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Enantiomer separation by chiral-phase liquid chromatography of urethane derivatives of natural diacylglycerols previously fractionated by reversed-phase liquid chromatography

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ABSTRACT

Enantiomers of diacylglycerols such as 3,5-dinitrophenyl isocyanate (urethane) derivatives previously fractionated by reversed-phase high-performance liquid chromatography (HPLC) were separated by HPLC on a chiral phase column [N-(*R*)-1-(α-naphthyl)ethylamino-carbonyl-(*S*)-valine chemically bound to γ-aminopropylsilanized silica]. In addition to the separation of commercial monoacid–diacylglycerol isomers, separation of diacid–diacylglycerol isomers obtained from peanut oil and cottonseed oil triacylglycerols by chemical hydrolysis is reported. Hexane–ethylene dichloride–ethanol mixtures were used for elution of the diacylglycerol derivative isomers, which were detected by their refractive indices or their UV absorption. The *sn*-1,2- and *sn*-2,3-isomers of a racemic mixture were well separated whereas the *sn*-1,3- and *sn*-1,2-isomers were eluted together. In complex mixtures the elution order varies as a function of chain length and unsaturation of the constituent fatty acids, additionally to their positioning. The enantiomer compositions of mixtures calculated from peak areas are similar in both types of detection and are assumed to be representative. The method can be applied to compositional analysis of diacylglycerol optical isomers during studies of the stereospecific distribution of fatty acids in natural triacylglycerols.

INTRODUCTION

In the stereospecific analysis of triacylglycerols, diacylglycerol stereoisomers, especially *sn*-1,2- and *sn*-2,3-diacylglycerols, have to be separately studied for fatty acid distribution [1]. In Brockerhoff's method, the two stereoisomers are differentiated by the stereospecific action of the snake venon phospholipase on synthetic phospholipids derived from the *sn*-1,2(2,3)-diacylglycerol mixture to be studied [2,3]. The method is time consuming and to simplify it several workers have worked out chromatographic separations of enantiomers.

Michelsen and Odham [4] explored the possibility of using capillary gas chromatography (GC) coupled to mass spectrometry to separate (and identify) short-chain diacylglycerol diastereoisomers [*sn*-1,2(2,3)-diethanoyl-, -dibutylroyl- and -didecanoylglycerols]. They observed that the diethanoylglycerol enantiomers were almost completely separated, but the separation rapidly decreased with increasing

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chain length. They then used high-performance liquid chromatography (HPLC) [5] and partially separated *sn*-1,2(2,3)-dilauroyl, -dimyristoyl-, -dipalmitoyl- and -distearoylglycerols as derivatives with 1-(1-naphtyl)ethyl carbamate.

Recently, Laakso and Christie [6] succeeded in separating diacylglycerols as chiral derivatives using (*R*)- or (*S*)-1-(1-naphthyl)ethyl isocyanate by HPLC on a silica column. Separations were of very good quality but with relatively high retention times as a result of the column length. On the other hand, Ôi and Kitahara [7] developed different chiral phases for HPLC that showed characteristic enantioselectivity toward derivatized amino acids, carboxylic acids, alcools and other compounds. On a phase with the chiral selector N-(*S*)-2-(4-chlorophenyl)isovaleroyl-D-phenylglycine (Sumipax OA-2100 from Sumitomo, Osaka, Japan), Itabashi and Takagi [8] succeeded in separating dialkyl- and diacylglycerol enantiomers as 3,5-dinitrophenylurethane (DNPU) derivatives but with retention times of several hours. Another phase with the chiral selector N-(*R*)-1-(α-naphthyl)ethylaminocarbonyl-(*S*)-valine (Sumipax OA-4100 from Sumitomo) appeared more convenient [9], showing the same good separations as above but with much shorter retention times.

These previous studies described enantiomer separations of monoacid–diacylglycerols. On the same last chiral phase, Takagi and Suzuki [10] presented separations of synthetic saturated and unsaturated diacid–diacylglycerols, those more likely to be found in stereospecific analysis of natural triacylglycerols. For this purpose, however, extremely long elution times (several hours) were required. Recently, Itabashi *et al.* [11] resolved enantiomeric diacylglycerols derived from natural sources with very short elution times on an (*R*)-1-(1-naphthyl)ethylamine column. Additionally, after collection of the enantiomers and their GC analysis as trimethylsilyl ether derivatives, Itabashi *et al.* [12] deduced the composition of enantiomeric diacylglycerols issued from triacylglycerols of three oils. In this work the diacylglycerols were tentatively identified by their equivalent carbon number (*ECN*). However, in spite of the narrow range of component fatty acids of these oils, the enantiomer mixtures were still too complex to allow the complete composition of molecular species of the oil triacylglycerols to be calculated.

To obtain much simpler mixtures of enantiomeric diacylglycerols, we first isolated pure triacylglycerols from peanut oil by combined argentation thin-layer chromatography (TLC) and reversed-phase HPLC. The diacylglycerols formed by partial chemical deacylation of these triacylglycerols were fractionated as DNPU derivatives by reversed-phase HPLC [13]. This paper describes the HPLC resolution of the previously isolated enantiomeric *sn*-1,2(2,3)-diacylglycerol derivatives on an N-(*R*)-1-(α-naphthyl)ethylaminocarbonyl-(*S*)-valine phase. The results show that after previous fractionation by reversed-phase HPLC, enantiomers of diacylglycerols derived by Grignard degradation of individual peanut oil triacylglycerols can be readily identified and determined.

