Journal of Chromatography, 209 (1981) 49–60
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,593

REVERSED-PHASE THIN-LAYER CHROMATOGRAPHY OF DIACYLGLYCEROLS AS THEIR LABILE DIMETHYLBORATE ESTERS

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(Received October 7th, 1980)

SUMMARY

In order to obtain a diacylglycerol mixture for reversed-phase chromatography, a triacylglycerol sample from native vegetable oils and having a pre-determined fatty acid composition was used. This sample, containing equimolar amounts of palmitic, stearic, oleic, linoleic and linolenic acids, was subjected to glycerolysis, and silica gel column chromatography of the products yielded a diacylglycerol mixture composed of 20 ± 3 mole-% of each of the above fatty acid residues. The mixture was separated into individual fractions by reversed-phase chromatography on plates with a permanent inert support layer using a methanol–trimethylborate–*n*-tetradecane system. The mixture was shown to consist of seven fractions with equivalent lipophilicities of 24–36, and the distribution of the residues of the individual fatty acids among these fractions is close to random.

INTRODUCTION

Although 1,2-*sn*-diacylglycerols (1,2-*sn*-DAGs) form the basis of the structure of natural glycerolipids[1], little is known about the composition and structure of the DAG moieties of these lipids, mainly because methods for the analysis of DAGs have been insufficiently developed[2]. In our laboratory, work is being conducted on devising a method for the chromatographic separation of natural DAG mixtures, formed by the hydrolysis of polar glycerolipids, into individual species. These mixtures, however, are not suitable for developing this method because in most instances they are not readily available and have limitations with respect to their fatty acid composition[1]. Therefore, we used an artificial mixture of pre-determined composition obtained by glycerolysis. This mixture serves as an adequate substitute for a natural mixture because the pattern of DAG mobility in liquid chromatography usually does not depend on the isomeric composition of the DAG species[3].

In addition to the preparation of a DAG mixture and the development of a chromatographic method to separate it into individual fractions, an aim of this work was also the preliminary identification of the isolated fractions as regards their equivalent lipophilicity[4], which is necessary for the possible future application of these

0021-9673/81/0000–0000/$02.50

fractions as standards in the investigation of DAGs of natural origin. Finally, in connection with the possibility of such an application of a standard DAG mixture, it was of interest to establish the rules governing the distribution of fatty acid residues among the individual DAG fractions in the mixture.

EXPERIMENTAL

Materials

Triacylglycerols (TAGs) from cacao butter recrystallized from a 10% (w/v) solution in *n*-hexane and TAGs from poppy seed[5] and linseed oils[6] were purified on an alumina column[7]. As shown by thin-layer chromatography (TLC), TAGs eluted from the column were devoid of more polar components. Tripalmitoyl- and tristearoylglycerols (C grade; California Foundation for Biochemical Research, Los Angeles, CA, U.S.A.) were used without further purification. The amount of the major component in the samples of these monoacid TAGs was assumed to be 100%. The TAG sample for glycerolysis was obtained by mixing TAGs from cacao butter (2.63 g), linseed oil (3.73 g) and poppy seed oil (2.17 g), and by adding tristearoyl- (0.84 g) and tripalmitoylglycerol (0.63 g).

TAG glycerolysis and isolation of DAG mixture

To 10.00 g (11.5 mmole) of the TAG sample 1.30 g (14.1 mmole) of anhydrous glycerol and 11 mg (0.3 mmole) of sodium hydroxide as a catalyst for glycerolysis were added. The mixture was incubated in a continuous stream of argon at low pressure (*ca.* 10 mmHg) at 200°C for 4 h, dissolved in diethyl ether, washed with 20% sodium chloride solution until neutral, dried and evaporated to dryness *in vacuo*. As a result, 10.78 g of an acylglycerol sample was obtained. To determine its composition both 100 μg of the sample and 100 μg of neutral lipid standards[8] were spotted at the starting points of a Silufol plate (Kavalier, Sklárny, Czechoslovakia). After TLC separation for 15 min using *n*-hexane–diethyl ether–acetic acid (50:50:1), the spots of the lipids were rendered visible with molybdophosphoric acid[8].

