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Analysis and fractionation of natural source diacylglycerols as urethane derivatives by reversed-phase high-performance liquid chromatography

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ABSTRACT

Reversed-phase high-performance liquid chromatography on a thermostatted octadecylsilyl column was used to separate and fractionate mixtures of diacylglycerols after derivatization with 3,5-dinitrophenyl isocyanate (urethane derivatives). In addition to the separation of commercial diacylglycerol species, the separation of diacylglycerols obtained from peanut oil and cottonseed oil triacylglycerols by chemical hydrolysis is reported. Acetonitrile–acetone mixtures were used for elution of the diacylglycerol urethane derivatives. Unsaturated and saturated derivatives were detected by their refractive indices. They were then collected and their fatty acids analysed as methyl esters by capillary gas chromatography. The elution order of diacylglycerol derivatives in complex mixtures varies as a function of chain length, unsaturation and positional isomerism of the constituent fatty acids. The elution order and resolution vary as a function of temperature. The diacylglycerol composition of mixtures calculated from peak areas is similar to the composition reconstituted from the fatty acid composition of the collected diacylglycerol fractions. The method can be applied to complete compositional analysis but is especially useful for the collection of pure fractions of diacylglycerols during studies of the stereospecific distribution of fatty acids in triacylglycerols.

INTRODUCTION

When studying the stereospecific distribution of fatty acids in triacylglycerol molecules, the fatty acids esterifying the internal *sn*-2 position are those of the *sn*-2-monoacylglycerols issuing from hydrolysis of triacylglycerols by pancreatic lipase [1–3]. The study of the distribution of fatty acids on the external *sn*-1 and *sn*-3 positions, stereospecifically distinct, was more generally performed to date using Brockerhoff's method [4,5]. This method is based on the snake venon phospholipase stereospecificity in the hydrolysis of phospholipid-like molecules synthesized from the mixture of *sn*-1,2- plus *sn*-2,3-diacylglycerols [denoted *sn*-1,2(2,3)-diacylglycerols] issuing from chemical hydrolysis of triacylglycerols [6]. The enzyme distinguishes the two stereoisomers. The present approach consists in separating the *sn*-1,2- and *sn*-2,3-diacylglycerols by high-performance liquid chromatography (HPLC), either as 3,5-dinitrophenyl urethane derivatives on a chiral column [7,8], or as chiral derivatives on a classical silica column [9,10]. However, diacylglycerols issuing from the hydrolysis

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of natural oil triacylglycerols are complex mixtures of molecules differing both in the nature of the component fatty acids and in their positioning. Analysis is difficult. The diacylglycerol mixtures have first to be fractionated into groups according to the positions of the fatty acids, the *sn*-1,3- and the mixture of *sn*-1,2(2,3)-diacylglycerols. The *sn*-1,2(2,3)-diacylglycerol group then has to be fractionated according to the nature of the fatty acids. For further accurate analysis the diacylglycerol fractions must be collected as pure as possible; they therefore have to be well separated to avoid minor contamination between fractions. The proportions of the different fractions in the mixture have additionally to be evaluated with high precision for further accurate calculations.

The diacylglycerol separation method chosen in this work was reversed-phase HPLC. Although widely used in the separation and fractionation of triacylglycerols [11], HPLC has to date rarely been applied to diacylglycerol mixtures. Krüger *et al.* [12] and Ryan and Honeyman [13] developed HPLC methods for the separation and determination of diacylglycerols after derivatization with α-naphthyl isocyanate and a fluorescent marker, N-dansyl (DNS) ethanolaminophosphate, respectively. However, these methods were not entirely convenient for the simultaneous determination of the homologous distribution and the ratio of positional isomers of diacylglycerols.

Kondoh and Takano [14] devised an original detection method for acylglycerols (acylglycerol selective post-column reaction detector), which they applied to the simultaneous determination of mono-, di- and triacylglycerols. However, the detection method involves destroying the partial acylglycerols and does not allow their collection for further analysis.