EXPERIMENTAL

Samples

The diacylglycerol samples used in this work were those reported in a previous study [13] dealing with their analysis by reversed-phase HPLC. They originated from two sources:

(i) Synthetic diacylglycerols were of commercial origin. They consisted of *rac*-1,2- and *sn*-1,3-dioleoylglycerols (18:1 18:1) from Serdary Research Labs. (London, Ontario, Canada); optically active *sn*-1,2-dioleoyl- and dipalmitoylglycerols (16:0 16:0) from Sigma (St. Louis, MO, USA). Prior to use the commercial *rac*-1,2-(18:1 18:1), partly isomerized, was separated into *rac*-1,2- and *sn*-1,3-(18:1 18:1), separately recovered, by TLC on borate-impregnated silica gel plates [14].

(ii) Natural-source diacylglycerols were prepared by Grignard deacylation [15] of triacylglycerols isolated from peanut and cottonseed oils by combined argentation TLC–reversed-phase HPLC [16]. These included palmitoyldioleoylglycerol (16:0 18:1 18:1), trioleoylglycerol (18:1 18:1 18:1), palmitoyloleoyllinoleoylglycerol (16:0 18:1 18:2), dioleoylinoleoylglycerol (18:1 18:1 18:2) and oleoyldilinoleoylglycerol (18:1 18:2 18:2) from peanut oil. The triacylglycerol 16:0 18:1 18:2 was also isolated from cottonseed oil for comparison. The *sn*-1,2(2,3)- and *sn*-1,3-diacylglycerols formed by hydrolysis were separated from the degradation products on borate-impregnated silica TLC [14].

Diacylglycerol derivatization

The commercial and natural-source diacylglycerols were analysed by reversed-phase and chiral-phase HPLC as urethane derivatives prepared as reported previously [13]. The acylglycerols reacted with 3,5-dinitrophenyl isocyanate (Sumitomo) in dry toluene in the presence of pyridine. The diacylglycerol derivatives were isolated from the reaction mixture either by TLC or by reversed-phase HPLC in a combined purification–fractionation procedure [13].

High-performance liquid chromatography

HPLC was used both to fractionate the diacylglycerol derivatives according to chain length, unsaturation and partly according to isomerism (reversed-phase HPLC) and to analyse the fractionated diacylglycerol derivatives according to positional isomerism (chiral-phase HPLC).

In both instances analyses were carried out using a Model 6000 A solvent-delivery system (Waters Assoc., Milford, MA, USA) connected either to a Model R 401 differential refractometer (Waters Assoc.) or to a Model 450 variable-wavelength UV detector (Waters Assoc.). The column and the conditions used in the reversed-phase HPLC analyses were as reported previously [13]. In the chiral-phase analyses, the column used was a Sumipax OA-4100 purchased from Sumitomo. The 250 mm × 4 mm I.D. stainless-steel column was packed with 5-μm particles of N-(*R*)-1-(α-naphthyl)ethylaminocarbonyl-(*S*)-valine chemically bound to γ-aminopropylsilanized silica. A LiChroCART 4-4 guard column filled with LiChrosorb Si 60 (Merck, Darmstadt, Germany) was attached to the column inlet.

The analyses were carried out isocratically at a constant flow-rate of either 0.9, 1.0 or 1.2 ml min^{-1} according to samples, at ambient temperature. The mobile phase was hexane–ethylene dichloride (or dichloromethane)–ethanol (80:20:1, v/v/v). Hexane of analytical-reagent grade was from SDS (Peypin, France). Ethylene dichloride (HPLC grade) was purchased from Fluka (Buchs, Switzerland). Dichloromethane (analytical-reagent grade) was obtained from Prolabo (Paris, France) and absolute ethanol from Carlo Erba (Rueil Malmaison, France). Solvents were filtered through a Millipore membrane (0.5 μm pore size) and the mobile phase mixtures were vacuum degassed for 2 min prior to use.

For quantitative determinations, peak areas were measured by means of an Enica 21 integrator–calculator (Delsi Instruments, Suresnes, France).

Gas chromatography

The fatty acid compositions of the diacylglycerols recovered from hydrolysis products and of the diacylglycerol derivatives fractionated by chiral-phase HPLC were determined by capillary GC of the methyl esters, as described previously [17]. The analyses were carried out on a Becker-Packard (Rungis, France) Model 417 gas chromatograph equipped with a laboratory-made 30 m × 0.4 mm I.D. glass capillary column, coated with Carbowax 20M (Applied Science Labs., State College, PA, USA), at a constant temperature of 195°C and a nitrogen flow-rate of 3 ml min^{-1}. The apparatus was fitted with a Ros injector [18] (Spiral, Dijon, France) and a flame-ionization detector (Becker-Packard). Peak areas were measured by means of an Enica 21 integrator–calculator (Delsi). Calibration factors for quantitative determinations were calculated using standard mixtures of fatty acids (Nu Chek Prep, Elysian, MN, USA).

Definitions

Different parameters were determined to characterize the chromatographic diacylglycerol separations. The partition number (*PN*) [1] or equivalent carbon number (*ECN*) [11] of the diacylglycerol fractions was calculated from the total acyl carbon number (*CN*) and the total number of double bonds (*DB*) of the two constituent fatty acids, according to the equation

$$PN = ECN = CN - 2\,DB$$

Peaks were characterized by their retention times, t_R (min), corrected from the column void volume and by their retention volumes, V_R (ml).

The separation between two peaks, 1 and 2 (in that elution order), was characterized by the separation factor, α [19], *i.e.* the ratio of their corrected retention times, t_{R2}/t_{R1}.

