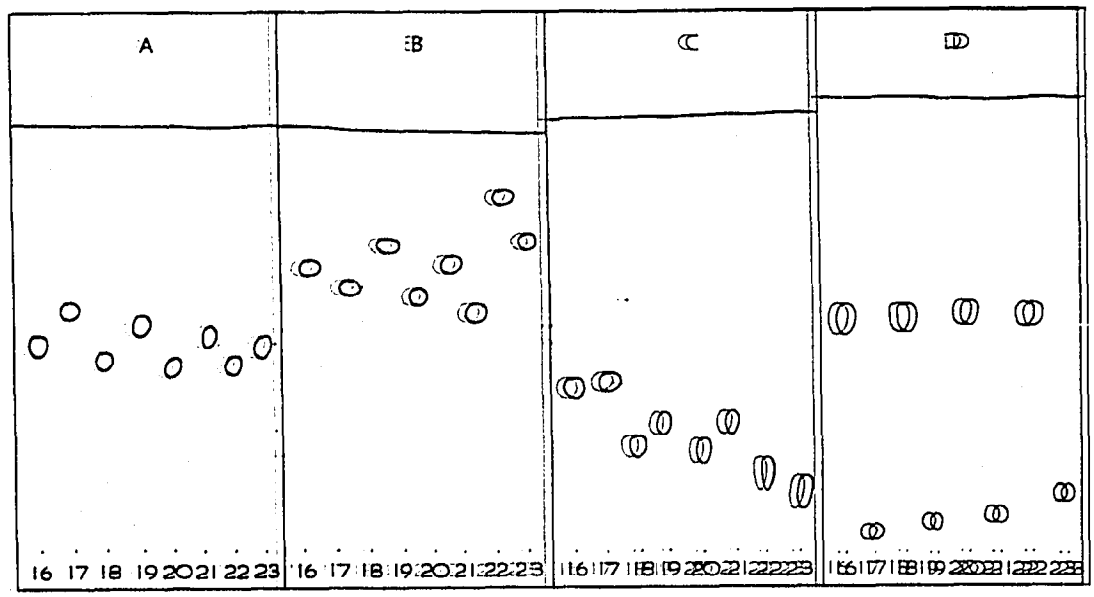

Fig. 3. Thin-layer chromatograms, on Silica Gel G (A), boric acid-impregnated Silica Gel G (B), sodium borate-impregnated Silica Gel G (C), and sodium arsenite-impregnated Silica Gel G (D), of methyl esters of the following tetrahydroxystearic acids (literature melting points of acids in parentheses): 16 = *erythro*-9,10-*erythro*-12,13- (177°); 17 = *erythro*-9,10-*erythro*-12,13- (156°); 18 = *threo*-9,10-*erythro*-12,13- (156°); 19 = *threo*-9,10-*erythro*-12,13- (136°); 20 = *erythro*-9,10-*threo*-12,13- (165°); 21 = *erythro*-9,10-*threo*-12,13- (122°); 22 = *threo*-9,10-*threo*-12,13- (148°); 23 = *threo*-9,10-*threo*-12,13- (122°). Developing solvents: A, B and C, methanol–chloroform (10:90); D, methanol–chloroform (4:96). Spots were located by spraying with aqueous sulphuric acid (1:1) and charring, and were reproduced by tracing.

On layers of Silica Gel G, impregnated with boric acid (Fig. 1B), sodium borate (1C), or sodium arsenite (1D), a clear separation of each diastereoisomeric pair is readily achieved. The lower melting *threo*-isomer of each pair has migrated faster on these impregnated layers.

Trihydroxy esters

The trihydroxy esters studied, like the dihydroxy esters, show little or no differences in rates of migration on untreated Silica Gel G that could be attributed to *erythro*- or *threo*-configuration of their glycol grouping. The 9,10,12-trihydroxystearates (9–12), however, do show a segregation of the diastereoisomers of each oxidation pair. The higher melting isomer (9 and 11), in each case, is more polar than the lower melting form (10 and 12). This resolution was utilised, in the preparation of these compounds for this work, to isolate the individual isomers from the pair of products resulting from hydroxylation. The 9,12,13-trihydroxystearate pairs (13,14) are not separable, in this way, into their high and low melting, diastereoisomeric forms. As expected, 9,10,16-trihydroxypalmitate (15) is more polar than the other trihydroxy esters studied, due to its shorter chain length and the fact that one of its hydroxyl groups is primary.