A column (42 × 2 cm I.D.) containing 60 g of Woelm silica gel for adsorption chromatography instead of the Davidson Grade 923 silica gel used earlier[9] was applied to isolate the DAG mixture (1.11 g). To determine the purity of the latter, a 100-μg aliquot was separated by TLC (see above); for comparison, 0.5 μg of TAGs were applied at one of the starting points. The colour intensity of individual chromatographic zones was evaluated visually.

DAG samples originating from cacao butter and poppy seed and linseed oils were prepared and evaluated in a similar manner. The fatty acid compositions of all acylglycerol samples and mixtures were determined by gas–liquid chromatography[10].

TLC plates and mobile phase

DAGs were separated on home-made glass plates designated as "plates with a permanent support layer". To prepare such plates, 20 g of Kieselguhr (Chromaton N; Chemapol, Prague, Czechoslovakia, particle size 0.100–0.125 mm) was silanized with dimethyldichlorosilane[11], mixed with 60 g of powdered glass[8] and suspended in 90 ml of water, and the slurry obtained was applied on to 20 × 10 cm plates using an SSG-1 automatic layer applicator (VEB Technisches Glas, Ilmenau, G.D.R.); the

applicator clearance was 0.5 μm. After the layer had been dried in air at 20°C the plates were heated at 675°C for 20 min[8]. These plates could be used for separation an unlimited number of times, because they can be purified from organic impurities by heating them in mixture of concentrated sulphuric and nitric acids and developing the support layer in methanol.

As the mobile phase for DAG separation we first used a mixture of methanol and water (95:5). Subsequently, a mixture of 475 g (14.8 mole) of absolute methanol and 111 g (1.8 mole) of reagent-grade boric acid was refluxed for 3 h, cooled and kept with 154 g (1.1 mole) of sodium sulphate for 45 h to remove water formed during esterification of the acid. The supernatant liquid was decanted and fractionally distilled, the methanol–trimethylborate (TMB) azeotrope, b.p. 54.6–55.0°C, n_D^{20} 1.3478, being collected[12]. A 1:9 mixture of this azeotrope with absolute methanol, plus a small excess of pure *n*-tetradecane, was used for chromatography.

Chromatographic separation and semi-quantitative evaluation of DAG mixture

To apply the stationary phase the plates were submerged in a 10% (v/v) benzene solution of tetradecane. Separate DAG samples and a mixture of them were applied on the starting line as a band (65 × 2 mm) with a GSG-1 automatic sample applicator (VEB Technisches Glass) fixed on the carriage of the SSG-1 instrument, or by a syringe as a series of single spots. At the end portions of the band (1–2 mm long) where the carriage changed the direction of its shuttle motion, a greater amount of DAG than in the middle portion of the band could be deposited, which subsequently resulted in significant distortion of the chromatographic zones. To avoid this, these portions of the layer were covered with paper strips when the sample had been applied.

DAGs (200 μg on the starting line or 20–50 μg as single spots) were separated for 2.5 h in a chamber for ascending TLC (Type OE-304; Labor, Budapest, Hungary) at 23 ± 0.5°C. *n*-Tetradecane was removed from the plates in a vacuum drying oven (Type LP-402; Labor) for 45 min at 140°C. After development of the spots[8], the chromatogram was photographed and a positive picture was obtained from the negative. The picture was scanned under transmitted light on a home-made densitometer. The peak areas on the densitogram were determined gravimetrically. When calculating the hR_F (R_F × 100) values the maximum of the peak on the densitogram was assumed to be the centre of the corresponding zone.