For our purpose of the stereospecific analysis of triacylglycerols, in this work we studied the HPLC separation of complex mixtures of diacylglycerols according to the nature of the constituent fatty acids (chain length and unsaturation) and their positioning (*sn*-1,3- and *sn*-1,2(2,3)-diacylglycerols). Analyses were carried out in part on underivatized diacylglycerols but particularly on the 3,5-dinitrophenyl isocyanate derivatives, which we expect to use in the separation of the *sn*-1,2- and *sn*-2,3-isomers on a chiral column.

EXPERIMENTAL

Samples

Synthetic *rac*-1,2 and *sn*-1,3-dioleoylglycerols (*rac*-1,2-18:1 18:1 and *sn*-1,3-18:1 18:1, respectively) were obtained from Serdary Research Labs.(London, Ontario, Canada). Synthetic optically active *sn*-,1,2-dioleoylglycerol (*sn*-1,2-18:1 18:1) and *sn*-1,2-dipalmitoylglycerol (*sn*-1,2-16:0 16:0) were purchased from Sigma (St. Louis, MO, USA). Prior to use the *rac*-1,2-18:1 18:1 from Serdary was purified and resolved into *rac*-1,2-18:1 18:1 and *sn*-1,3-18:1 18:1 (impurity), separately recovered by thin-layer chromatography (TLC) on borate-impregnated silica gel (5%, w/w) using light petroleum–diethyl ether (50:50, v/v) as the developing solvent [15].

Natural source diacylglycerols (DGs) were prepared by Grignard degradation with ethylmagnesium bromide [16] or pancreatic lipase hydrolysis [17] of triacylglycerols (TGs) from peanut oil and cottonseed oil. The oil TGs were first fractionated by argentation TLC according to their unsaturation and then fractionated by reversed-phase HPLC [18]. The DGs were separated from the hydrolysis mixture by

TLC on borate-impregnated silica gel into *sn*-1,3- and *sn*-1,2(2,3)-diacylglycerols according to decreasing R_F values. The TGs fractionated by combined TLC-HPLC were palmitoyldioleoylglycerol (16:0 18:1 18:1), a complex mixture of stearoyldioleoylglycerol plus palmitoyloleoyleicosenoylglycerol (18:0 18:1 18:1 + 16:0 18:1 20:1), trioleoylglycerol (18:1 18:1 18:1), palmitoyloleoyllinoleoylglycerol (16:0 18:1 18:2), dioleoyllinoleoylglycerol (18:1 18:1 18:2) and oleoyldilinoleoylglycerol (18:1 18:2 18:2) from peanut oil. Palmitoyloleoyllinoleoylglycerol (16:0 18:1 18:2) was also isolated from cottonseed oil in order to compare it with peanut oil and with previous results obtained by a different method [19].

The diacylglycerols prepared after hydrolysis of these natural triacylglycerols were the two series of *sn*-1,3- and *sn*-1,2(2,3)-isomers of dilinoleoylglycerol (18:2 18:2), oleoyllinoleoylglycerol (18:1 18:2), palmitoyllinoleoylglycerol (16:0 18:2), dioleoylglycerol (18:1 18:1), palmitoyloleoylglycerol (16:0 18:1), oleoyleicosenoylglycerol (18:1 20:1) and stearoyloleoylglycerol (18:0 18:1).

They were identified during HPLC analysis by comparison with the above commercial diacylglycerols and with the series of natural *sn*-1,2(2,3)-diacylglycerols identified after fractionation by gas chromatographic (GC) analysis of their constituent fatty acids and by HPLC on a chiral stationary phase to confirm the type of isomerism.

Preparation of 3,5-dinitrophenyl isocyanate derivatives of diacylglycerols

The procedures used to prepare the 3,5-dinitrophenyl isocyanate derivatives of DGs was derived from that employed by Ôi and Kitahara [20] for chiral alcohols and adapted to DGs by Itabashi and Takagi [7,8].