Boric acid-impregnated Silica Gel G gives a somewhat different pattern of migration of the trihydroxy esters (Fig. 2B). All compounds have migrated rather further than on untreated Silica Gel G with the same solvent system. This was also shown with the di- and tetrahydroxy esters and indicates that modification of the layer by incorporating boric acid in the adsorbent reduces its adsorbability. There is,

however, no obvious pattern of separation as a function of *threo*- or *erythro*-configuration of the glycol grouping, as was the case with the dihydroxy esters. Such an effect seems to be shown by samples 13,14 and 15, where the *threo*-9,12,13-trihydroxystearate pair and the *threo*-9,10,16-trihydroxypalmitate have migrated somewhat faster than and are clearly resolved from the *erythro*-9,12,13-trihydroxystearate pair. This last named pair of diastereoisomers (15) has been resolved into individual isomer spots although the *threo*-pair (14) has not. The 9,10,12-trihydroxystearates, however, show no similar separation according to *threo*- or *erythro*-configuration, the *threo*-compounds in fact being slightly more polar than their *erythro*-isomers. Also, the greater polarity of the high melting isomer of each pair has been lost and these compounds (9 and 11), are, if anything, slightly less polar on this medium than their lower melting isomers (10 and 12).

Sodium borate impregnation gives an even less predictable pattern with the trihydroxy esters (Fig. 2C). The *threo*-9,12,13-trihydroxystearate pair (14) and *threo*-9,10;16-trihydroxypalmitate (15) are only slightly less polar than the *erythro*-9,12,13-trihydroxy pair, which in this case also shows some resolution into individual isomers. The *threo*-9,10,12-trihydroxystearates (11 and 12), on the other hand, are held back appreciably, relative to their *erythro*-analogues (9 and 10), which is the reverse effect to that shown with the dihydroxy esters on the same adsorbent.

The separations of the trihydroxy isomers obtained on sodium arsenite-impregnated Silica Gel G (Fig. 2D) are more dramatic and potentially more useful. The higher melting isomer of each 9,10,12-trihydroxystearate pair (9 and 11) has migrated very much faster than the corresponding lower melting form (10 and 12). The *erythro*- and *threo*-9,12,13-trihydroxystearate pairs (13 and 14) have also been clearly resolved. The higher melting *erythro*-9,12,13-trihydroxy isomer was again the least polar and in the *threo*-9,12,13-trihydroxy pair the lower spot represents the ester of the acid melting at 89°, the melting point of the acid isomeric to this one is as yet undetermined[7]. In addition to these "major" separations there has also been shown some differentiation between comparable analogues (*e.g.* 9 and 11, 10 and 12) as a function of the *erythro*- or *threo*-configuration of their glycol group. The *threo*-isomer has in each case migrated slightly further than the *erythro*-isomer, as was the case with the dihydroxy esters. These two types of differences in migration are sufficient to allow complete resolution of all four diastereoisomers of 9,10,12-trihydroxystearate and of 9,12,13-trihydroxystearate on sodium arsenite-impregnated silica gel.

Tetrahydroxy esters

On untreated Silica Gel G (Fig. 3A), the 9,10,12,13-tetrahydroxystearates behave similarly to the 9,10,12-trihydroxy compounds. No differentiation has occurred on the basis of *threo*- or *erythro*-configurations of glycol groups but the higher melting isomer of each oxidation pair is somewhat more polar than the lower melting form. This difference in mobility was again utilised for the isolation of the individual isomers from these oxidation pairs, by preparative thin-layer chromatography.

A reversal of mobilities of the isomers in each oxidation pair is the result of impregnating the Silica Gel G with boric acid (Fig. 3B) and the higher melting form migrates the faster in each case. There is again no obvious separation as a result of the *erythro*- or *threo*-configurations of the glycol groups, although the di-*threo*-isomers (22 and 23) are somewhat less polar than the other six isomers.

On sodium borate-impregnated layers a further difference in migrating pattern is evident (Fig. 3C). The tetrahydroxystearates here show a pattern which could be attributed, at least in part, to the configurations of the glycol groups, but this has operated in the reverse direction to that found with the dihydroxy esters on the arsenite-impregnated layer. Thus the two di-*erythro*-compounds (16, 17) are least polar, the four compounds having one *erythro*- and one *threo*-glycol group (18–21) are more polar, and the two di-*threo*-compounds (22 and 23) are more polar still. The relative positions of the two isomers of each oxidation pair is also rather anomalous. The *threo*-*erythro* pair and the *erythro*-*threo* pair have maintained the same pattern as on untreated silica gel, with the higher melting form being more polar, but this pattern has been largely lost with the di-*erythro*-pair and has been inverted with the di-*threo*-pair.

As with the trihydroxy esters, the separation pattern of the tetrahydroxystearates on sodium arsenite-impregnated Silica Gel G (Fig. 3D) is remarkable but consistent. The higher melting isomer of each oxidation pair has migrated much faster and, with the more polar, low melting isomers at least, there is a subsidiary separation effect which could be attributed to *threo*- or *erythro*-conformations of glycol groups, di-*threo*- (23) migrating faster than *threo*-*erythro*-, or *erythro*-*threo*-, (19, 21) and these in turn migrating faster than the di-*erythro*-isomer (17).