RESULTS

Composition of the mixed TAG sample for glycerolysis

A DAG mixture in which molar contents of individual DAG species are as similar to each other as possible is desirable as a chromatographic standard. As can be calculated[13], this condition is most readily met when each fatty acid in the starting TAG sample is present in equal molar concentrations. On the other hand, as this standard was designed primarily for investigating common plant lipids, it had to be similar to them in its qualitative fatty acid composition. It is known that the individual aliphatic acids present in these lipids are very numerous, but in almost all instances residues of the five major acids palmitic, stearic, oleic, linoleic and linolenic acid are present. Therefore, we decided to limit ourselves to the preparation of a DAG mixture containing only these five acids.

Thus, the optimal starting material for glycerolysis in our work would be an equimolar mixture containing 20% of each of the monoacid TAGs of the five major acids. However, reagent-grade samples of such TAGs, especially unsaturated ones, are often unavailable, so we decided to prepare a TAG sample from the total TAGs of common, readily available vegetable oils; these TAGs could be easily isolated from the oils by adsorption chromatography. Cacao butter rich in saturated and oleic acids, poppy seed oil with predominantly linoleic acid and linseed oil as a source of linolenic acid were used (Table I). It can be seen that the oleic content in the cacao butter sample was about 35% and in two other oils it was close to 20%, a pre-determined value for the whole sample. Therefore, to attain this value in the sample it was necessary to include additionally small amounts of the simple saturated TAGs tripalmitoyl- and tristearoylglycerol.

TABLE I
FATTY ACID COMPOSITION OF ACYLGLYCEROLS (MOLE-%)

Fatty acid	*TAGs from the fat of*			*TAG sample before glycerolysis*	*Acylglycerol sample*	*DAG mixture*
	Cacao	*Poppy seed*	*Linseed*			
Palmitic	27.1	9.3	5.7	17.2	17.4	19.7
Stearic	35.8	4.4	3.8	17.6	18.9	18.4
Oleic	34.9	16.3	19.6	21.6	20.6	19.8
Linoleic	2.2	70.0	11.6	23.3	23.5	22.9
Linolenic	0.0	0.0	59.3	20.3	19.6	19.2

For direct preparation of the sample (total weight 10.00 g) it was necessary to determine the weights (g) of TAGs of cacao butter, poppy seed and linseed oils, and of tripalmitoyl- and tristearoylglycerol (X, Y, Z, V and W, respectively), which would ensure the pre-determined fatty acid composition. X g of cacao TAGs contain

$$X' = X/\bar{M}_x \tag{1}$$

mole of TAGs, where $\bar{M}_x = \sum_i [(a_i)_x M_i] \cdot 10^{-2}$ = average molecular weight of cacao butter TAGs, $(a_i)_x$ (mole-%) = content of the ith acid (Table I) and M_i = molecular weight of the monoacid TAG of the ith acid[6]; values of $Y' = Y/\bar{M}_y, \ldots, W' = W/\bar{M}_w$ (mole) are expressed similarly.

Using these values, one can calculate, as a dimensionless quantity, the molar proportion of cacao TAGs in the sample:

$$x = X'/(X' + \ldots + W') \tag{2}$$

The values of y, z, v and w are expressed similarly ($x + y + z + v + w = 1$).

The pre-determined content of stearic acid (20%) in the TAG sample to be prepared is made up of the amounts of this acid in TAGs from each of the oils:

$$35.8x + 4.4y + 3.8z + 100w = 20 \tag{3}$$

Similarly, for oleic and linoleic acids:

$$34.9x + 16.3y + 19.6z = 20 \quad (4)$$

and

$$2.2x + 70.0y + 11.6z = 20 \quad (5)$$

Preliminary experiments showed that the content of palmitic acid in the DAG mixture obtained by glycerolysis is higher and that of linolenic acid is lower than the contents in the starting TAG sample. Therefore, we decided to mix TAGs so that the calculated amounts of these acids in the starting sample were 18 and 22%, respectively:

$$27.1x + 9.3y + 5.7z + 100v = 18 \quad (6)$$

and

$$59.3z = 22 \quad (7)$$

From eqns. 3–7, $x = 0.263$, $y = 0.216$, $z = 0.371$, $v = 0.068$ and $w = 0.082$. By substituting the values of $x, \ldots, w$ into eqns. 1 and 2 for X and X', and into similar equations for $Y, \ldots, W$ and $Y', \ldots, W'$, and by combining this equation system with the expression $X + Y + Z + V + W = 10$ g, one can solve it and obtain the TAG weights: $X = 2.63$ g, $Y = 2.17$ g, $Z = 3.73$ g, $V = 0.63$ g and $W = 0.84$ g.