Amounts of 1–5 μmol (0.6–3 mg) of DGs were dissolved in 4 ml of dry toluene in an 8-ml glass tube with a PTFE-lined screw-cap; 10–50 μmol (2–10 mg) of 3,5-dinitrophenyl isocyanate (Sumitomo, Osaka, Japan) and 40 μl of dry pyridine were added to the solution and the mixture was left for 1 h at ambient temperature with occasional shaking. At the end of the reaction the solvent was removed under nitrogen and the residue was dissolved in 0.2 ml of chloroform. The DG derivatives were isolated by TLC on silica gel $60F_{254}$-precoated plates (Merck, Darmstadt, Germany). The plates containing a fluorescence indicator were previously activated at 110–120°C for 1 h in an oven. The developing solvent was hexane–ethylene dichloride–ethanol (40:10:3, v/v/v). After drying under nitrogen the diacylglycerol derivative bands were delineated under UV light (254 nm),the corresponding silica gel was scraped off the plate and the urethane derivatives were extracted with diethyl ether.

Alternatively, the crude diacylglycerol urethane derivatives were purified by reversed-phase HPLC instead of TLC. In this instance, at the end of the derivatization reaction the mixture was decanted. The limpid upper phase was filtered through hyperfine glass-wool into another vial. The solvent was evaporated under nitrogen and the urethane derivatives were dissolved in chloroform for storage, in acetonitrile or in the solvent used for reversed-phase HPLC for fractionation.

Purification of crude diacylglycerol derivatives was coupled with fractionation according to carbon number, degree of unsaturation and positional isomerism (*sn*-1,3-diacylglycerol derivatives were separated from *sn*-1,2(2,3)-isomers). Isocratic operation was applied with the solvent system acetonitrile–acetone (55:45 and 60:40, v/v) at different temperatures. The temperature was ambient (16–18°C) for the three

diacylglycerol mixtures: *sn*-1,2(2,3) 18:1 18:1 and 16:0 18:1, *sn*-1,2(2,3) 18:1 18:2 and 18:1 18:1 and *sn*-1,2(2,3) 18:2 18:2 and 18:1 18:2. They were isolated after hydrolysis of the natural triacylglycerols 16:0 18:1 18:1, 18:1 18:1 18:2 and 18:1 18:2 18:2, respectively. A constant subambient thermostatically controlled temperature [21] of 10–12°C was used to improve separation of the critical pairs (18:1 18:2 and 16:0 18:2) in the mixture *sn*-1,2(2,3)-18:1 18:2, −16:0 18:2 and −16:0 18:1 issuing from hydrolysis of the peanut oil or cottonseed oil 16:0 18:1 18:2 triacylglycerol. An ambient temperature of *ca.* 20°C was used for the mixture of all these diacylglycerols and their *sn*-1,3-isomers.

Column liquid chromatography

HPLC was carried out using a Model 6000 A solvent-delivery system connected either to a Model R 401 differential refractometer or to a Model 450 variable-wavelength UV detector (Waters Assoc., Milford, MA, USA). Separations were achieved on two stainless-steel columns: a Superspher RP-18 and a chiral column. A prepacked 250 mm × 4 mm I.D. Hibar LiChroCART HPLC cartridge, LiChrospher 100 CH-18/II SUPER (4 μm particles) column was obtained from Merck. The 250 mm × 4 mm I.D. chiral column used in several controls, packed with 5-μm particles of N-(*R*)-1-(α-naphthyl)ethylaminocarbonyl-(*S*)-valine chemically bonded to δ-aminopropylsilanized silica (Sumipax OA-4100) was purchased from Sumitomo. A Guard-Pac precolumn module was attached to the inlet of each column: a LiChroCART 4-4 filled with LiChrosorb 100 RP-18 (Merck) for the RP-18 column and a LiChroCART 4-4 filled with LiChrosorb Si 60 (Merck) for the chiral column.

The analyses were carried out isocratically at a constant flow-rate of 0.9, 1 or 1.2 ml min^{-1} at ambient temperature (chiral OA-4100 column and LiChrospher RP-18) or at a constant controlled temperature [21] below or above ambient temperature (LiChrospher RP-18).