The resolution between two peaks, 1 and 2, was characterized by the resolution factor R_s calculated from the retention times t_R and the peak widths at the baseline (*W*) according to the equation [19]

$$R_s = 2\,(t_{R2} - t_{R1}) / (W_2 + W_1)$$

From $R_s = 1$ two peaks are reasonably well separated.

RESULTS AND DISCUSSION

Qualitative analysis

Fig. 1 shows four chromatograms registered during the analysis of dioleoylglycerol urethane derivatives by chiral-phase HPLC. The first (A) refers to a mixture of the three *sn*-1,2-, *sn*-2,3- and *sn*-1,3-dioleoylglycerol stereoisomers isolated after chemical deacylation of peanut oil trioleoylglycerol. Chromatograms B and D were obtained with the mixture of *sn*-1,2(2,3)- and *sn*-1,3-dioleoylglycerols, respectively,

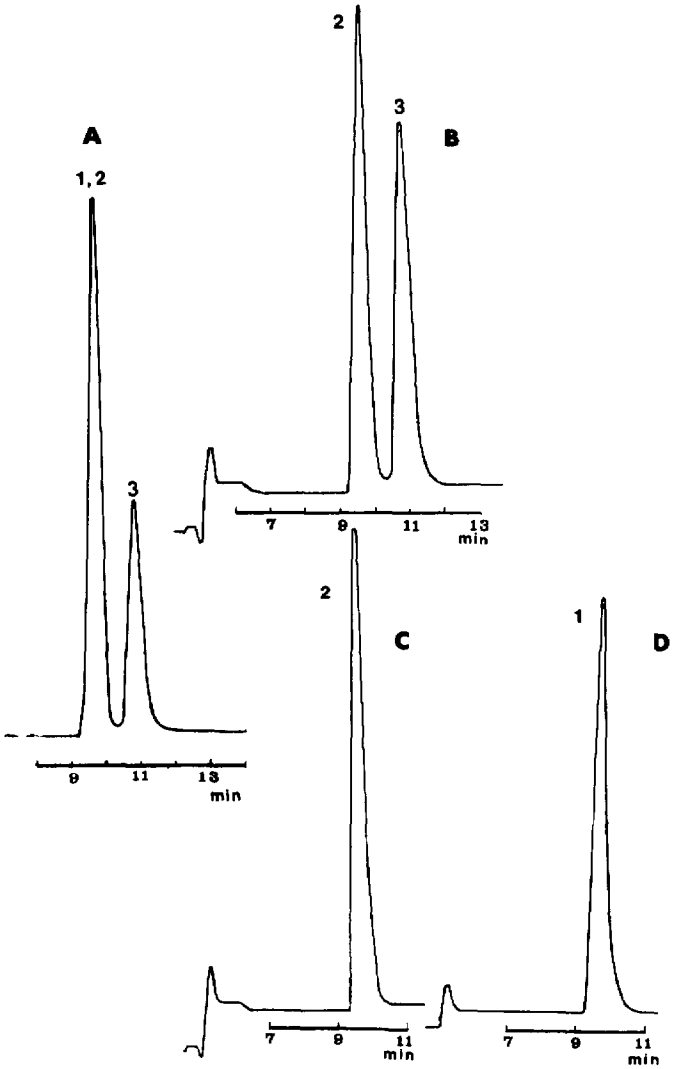

Fig. 1. Chiral-phase HPLC analysis as 3,5-dinitrophenyl isocyanate derivatives, of (1) *sn*-1,3-dioleoylglycerol (*sn*-1,3-18:1 18:1), (2) *sn*-1,2-18:1 18:1 and (3) *sn*-2,3-18:1 18:1. Mixture A contained the three isomers formed by partial chemical hydrolysis of peanut oil trioleoylglycerol. Mixture B was the enantiomeric *sn*-1,2(2,3)-dioleoylglycerol and D the *sn*-1,3-dioleoylglycerol isolated from mixture A. C was commercial *sn*-1,2-dioeoylglycerol. Mobile phase, hexane–ethylene dichloride–ethanol (80:20:1, v/v/v) at a flow-rate of 1 ml min^{-1}; analysis temperature, ambient (20.5°); detection, refractive index. Other HPLC conditions as in the text.

after separation by TLC on borate-impregnated silica plates of the mixture A diacylglycerols. Chromatogram C concerns the synthetic commercial *sn*-1,2-dioleoylglycerol. The mixture of the three stereoisomers (A) was resolved into two peaks with an approximate area ratio of 2:1. The mixture of *sn*-1,2(2,3)-isomers (B) was resolved

into two peaks with an area ratio of *ca.* 1:1. Comparison with chromatogram C shows that the retention time of the *sn*-1,2-dioloylglycerol corresponds to that of the first eluted peak in B, the second thus corresponding to *sn*-2,3-dioleoylglycerol. Comparison with chromatogram D demonstrates that, under the analytical conditions used, *sn*-1,3- and *sn*-1,2-dioleoylglycerol had approximately the same retention times (9,7 and 9.8 min from the injection point, respectively). They were not resolved. On the same chiral phase (Sumipax OA-4100), Takagi and Itabashi [9], using the same conditions as ours, partly separated *sn*-1,3- and *sn*-1,2-dioleoylglycerols (separation factor 1.04). The elution order was that which we observed, namely *sn*-1,3-, *sn*-1,2- and *sn*-2,3- according to increasing retention time. On another type of chiral phase, consisting of (*R*)-(+)-1-(1-naphthyl)ethylamine, which has a higher enantioselectivity, Itabashi *et al.* [11] obtained the complete resolution of *sn*-1,3- and *sn*-1,2-dioleoylglycerols (separation factor 1.23) with the same elution order as above.