The fatty acid composition of the starting TAG sample is shown in Table I; it is close to the pre-determined value.

Preparation of the DAG mixture

As suggested by the preliminary data available[3], individual DAG species, on reversed-phase chromatography and on liquid chromatography in the presence of complex-forming agents, are not separated into positional isomers [1,2(2,3)- and 1,3-diacyl-*sn*-glycerols] of the same fatty acid composition[1]. Hence, any reaction that gives rise to all of these isomers or to only some of them generally can be used to prepare model DAGs for reversed-phase chromatography, because individual DAG species of the mixture will have the same mobility as corresponding 1,2-diacyl-*sn*-glycerols of natural origin.

In this work we obtained the DAG mixture by glycerolysis, which is largely free from limitations inherent in other methods of DAG synthesis on the micro-scale[13,14]. In an anhydrous medium the glycerolysis reaction, *i.e.*, transesterification of TAGs with glycerol in the presence of a catalyst[15–17], involves TAG deacylation and continuous acyl migration between TAGs, DAGs, monoacylglycerols (MAGs) and glycerol; as a result, dynamic equilibrium between all of these compounds is established. As can be seen from the Table I, there is little, if any, difference between the overall acylglycerol sample formed by TAG glycerolysis and the original TAGs as regards their fatty acid composition. The TLC data (not shown) suggest that DAGs comprise almost half of the sample, whereas free fatty acids which would interfere with the isolation of the DAG mixture are virtually absent.

The isolation itself was carried out using a silica gel column, and the degree of purity of the mixture obtained was determined by TLC. The DAG content in the final mixture was >99.5%, and the yield was about 50%. The fatty acid composition of DAGs is shown in the last column in Table I. It can be seen that the actual concentrations of individual acids are around 20 ± 3 mole-%.

Reversed-phase TLC of the DAG mixture

DAGs were separated chromatographically in a reversed-phase system[18]. Instead of paper chromatography[19], TLC was used because of its greater separation efficiency. However, under the usual conditions the solid support containing a hydrophobic stationary phase is poorly retained on the surface of a glass plate and is easily washed off by the mobile phase[20]. Therefore, plates with a permanent inert support (Kieselguhr) layer were used for TLC. These plates were prepared by the method developed previously for adsorption plates[8].

As aqueous water-soluble organic solvent–hydrophobic phase systems have been used successfully for separating free fatty acids[4], TAGs[15], MAGs[21] and other neutral lipids, we carried out experiments on DAG fractionation in these systems. The hydrophobic phases tested included higher hydrocarbons, long-chain esters and ethers and aliphatic methyl ketones, and the hydrophilic components used included acetic acid, methanol and acetone. In no instance, however, was a satisfactory DAG separation achieved. As an example, Fig. 1 shows the results of the analysis of a standard DAG mixture in the aqueous methanol–*n*-tetradecane system.

We then considered the adsorption chromatography of hydroxy lipids. In some of these studies, to increase the selectivity and quality of the separation of hydroxy fatty acids[22], and positional isomers of MAGs and DAGs[23,24], other workers used adsorbents impregnated with boric acid, which is known[25] to form labile complexes with free hydroxyl groups. It could be assumed that in a reversed-phase system the