Several solvent systems were used as mobile phase, depending on the separation desired: acetone–acetonitrile (45:55 or 40:60, v/v) for underivatized or 3,5-dinitrophenyl isocyanate-derivatized diacylglycerols on the RP-18 column; hexane–ethylene dichloride (or methylene chloride)–ethanol (80:20:1, v/v/v) for diacylglycerol enantiomer resolution on the chiral column OA-4100, as 3,5-dinitrophenyl urethane derivatives. Acetonitrile (Far UV HiperSolv) was from BDH. Acetone, hexane (SDS, Peypin, France) were of analytical-reagent grade. Ethylene dichloride (HPLC grade) was purchased from Fluka (Buchs, Switzerland). Dichloromethane of analytical-reagent grade was obtained from Prolabo (Paris, France) and ethanol of the same grade from Carlo Erba (Milan, Italy). Water was doubly distilled. Solvents were filtered through a Millipore membrane (pore size 0.5 μm) and the mobile phase mixtures were vacuum degassed for 2 min before use.

Samples for HPLC separations initially in chloroform were generally dissolved in pure acetonitrile or in the solvent used as the mobile phase for injection onto the HPLC column.

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

Gas chromatography

The fatty acid composition of underivatized diacylglycerols recovered after hy-

drolysis and the fatty acid composition of the 3,5-dinitrophenyl isocyanate derivatives of the HPLC-collected diacylglycerols (RP-18 column) were determined by GC of the methyl esters prepared from methanol–boron trifluoride [22]. The analyses were carried out on a Becker-Packard 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 equipped with an ROS injector [23] (Spiral, Dijon, France) and a flame ionization detector. Peak areas were measured with an Enica 21 integrator–calculator. 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 equivalent carbon number (*ECN*) [11] of the diacylglycerol fractions, equivalent to the partition number (*PN*) [3], was calculated from the total acyl carbon number (*CN*) and total number of double bonds (*DB*) of the two constituent fatty acids, according to [11]

$$ECN = CN - 2DB$$

Peak elution was characterized by the retention time, t_R (min), corrected for the column void volume.

Separation between two peaks 1 and 2 (in order of elution) was characterized by the separation factor α, that is, the ratio of their corrected retention times, t_{R2}/t_{R1}. Separation was considered to be complete from $\alpha = 1.10$.

Resolution between two peaks 1 and 2 was characterized by the resolution factor R_s calculated from the retention times and the peak widths at the baseline (w) according to [24]

$$R_s = 2(t_{R2} - t_{R1})/(w_2 + w_1)$$

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

RESULTS AND DISCUSSION

Separation of underivatized diacylglycerols

Mixtures of underivatized dipalmitoyl- and dioleoylglycerol stereoisomers were separated by reversed-phase HPLC using different acetonitrile–acetone mixtures as mobile phase: 50:50, 55:45 or 60:40 (v/v). Diolcoylglycerols were easily eluted as two well separated peaks corresponding to the first-eluted single *sn*-1,3-isomer followed by the mixture of the two *sn*-1,2- and *sn*-2,3-isomers eluted as a single unshouldered peak (Fig. 1B). The retention times under the conditions used [19°C with acetonitrile–acetone (60:40, v/v) at 1 ml min^{-1}] were 14.7 and 16.2 min from the injection point, respectively. In contrast, dipalmitoylglycerol isomers were incompletely separated whatever the chromatographic conditions, probably because of poor dissolution of the saturated acylglycerols in the mobile phase and in the injection solvent. It was

nevertheless possible to determine the elution order of the different isomers. The *sn*-1,3-isomers were eluted first, followed by the *sn*-1,2(2,3)-isomer mixture, and the unsaturated dioleoylglycerols preceded the saturated dipalmitoylglycerols. This corresponds to what was previously observed by Kondoh and Takano [14], although using other analytical conditions.