As evident in chromatogram B, the two *sn*-1,2- and *sn*-2,3-stereoisomers of dioleoylglycerol were eluted as symmetrical and well separated peaks in less than 11 min after injection and with a resolution factor of 2.15. The observed elution order demonstrates that the *sn*-2,3-dioleoylglycerol showed a stronger diatereoisomeric interaction than the *sn*-1,2-isomer with the chiral stationary phase, in agreement with what was observed previously [11]. The earlier elution of the *sn*-1,3-dioleoylglycerol shows that this molecule is less polar than the other two, as was also shown by TLC on silica gel plates [20].

These results show that the analytical conditions used in this work were suitable for the separation of a mixture of enantiomers (*sn*-1,2- and *sn*-2,3-diacylglycerols) but not for the separation of the third possible *sn*-1,3-isomer. However, this drawback is not a problem in the stereospecific analysis of triacylglycerols as the two groups of diacylglycerols can be easily separated by TLC [14,21] or reversed-phase HPLC [13].

During their study on the separation of diacylglycerol enantiomers on the chiral column Sumipax OA-2100, Itabashi and Takagi [8] observed that the retention time of a diacylglycerol sample analysed consecutively several times varied over a range of 5–30 min. This situation could be due to a lack of equilibrium between the mobile phase and the chiral stationary phase, which may persist despite prolonged conditioning of the column. Moreover, the column stability is also influenced by the water content of the silica which binds the chiral compound and by the ambient temperature. Such variations were also observed in this work on another type of chiral column (Sumipax OA-4100). For example, with the dioleoylglycerol enantiomers the following extreme values of retention times were observed: for the *sn*-1,2-isomer 9.2 and 12.5 min and for the *sn*-2,3-isomer 10.3 and 14.0 min, under the same apparent analytical conditions. With other diacylglycerols the variations were more pronounced.

In spite of these difficulties, the analysis of a wide range of diacylglycerols produced by natural triacylglycerol hydrolysis could be performed and the elution order established by individual analyses of each molecular species.

In Table I are reported several characteristics observed in the analysis of twelve stereoisomers of mixed diacylglycerol urethane derivatives on the Sumipax OA-4100 chiral column. For each type of *sn*-1,2(2,3)-diacylglycerol the two enantiomers were well separated in less than 12 min from the injection time. The separation factor (α) between the two stereoisomers was identical (1.16) for the six diacylglycerols ana-

TABLE I

SOME CHROMATOGRAPHIC CHARACTERISTICS OBSERVED IN THE SEPARATION OF *sn*-1,2- AND *sn*-2,3-DIACYLGLYCEROL URETHANE DERIVATIVES BY CHIRAL-PHASE HPLC

Diacylglycerol[a]	Enantiomer[b]	*CN*[c]	*DB*[d]	*PN*[e]	t_R[f]	α[g]		R_s[h]	
						A	B	A	B
18:0 18:1	sn-1,2	36	1	34	6.9	1.16		2.09	
	sn-2,3				8.0				0.09[i]
16:0 18:1	sn-1,2	34	1	32	7.2	1.16	1.04	2.19	0.60
	sn-2,3				8.3		1.05		0.69
18:1 18:1	sn-1,2	36	2	32	7.2	1.16	1.00	2.15	0.05
	sn-2,3				8.4		1.01		0.09
16:0 18:2	sn-1,2	34	2	30	7.5	1.16	1.03	2.29	0.50
	sn-2,3				8.7		1.03		0.47
18:1 18:2	sn-1,2	36	3	30	7.6	1.16	1.02	1.97	0.31
	sn-2,3				8.8		1.02		0.25
18:2 18:2	sn-1,2	36	4	28	7.9	1.16	1.04	2.11	0.52
	sn-2,3				9.2		1.04		0.57

[a] *sn*-1,2(2,3)-Diacylglycerols represented by their two constituent fatty acids.
[b] *sn*-1,2- and *sn*-2,3-stereoisomers of the *sn*-1,2(2,3)-diacylglycerols.
[c] Carbon number = total acyl carbon atoms of the two constituent fatty acids.
[d] Total number of double bonds of the two constituent fatty acids.
[e] Partition number calculated according to $PN = CN - 2DB$.
[f] Retention time corrected for the column void volume.
[g] Separation factor between two peaks 1 and 2 represented by the ratio of their corrected retention times ($\alpha = t_{R2} / t_{R1}$); (A) between the two enantiomers *sn*-1,2 and *sn*-2,3 of the same diacylglycerol; (B) between the same enantiomer (*sn*-1,2 or *sn*-2,3) of two successive diacylglycerols as listed in the first column.
[h] Resolution factor between two peaks 1 and 2 according to $R_s = 2(t_{R2} - t_{R1}) / (w_2 + w_1)$, in which t_R represents the retention time (corrected or not) and w the peak width at the baseline.
[i] Resolution factor between *sn*-2,3-(18:0 18:1) and *sn*-1,2-(18:2 18:2).

lysed. Therefore, the separation efficiency was independent of the nature of the component fatty acids. Tagaki and Itabashi [9] also observed almost the same separation factor (1.15) between the *sn*-1,2- and *sn*-2,3-dioleoyl- and -dipalmitoylglycerols on the same chiral phase. On a more selective chiral phase, Itabashi *et al.* [11] observed a higher separation factor (1.44) but it was identical for dipalmitoyl- and dioleoylglycerols.

The resolution between the two optical isomers that we observed in this work varied from 1.97 to 2.29 according to the diacylglycerols, demonstrating very good resolution.