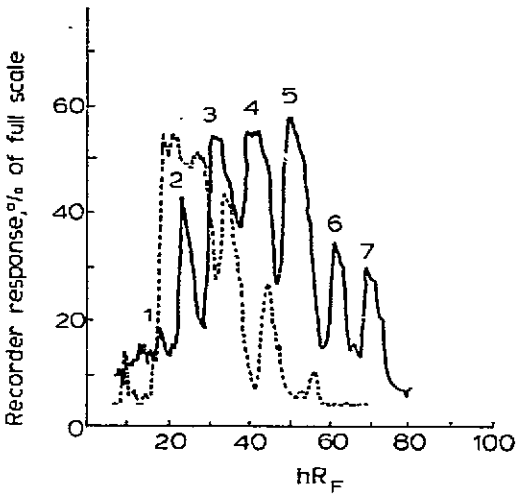


Fig. 1. Densitograms of reversed-phase chromatograms of diacylglycerols. Mobile phases: methanol–water (95:5) saturated with *n*-tetradecane (broken line) and methanol–trimethylborate (92.7:7.3) saturated with *n*-tetradecane (solid line).

addition of this acid or its derivatives to the mobile phase would also result in an improvement of the chromatographic parameters, in a similar manner to the results with Ag^+ ions, which initially were applied only for the adsorption chromatography of neutral lipids[2] but were later used in their reversed-phase chromatography[26].

To verify this assumption, experiments on DAG separation were conducted in a system containing methanolic solutions of either boric acid (5%) or an equimolar amount of TMB as the mobile phase. Under these conditions, a highly selective and efficient separation of DAGs into seven fractions was achieved, and when the TMB system devoid of free water was used the chromatographic mobility of separate DAG fractions, especially the fully saturated ones, increased even further (Fig. 1). Therefore, only the latter system was used in subsequent work. The results of the semi-quantitative densitometric determination of the concentration of each of these fractions in the DAG mixture are presented in Table II.

TABLE II
DENSITOMETRIC ANALYSIS OF THE CONTENT OF SEPARATE FRACTIONS IN A MIXTURE OF DIACYLGLYCEROLS

*Fraction No.**	hR_F	*Content (wt.-%)*
1	20	2– 6
2	28	9–11
3	36	14–21
4	44	23–27
5	52	21–28
6	60	8–14
7	68	7–12

* DAG peak numbers in Fig. 1.

Tentative identification of DAG fractions

For the tentative identification of the isolated DAG fractions (Fig. 1), it is first necessary to establish the distribution pattern of DAGs in a reversed-phase chromatographic system of moderate efficiency. It can be assumed that DAGs, being neutral lipids, do not differ in this respect from other lipids of this kind, such as TAGs and fatty acids. The distribution of these lipids is known to be determined by their theoretical equivalent lipophilicity, $L_1 = m - 2e$, where m and e are the number of carbon atoms and the number of double bonds, respectively, in the aliphatic chains of a given molecule[27]. As a result, neutral lipids belonging to the same class and with identical L_1 values also have identical hR_F values in a reversed-phase system.

Identification of the fractions could also be based on the assumption that the composition of DAGs produced in the reaction of the glycerolysis of oils is "random", *i.e.*, it reflects the random distribution of fatty acid residues among the individual DAG species. In order to establish the extent to which the data in Fig. 1 and Table II correspond to this composition, it is necessary, proceeding from the number (n) and the concentration (a, b, ... %) of the individual fatty acid residues in the DAG mixture (Table I), to calculate the random number of DAG species (N) and the random concentration of each species (A_1, A_2, ... %), and then to compare the calculated results with the experimental ones (Table II).

To derive the equations for the calculation of N, and also of A_1, A_2, ..., we modified the available probability equations for TAGs[15]. It is clear that N is made up of the numbers of monoacid (n) and diacid [$n(n-1)/2$] species of DAGs, *i.e.*, $N = n(n+1)/2$; in our mixture, where $n = 5$, $N = 15$. In a similar way, for each monoacid DAG species $A_1 = a^2/100$, and for the diacid species $A_2 = 2ab/100$; the factor of 2 has been introduced into this equation to take into account both positional isomers of DAGs. On considering the random composition of DAG species calculated from the qualitative composition and the quantitative content of fatty acid residues in their mixture (Table I) and presented in Table III, one can see that in the number of fractions of definite lipophilicity the DAG mixture isolated here (Table II) conforms to the random composition, and the quantitative content of each of these fractions is also sufficiently close to random (± 1–10%).