DISCUSSION

The results described above demonstrate that the migration characteristics of polyhydroxy compounds on thin-layer chromatograms can be markedly altered by incorporating various inorganic materials in the adsorbent layer. These changes in migration characteristics are such that clear separation of several diastereoisomeric compounds, on the basis of *threo* or *erythro* configuration of glycol groups or of other stereochemical differences between isomers, has been achieved.

threo- and *erythro*-Dihydroxy isomers can be readily differentiated, on the micro scale, by TLC on Silica Gel G impregnated with boric acid, sodium borate or sodium arsenite. The separation of oxidation pairs of tri- and tetrahydroxystearates is possible, in some cases, on untreated, boric acid-impregnated or sodium borate-impregnated Silica Gel G layers and, in all cases studied, on sodium arsenite-impregnated layers. These separations may also be carried out on the preparative scale so that the tedious fractional crystallisation procedures required for separation of such isomers[5–7], may be obviated. By suitable combination of these chromatographic methods, the identity of a micro-sample of any one of the tri- or tetrahydroxy esters described in this work could be positively determined, if the whole series were available for comparison.

Insufficient is known, as yet, about the type of interaction between migrating substances and impregnated adsorbent, to enable valid conclusions to be drawn as to the relative conformations of diastereoisomers separated in this way. The use of thin-layers impregnated with glycol-complexing agents has been shown to result in changes in migration characteristics and consequent resolution of some sugars[9,10] and of some phenol-carboxylic acids (1-carboxy-3,4-dihydroxybenzene derivatives)[11]. In this last work, several impregnating agents were used and it was assumed that chelate formation occurred and was responsible for the observed changes in migration

patterns. Although this assumption may well be correct, not enough is yet known about this type of interaction on thin-layer chromatograms to allow its unqualified acceptance as the basis of detailed stereochemical interpretation of the results described in this paper. A purely physical interaction between active sites introduced into the layer by impregnation and preferred conformations of the hydroxy groups in the polyhydroxy ester isomers could conceivably result in the migration patterns achieved. In this connection, it should be noted that the resolution of the oxidation pairs of 9,10,12-tri- and 9,10,12,13-tetrahydroxystearates on untreated Silica Gel G must be due to some such purely physical interaction, since chemical chelating is clearly not possible in this case.

It is, in fact, considered that some form of chemical chelating with the impregnating agents is at least one factor, and probably the main factor, in producing the observed migration patterns. Several lines of evidence suggest that this is so, including the qualitative similarity of some the of results to those obtained in the electrophoretic system of FRAHN AND MILLS[3], where chemical chelating of glycols with borate was demonstrably the factor causing separation. Also, in the present work, it was found that sodium borate-impregnated Alumina G layers afforded a similar degree of separation, with the same solvent system, of the dihydroxy esters (1–8) as did borate-impregnated Silica Gel G, but in the reverse direction; *i.e.* the *threo*-dihydroxy esters were held back relative to the *erythro*-isomers. The only relevant difference in conditions is that the Silica Gel G layer has an acidic character whereas the Alumina G layer is basic. Thus, the observed reversal of pattern may be due either to (1) formation of boric acid esters on the acidic Silica Gel G layer and borate complexes on the basic Alumina G layer (*cf.* ref. 12), the different products then having lesser and greater polarities respectively than the unaffected *erythro*-isomers, or to (2) formation of borate complexes on both layers, which are then influenced by opposite ion-exchange effects on the acidic and basic layers. From consideration of other results, (2) seems more likely but either possiblity would indicate that chemical, rather than physical, interactions occur and are responsible for the separation of stereoisomers.

These conditions are, at present, of no great assistance in determining the relative configurations of the hydroxy groups of isomeric tri- and tetrahydroxystearates from the observed chromatographic behaviour. A detailed study of the migration characteristics of model dihydroxy compounds of known configurations, under the conditions described here and more strictly controlled conditions of pH, will be necessary before these phenomena may be understood sufficiently to be utilised for conformational analysis of more complicated molecules. This work is currently proceeding and will be reported in detail elsewhere. In the meantime, the practical utility of this method for separation of suitable diastereoisomeric polyhydroxy compounds has been amply demonstrated.

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SUMMARY

The migration characteristics, on silica gel thin-layer plates, of a series of dihydroxy, trihydroxy and tetrahydroxy long-chain fatty acid methyl esters have been examined. By incorporating various inorganic glycol-complexing agents in the adsorbent layers, considerable variations in migration characteristics were produced. Diastereoisomeric compounds were clearly resolved on the basis of *threo* or *erythro* configurations of glycol groups or of other stereochemical differences between such isomers.

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