In addition to the separation between enantiomers due to the chiral selector, the separation of the different molecular species occurred according to chain length and unsaturation. This is due to the silica to which the chiral molecules are bound. The elution order was that of decreasing partition numbers (*PN*) or equivalent carbon number (*ECN*). When two isomers presented the same *PN* or *ECN*, the more unsaturated was eluted later. The observed elution order can be explained mainly by increasing polarity with increasing unsaturation and also by decreasing chain length.

Fig. 2 [log(retention volume) *versus* partition number] shows that for either type

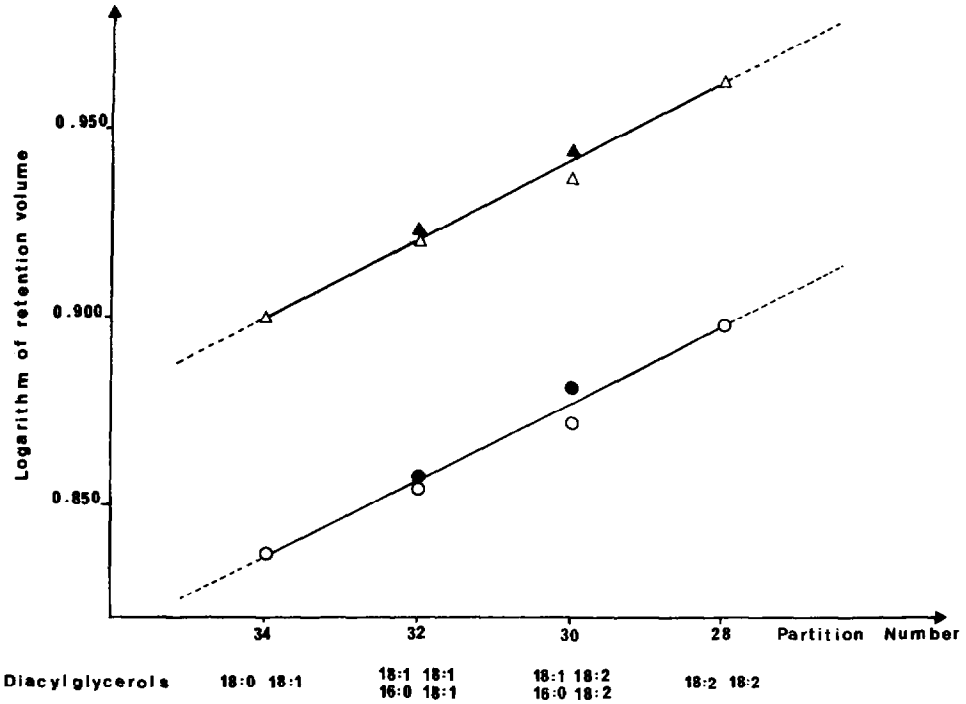

Fig. 2. Plot of logarithm of retention volume *versus* partition number of enantiomers of diacylglycerol urethane derivatives analysed by chiral-phase HPLC. Retention volume (ml) = retention time (min) corrected for the column void volume × solvent flow-rate (ml min^{-1}). Partition number: $PN = CN - 2DB$, where CN is the total number of acyl carbon atoms and DB the total number of double bonds of the two constituent fatty acids. △, *sn*-2,3-Enantiomer; ○, *sn*-1,2-enantiomer. Solid symbols for the more unsaturated and open symbols for the less unsaturated enantiomer (PN = 32 and 30).

of enantiomer an inverse proportionality was observed. A small variation was observed between the two critical pairs (16:0 18:1, 18:1 18:1 and 16:0 18:2, 18:1 18:2), the more unsaturated being eluted slightly later than the other (Table I). The two straight lines corresponding to the *sn*-1,2- and *sn*-2,3-isomers were parallel and related to each other by the equation

$$\log V_R \text{ (}sn\text{-2,3)} = \log V_R \text{ (}sn\text{-1,2)} + 0.065$$

where V_R is the retention volume. Extrapolation of the straight lines allows the retention volumes of known diacylglycerol isomers to be deduced or conversely the partition numbers of diacylglycerol isomers to be deduced from their retention volumes. Moreover, the above equation allows the retention volume of an enantiomer to be calculated when that of the other enantiomer is experimentally known.

The same observation was made by Itabashi and Takagi [8] in the separation of the enantiomers of dilauroyl-, dimyristoyl-, dipalmitoyl-, distearoyl- and dioleoylglycerols on Sumipax OA-2100 and OA-4100 chiral columns, and by Itabashi *et al.* [11] for natural diacylglycerols from linseed oil on a A-K03 chiral column (Sumitomo).

On a non-chiral silica column the same characteristics of elution were previously observed [22], indicating that it is a property of the silica support in the chiral columns.

Table I and Fig. 2 also show that the *sn*-1,2- and *sn*-2,3-enantiomers of two diacylglycerols exhibiting the same *PN* or *ECN* were virtually unseparated (separation factor $\approx$ 1 and resolution factor $\approx$ 0). Changing the mobile phase could possibly resolve the critical pairs by increasing the column efficiency, as was observed by Itabashi *et al.* [11] on another chiral phase.