TABLE III
RANDOM SPECIES COMPOSITION OF A MIXTURE OF DIACYLGLYCEROLS

Species and fractions of DAGs	*m**	*e**	L_1	*Random content of DAGs (wt.-%)***	
				Species	*Fractions*
Distearoylglycerol	36	0	36	3.6	3.6
Stearoylpalmitoylglycerol	34	0	34	6.9	14.5
Stearoyloleoylglycerol	36	1		7.6	
Dipalmitoylglycerol	32	0	32	3.3	23.6
Palmitoyloleoylglycerol	34	1		7.4	
Stearoyllinoleoylglycerol	36	2		8.8	
Dioleoylglycerol	36	2		4.1	
Palmitoyllinoleoylglycerol	34	2	30	8.5	25.2
Stearoyllinolenoylglycerol	36	3		7.3	
Oleoyllinoleoylglycerol	36	3		9.4	
Palmitoyllinolenoylglycerol	34	3	28	7.1	20.3
Oleoyllinolenoylglycerol	36	4		7.8	
Dilinoleoylglycerol	36	4		5.4	
Linoleoyllinolenoylglycerol	36	5	26	9.0	9.0
Dilinolenoylglycerol	36	6	24	3.8	3.8

* m and e are the number of carbon atoms and number of double bonds, respectively, in the aliphatic chains of DAG molecules.

** Random contents of DAGs are expressed in weight-% and calculated in accordance with the theory of random distribution from the fatty acid composition of the model DAG mixture (Table I).

For an even more accurate identification of separate fractions in the standard DAG mixture by the L_1 value, the composition of this mixture was compared with those of DAG samples isolated from the products of glycerolysis of cacao butter and poppy seed and linseed oils, which differ greatly in their fatty acid compositions (Table I). To carry out this comparison each of the three samples, and also the DAG mixture, were applied on the same TLC plate, separated, detected with molybdophos-

phoric acid and quantified by densitometry. The found and random contents of separate DAG fractions in the samples are shown in Table IV. The number of fractions found in each sample (four for cacao and poppyseed, six for linseed) and in the mixture (seven) depends on the number of individual acids in the corresponding original oil (four, four and five, respectively; Table I). As in the case of a standard DAG mixture, the number of separate fractions conforms in all instances to the random composition and their content is close to that calculated using the equations of random distribution.

TABLE IV
FOUND AND RANDOM CONTENTS OF SEPARATE FRACTIONS IN DIACYLGLYCEROL SAMPLES OBTAINED BY GLYCEROLYSIS OF CACAO BUTTER AND POPPYSEED AND LINSEED OILS (WT.-%)
Found values, obtained by densitometry; random values, calculated in accordance with the theory of random distribution from the fatty acid composition of the corresponding fats (Table I) expressed in weight-%.

hR_F	*Cacao*		*Poppyseed*		*Linseed*		*Identification of fractions by* L_1
	Found	*Random*	*Found*	*Random*	*Found*	*Random*	
20	1– 3	9.0	–	0.2	–	0.2	36
28	16–33	39.6	2– 3	2.3	0– 3	2.2	34
36	52–55	45.8	14–18	12.6	9–18	6.7	32
44	14–25	5.4	34–37	35.3	13–21	11.0	30
52	–	0.2	45–47	49.6	23–27	29.9	28
60	–	0.0	–	0.0	13–18	13.4	26
68	–	0.0	–	0.0	27–42	36.6	24

As can be seen from the chromatogram in Fig. 2, this mixture has almost the same hR_F values of the corresponding DAG fractions as the DAG samples. With decreasing lipophilicity of the fractions these values increase, and relationship between hR_F and L_1 in Table IV is linear: $hR_F = 164 - 4\,L_1$. This equation, reflecting the polarity of the mobile phase in a TMB chromatographic system, can be used in the L_1 range 16–41. The final results of the identification of the fractions, characterized by certain hR_F values, by their lipophilicities are given in Table IV.