For the *sn*-1,2- or *sn*-2,3-enantiomers differing by two carbon atoms (18:0 18:1 and 16:0 18:1) or by one double bond (16:0 18:1 and 16:0 18:2), the observed separation and resolution factors were low. The two molecular species were poorly separated. The same was true for the enantiomers showing an identical carbon number and an increasing degree of unsaturation (*e.g.*, 18:0 18:1, 18:1 18:1, 18:1 18:2 and 18:2 18:2 with respective *PN* or *ECN* of 34, 32, 30 and 28). Two molecular species differing in one double bond were poorly separated. In constrast when the *PN* differed by 4 units (four carbon atoms or two double bonds), the corresponding enantiomers were completely separated (*e.g.*, the two pairs 18:0 18:1, 18:1 18:2 and 18:1 18:1, 18:2 18:2 with respective resolution factors of 1.39 and 1.33 for the *sn*-1,2-isomers). The same was still more true when the molecular species differed by three double bonds (*e.g.*, the pair 18:0 18:1, 18:2 18:2 with respective resolution factors of 1.94 and 2.07 for the *sn*-1,2- and *sn*-2,3-isomers). These last values are very close to the average resolution factor observed between the two enantiomers of an *sn*-1,2(2,3)-diacylglycerol (between 1.97 and 2.29, as indicated in Table I). This means that the separation between two optical isomers *sn*-1,2- and *sn*-2,3- is identical with that between the *sn*-1,2- or the *sn*-2,3-enantiomers of two diacylglycerols differing by three double bonds. The same could probably be true with diacylglycerols differing by six carbon atoms or any combination of chain length and unsaturation to provide partition numbers differing by 6 units.

The different parameters calculated in the separation on the Sumipax OA-4100 chiral column of the twelve stereoisomers listed in Table I and partly reported in this table are different of those reported by Itabashi and Takagi [8] with the Sumipax OA-2100 chiral column. The chiral selector of this last column was adapted to the separation of monoacylglycerol enantiomers. However, the separation of diacylglycerol enantiomers required the use of three 25-cm columns in series and the retention times were increased. Recently, the same group [9] succeeded in separating diacylglycerol enantiomers on the same type of column as used in this work. With the more enantioselective A-K03 column Itabashi *et al.* [11] observed higher separation factors between enantiomers (1.23 compared with 1.16).

In Table II the twelve stereoisomers are listed according to their elution order and the separation and resolution factors between each peak and the preceding one are reported.

All the *sn*-1,2-enantiomers of the six diacylglycerols studied (Table I) were eluted earlier than their *sn*-2,3-isomers, a property also observed with still more complex mixtures of natural diacylglycerols [11]. In addition to the usual critical pairs whose enantiomers were eluted together (16:0 18:1, 18:1 18:1 and 16:0 18:2, 18:1 18:2), two stereoisomers were not easily separated, namely two different enantiomers, *sn*-1,2-(18:2 18:2) and *sn*-2,3-(18:0 18:1), with respective partition numbers of 28 and 34.

TABLE II

SEPARATION (α)[a] AND RESOLUTION FACTORS (R_s)[b] OF DIACYLGLYCEROL ENANTIOMER URETHANE DERIVATIVES ANALYSED BY CHIRAL-PHASE HPLC

Isomer	Parameter	Diacylglycerol[c]					
		18:0 18:1	16:0 18:1	18:1 18:1	16:0 18:2	18:1 18:2	18:2 18:2
sn-1,2-[d]	α		1.04	1.00	1.03	1.02	1.04
	R_s		0.60	0.05	0.50	0.31	0.52
sn-2,3-[d]	α	1.01	1.05	1.01	1.03	1.02	1.04
	R_s	0.09	0.69	0.09	0.47	0.25	0.57

[a] α = Separation factor between two successive peaks expressed as the ratio of their respective retention times corrected for the column void volume.

[b] R_s = resolution factor between two successive peaks 1 and 2 calculated from their respective retention times (t_R) and their widths (w) at the baseline according to $R_s = 2(t_{R2} - t_{R1}) / (w_2 + w_1)$.

[c] *sn*-1,2(2,3)-Diacylglycerols.

[d] *sn*-1,2- and sn-2,3-enantiomers of the diacylglycerols listed according to elution order.

Between two successive peaks, the separation factor was very low, hardly greater than 1, as was the resolution factor, which lay between 0.05 (peaks eluted together) and 0.60 (half-separated peaks). Stereospecific analysis of such a complex mixture of diacylglycerols cannot be correctly achieved on this type of column. This is generally also true for simpler mixtures of *sn*-1,2(2,3)-diacylglycerols produced by hydrolysis of natural oil triacylglycerols. When these triacylglycerols consist of three different fatty acids (*e.g.*, 16:0 18:1 18:2), six stereoisomers have to be properly separated to estimate their proportions accurately. When only two different fatty acids are present (*e.g.*, 18:1 18:1 18:2 or 18:1 18:2 18:2), four stereoisomers have to be separated.

We tried to analyse three mixtures of *sn*-1,2(2,3)-diacylglycerols formed by hydrolysis of the three peanut oil triacylglycerols cited above in parentheses. In the first instance (three different fatty acids) only two peaks (out of six) were readily distinguishable. Shoulders showed that minor peaks were partly separated. In the second instance (only two different fatty acids), two groups of two poorly separated peaks were registered on the chromatogram. Such poor resolutions preclude an accurate determination of enantiomer composition.

The resolution can probably be improved by varying the analytical conditions such as column length, solvent polarity and analysis temperature. However, the retention times probably increase considerably, as was observed by others [9]. The use of a chiral column with higher enantioselectivity can improve the resolution of enantiomers [11], although it was not evident when analysing complex mixtures of natural diacylglycerol enantiomers (from corn oil, linseed oil and menhaden oil). The alternative we preferred was to proceed to a previous fractionation (coupled with purification) of the diacylglycerol derivatives by reversed-phase HPLC to obtain much simpler mixtures with short retention times. These simple mixtures were easily resolved into their constituent enantiomers by chiral-phase HPLC, as shown in Fig. 3.

Quantitative analysis

Good separations between enantiomers such those reported in Fig. 3 are a

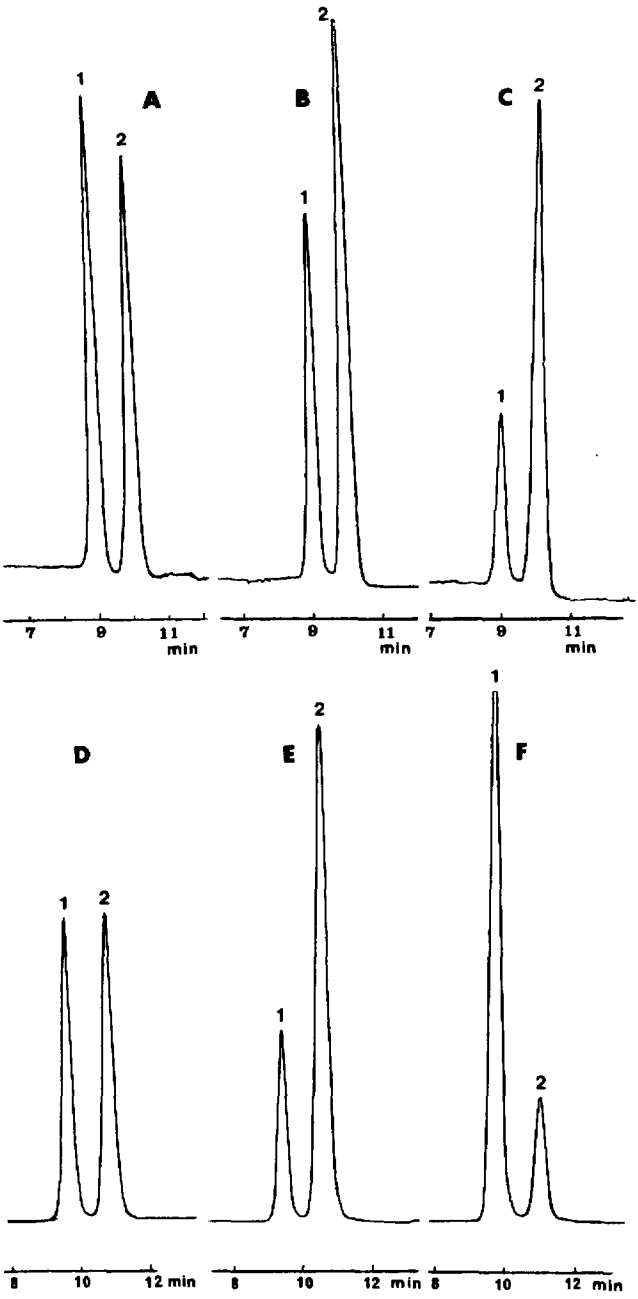

Fig. 3. HPLC separation of diacylglycerol enantiomers as 3,5-dinitrophenyl isocyanate derivatives on the chiral OA-4100 column. Diacylglycerols derived from peanut oil and cottonseed oil triacylglycerol by chemical (B, C, E and F) and enzymatic (A, D) hydrolysis. They were previously fractionated by reversed-phase HPLC. (A) 18:0 18:1; (B) 16:0 18:1; (C) 18:1 18:1; (D) 16:0 18:2; (E) 18:1 18:2; (F) 18:2 18:2. Peaks: 1 = *sn*-1,2-Enantiomer and 2 = *sn*-2,3-enantiomers in each chromatogram. Chromatograms B, C, E and F correspond to enantiomeric diacylglycerols B, C, E and F reported in Table III. Detection, UV absorption (254 nm). Other conditions as in Fig. 1.

prerequisite for compositional analysis. The second condition is quantitative detection of the molecules eluted by HPLC. This could be checked with commercial standard mixtures. As commercial standards of diacylglycerol urethane derivatives were not available, the quantitative aspects of their detection were studied in another way. The diacylglycerol derivatives analysed by chiral-phase HPLC are generally detected by their UV absorption [8,9,11], a very sensitive mode of detection. As these derivatives were quantitatively detected by their refractive indices when previously separated by reversed-phase HPLC [13], we compared, in the present enantiomer analysis, peak areas registered after detection by a variable-wavelength UV detector and a

TABLE III

QUANTITATIVE ANALYSIS OF ENANTIOMERS OF *sn*-1,2(2,3)-DIACYLGLYCEROL URETHANE DERIVATIVES BY CHIRAL-PHASE HPLC DETECTED BY THEIR REFRACTIVE INDICES OR UV ABSORPTION