Thus, the standard mixture of DAGs includes seven separate fractions having hR_F values of 20, 28, 36, 44, 52, 60 and 68 and L_1 values of 36, 34, 32, 30, 28, 26 and 24, respectively. The distribution of the residues of individual fatty acids among these fractions appears to be close to random.

DISCUSSION

The glycerolysis conditions specified under Experimental appear to be optimal because the DAG yield was higher than in most instances reported by other workers[28,29]. At lower temperatures transesterification would be markedly slower[16], whereas higher temperatures would decrease the yield of reaction products owing to their thermal degradation, polymerization and removal of glycerol by distillation[30]. At the chosen temperature the highest glycerolysis rate is attained on adding 0.1–0.2% of sodium hydroxide to the reaction mixture[31]; sodium hydroxide is also ad-

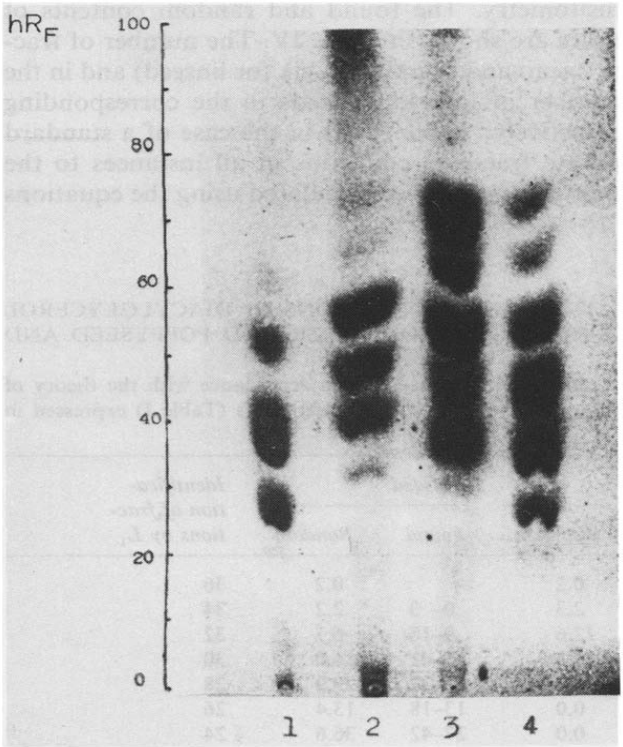


Fig. 2. TLC of diacylglycerols in the reversed-phase system. Mobile phase: methanol–trimethylborate saturated with *n*-tetradecane (Fig. 1). 1–3 = DAG samples (20 μg each) from cacao butter and poppy seed and linseed oils, respectively; 4 = DAG mixture (50 μg).

vantageous as a catalyst because it lacks marked fatty acid specificity in the transesterification reaction[16]. The highest DAG content in the products of this reaction (>40 mole-%) was obtained when the TAG:glycerol molar ratio was 7–17[17], compared with 7.7 in our sample. We also reproduced the gas-phase conditions of glycerolysis, *i.e.*, a slight vacuum[31] and an inert atmosphere[16].

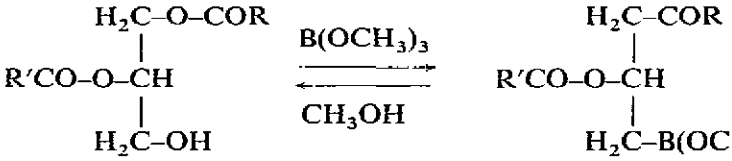
If natural lipids having qualitative and quantitative fatty acid compositions other than those used here were to be employed as starting materials for this reaction, then, using the scheme for TAG sample preparation suggested above, it would be possible to obtain acylglycerol mixtures with a virtually unlimited range of fatty acid compositions. Such mixtures could be useful as standards for reversed-phase chromatography, and also for creating various desirable fatty compositions for biochemical, chemical and technological lipid studies.

Our use of reversed-phase chromatography for the fractionation of DAG mixtures is uncommon, because in recent years DAGs have been separated into individual components almost exclusively by the GLC of their acetyl, trimethylsilyl and other derivatives, and by adsorption TLC of these derivatives with silver ions[1]. However, these methods involve a number of difficulties, especially the need to synthesize the derivatives. The yields of these derivatives, especially when obtained on the micro-

scale, have usually not been cited, and the derivatives themselves often appear to be insufficiently stable[32]. Moreover, when performing such syntheses there is inevitably migration of acyl residues in DAGs[1]. Finally, the selectivity of the chromatographic fractionation of DAG derivatives by the methods used in the past is limited: in GLC effective separations and quantitative determinations can be accomplished only for compounds with different chain lengths, and in Ag^+ TLC only for derivatives with different numbers of double bonds[2].

Reversed-phase chromatography has been used for a long time to determine the species composition of many classes of neutral lipids, primarily TAGs[2], as it is characterized by mild fractionation conditions and a high efficiency and selectivity of separation. At the same time, this method has been applied to the analysis of DAG composition only in the early period of the development of reversed-phase chromatography[19,33] and, therefore, it was of interest to test it again for this purpose, taking into account recent achievements in this type of chromatography.

Our results show that the reversed-phase fractionation of DAGs became possible only when TMB was used, which resulted in the substitution of the free hydroxyl group due to the formation of dimethylborate (DMB) esters of DAGs in this system:



Previously DMB esters of MAGs were identified as the products of the reaction between acetonides of monoacylglycerols and boric acid[25]. DMB esters of DAGs were formed upon detritylation of trityl-DAG in the presence of boric acid[32] and upon adsorption chromatographic separation of positional isomers of DAGs in a solvent system containing TMB[24]; in both instances the formation of DMB esters effectively inhibited acyl migration in DAGs.

An important advantage of DMB esters is that in the presence of water they are very rapidly hydrolysed, yielding initial acylglycerols[25], whereas acetates and other DAG derivatives (see above) cannot be used for recovering native DAGs. Another advantage is that the chromatographic fractionation of DMB esters is not accompanied by the additional separation of the zones of individual DAGs into positional isomers (Fig. 1), although these isomers are known to be present in the DAG mixture under study because it was obtained by glycerolysis of TAGs. Hence the use of these esters makes it possible to perform with equal success the analysis of any DAG mixture, irrespective of its isomeric composition, without preliminary isolation of individual isomers.

The separation of the mixture of the DAGs as their DMB esters by reversed-phase chromatography showed that the concentrations of the separate fractions in this mixture are close to those calculated according to the random distribution pattern. Thus, such a distribution of fatty acyls is established not only in TAGs formed by glycerolysis[13] but also in other products of this reaction, especially in DAGs. This pattern of DAG composition is also supported by the similarity between DAG mixtures and the original TAGs with respect to their fatty acid composition; if DAG formation proceeded in accordance with a selective mechanism this similarity would not exist. It was previously shown that under the chosen conditions of glycerolysis the reaction does not reach the equilibrium state and, as a result, the DAG concentration

(mole-% of the total acylglycerols) is considerably higher than the random concentration[34]. Consequently, during glycerolysis of TAGs a random distribution of acyl residues first appears inside the DAG class and only later between the other classes of acylglycerols.

This kind of distribution in the artificial DAG mixture will serve as an important advantage in the use of this mixture as a chromatographic standard because it will ensure the comparison of 1,2-*sn*-DAGs of natural origin with the widest range of DAG species, *i.e.*, with all the DAG species possible for a given fatty acid composition in a mixture.

CONCLUSION

The proposed method of reversed-phase chromatography of the DMB derivatives of DAGs in the methanol–trimethylborate–*n*-tetradecane system appears to be suitable for the analysis not only of model DAG mixtures but also of DAG samples obtained by the enzymatic hydrolysis of polar glycerolipids, and the standard mixture can be used as a standard for the chromatographic identification of DAGs with L_1 = 24–36 in terms of their lipophilicities and for the three DAG species with L_1 = 24,26 and 36 according to their structures.

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