Oil	Hydrolysed triacylglycerol[a]	*sn*-1,2(2,3)-Diacylglycerol[b]	Enantiomer[c]	Peak area (%)[d]		Variation (%)[e]
				RI	UV	
Peanut	16:0 18:1 18:1	18:1 18:1	*sn*-1,2-	37.6	38.2	1.6
			sn-2,3-	62.4	61.8	
		16:0 18:1	*sn*-1,2-	58.9	59.0	0.2
			sn-2,3-	41.1	41.0	
	18:1 18:1 18:1	18:1 18:1	*sn*-1,2-	50.1	49.6	1.0
			sn-2,3-	49.9	50.4	
	16:0 18:1 18:2	18:1 18:2	*sn*-1,2-	38.0	39.0	2.6
			sn-2,3-	62.0	61.0	
		16:0 18:2	*sn*-1,2-	68.5	66.0	7.3
			sn-2,3-	31.5	34.0	
		16:0 18:1 (B)[f]	*sn*-1,2-	37.3	35.5	5.1
			sn-2,3-	62.7	64.5	
	18:1 18:1 18:2	18:1 18:2	*sn*-1,2-	57.6	55.8	4.3
			sn-2,3-	42.4	44.3	
		18:1 18:1 (C)[f]	*sn*-1,2-	22.9	23.6	3.0
			sn-2,3-	77.1	76.4	
	18:1 18:2 18:2	18:2 18:2 (F)[f]	*sn*-1,2-	82.2	81.8	2.2
			sn-2,3-	17.8	18.2	
		18:1 18:2 (E)[f]	*sn*-1,2-	22.3	24.3	4.1
			sn-2,3-	77.7	75.7	
Cottonseed	16:0 18:1 18:2	18:1 18:2	*sn*-1,2-	48.2	47.6	1.3
			sn-2,3-	51.8	52.4	
		16:0 18:2	*sn*-1,2-	49.9	50.1	0.4
			sn-2,3-	50.1	49.9	
		16:0 18:1	*sn*-1,2-	53.8	53.2	1.3
			sn-2,3-	46.2	46.8	

[a] Triacylglycerols isolated from peanut oil and cottonseed oil by coupled argentation TLC–reversed-phase HPLC and deacylated by Grignard reagent.

[b] *sn*-1,2(2,3)-Diacylglycerols isolated from hydrolysis products by borate-impregnated silica TLC and fractionated by reversed-phase HPLC as urethane derivatives.

[c] *sn*-1,2- and *sn*-2,3-diacylglycerol derivatives separated by chiral-phase HPLC.

[d] Peak areas (%) of the two enantiomers detected by their refractive indices (RI) or UV absorption (UV).

[e] Maximum % variation between the two series of figures.

[f] The enantiomeric diacylglycerols B, C, E and F correspond to chromatograms B, C, E and F in Fig. 3.

differential refractometer. Samples were prepared from chemical hydrolysis products of peanut oil and cottonseed oil triacylglycerols. The *sn*-1,2(2,3)-diacylglycerols isolated by TLC were derivatized and fractionated by reversed-phase HPLC [13] according to chain length and unsaturation. The two enantiomers of the diacylglycerol derivatives were separated by chiral-phase HPLC, first detected by means of the differential refractometer (sensitivity 25–50 μg per peak, attenuation 8), and then detected, under strictly the same analytical conditions, by the UV detector (sensitivity 1 μg per peak, attenuation 2 a.u.f.s.).

The percentages of the two enantiomers were calculated from the peak areas. The two series of values reported in Table III were compared and the maximum percentage variation was calculated from the difference observed for the lower percentages. In most instances the variation between the two figures was very low (< 5%). The average variation was 2.6%. Relatively high variations were observed in the analysis of the three diacylglycerol mixtures formed by deacylation of peanut oil 16:0 18:1 18:2 (7.3% maximum). This is probably due to a defective peak integration in this instance, as the same three diacylglycerol mixtures formed by deacylation of the same triacylglycerol but isolated from cottonseed oil (last column) exhibited much smaller differences (1.3% maximum).

As detection by refractometry was found to be quantitative, at least for the enantiomer mixtures and in the range of the diacylglycerols checked [13], we can reasonably conclude that detection of the separate enantiomers by the UV detector, comparable to that by refractometry, is also quantitative. Peak areas so determined can be used to calculate the percentages of the two enantiomers in the mixture. As the two enantiomers contain the same two fatty acids, peak-area percentages also represent weight or molar percentages.

Another proof of the accuracy of the enantiomer determination can be found in the analysis of the *sn*-1,2(2,3)-diacylglycerols formed by chemical deacylation of trioleoylglycerol (18:1 18:1 18:1) whose racemic character is obligatory. The values found with both types of detection were very close to the expected values of 50:50.

Another sample was unexpectedly found to be racemic in these analyses. This was the diacylglycerol 16:0 18:2 produced by hydrolysis of the cottonseed oil 16:0 18:1 18:2, whereas this did not occur with the identical triacylglycerol isolated from peanut oil, whose enantiomer proportion was close to 2:1. This shows that depending on its origin, a given triacylglycerol can present different stereospecific distributions of the same three fatty acids between the three positions of the glycerol moiety. These differences probably reflect species specificity in triacylglycerol biosynthesis in seeds.

The results obtained with cottonseed oil 16:0 18:1 18:2 in this work were different from those obtained in another study using Brockerhoff's method [23]. The percentages of the two 16:0 18:2 diacylglycerol enantiomers were found to be *ca.* 40 and 60 instead of *ca.* 50 and 50 in this work. Several features may be responsible for this difference. First, the cottonseed oil sample used to prepare the triacylglycerol was not the same in both studies. This could mean that in the same vegetable species, stereospecific distribution of fatty acids in the glycerol molecules can vary according to particular factors. Second, the method used for stereospecific analysis of the triacylglycerols was different in the two instances. Comparison between the two methods would be of great interest.

The results obtained in this work with a relatively wide range of mixed di-

acylglycerols confirm and extend those reported by Takagi and Itabashi [9] for racemic simple diacylglycerols, by Tagaki and Suzuki [10] for synthetic mixed diacylglycerols and by Itabashi *et al.* [11] for natural complex diacylglycerol mixtures. They show that accurate stereospecific analyses of natural triacylglycerols can be achieved by chiral-phase HPLC provided that the original complex diacylglycerol mixtures were divided into simpler mixtures by reversed-phase HPLC fractionation [13].

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