Because of the difficulties encountered in the separation of the saturated diacylglycerols and as the objective was to collect pure mixtures of *sn*-1,2(2,3)-isomers to be separated further into optical isomers on a chiral column as 3,5-dinitrophenyl isocyanate derivatives, these derivatives were henceforth used with several additional advantages. First, derivatization improves the solubility of the more saturated diacylglycerols in the solvents used in HPLC with refractometric detection, *i.e.*, acetonitrile and acetone. Second, with derivatized diacylglycerols, fractionation according to the nature and the positions of fatty acids in the glycerol moiety can be easily combined. Third, the previous purification step by TLC of the DG derivatives before analysis on a chiral column with UV detection, as recommended by Itabashi and Takagi [7,8], is avoided. Last, derivatization of diacylglycerols can be carried out more rapidly after hydrolysis of triacylglycerols and just after their isolation on borate-impregnated silica gel TLC plates, decreasing the extent of possible isomerization due to the free hydroxyl group of glycerol. Fig. 1A illustrates the good separation

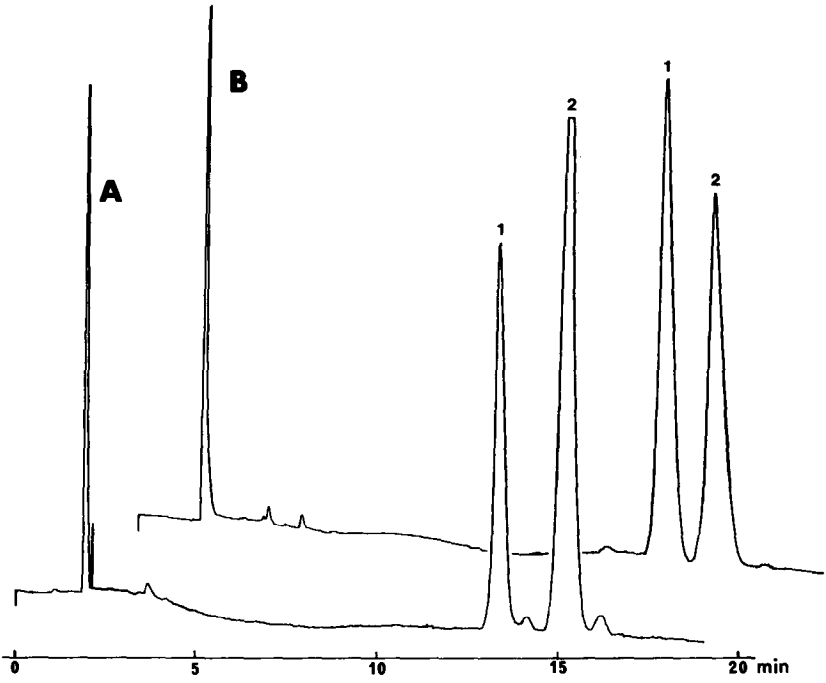

Fig. 1. HPLC separation of dioleoylglycerol isomers (A) as 3,5-dinitrophenyl isocyanate derivatives and (B) underivatized on an RP-18 column. 1 = *sn*-1,3-Dioleoylglycerol (*sn*-1,3-18:1 18:1); 2 = *sn*-1,2(2,3)-dioleoylglycerol [*sn*-1,2(2,3)-18:1 18:1]. The minor satellite peaks emerging after the major peaks in A were not identified. Analytical conditions: stainless-steel column (250 mm × 4 mm I.D.) packed with 4-μm octadecylsilyl (C_{18}) reversed-phase material; eluent, acetonitrile–acetone (60:40, v/v) at 1 ml min^{-1}; temperature thermostatically controlled at 19°C; refractive index detection; isocratic analysis.

of the two pairs of derivatized *sn*-1,3- and *sn*-1,2(2,3)-dioleoylglycerols in 13.2 and 15.0 min, respectively. The retention times were slightly shorter than those for the underivatized diacylglycerols and the separation was better with narrower peaks. The separation and the resolution factors were 1.13 and 2.77, respectively, with the derivatives and only 1.10 and 1.83, respectively, with underivatized dioleoylglycerols.

Properties of diacylglycerol derivatives

Several problems were encountered in the preparation, purification and conservation of the DG derivatives. The first problem was the poor solubility of the more saturated diacylglycerols in toluene. In several instances it was necessary to heat the reaction mixture slightly. The second problem was the fractionation of pure DG derivatives from the reaction mixture on a TLC plate as proposed by Itabashi and Takagi [7,8]. In the purification of *rac*-1,2-dipalmitoylglycerol, they observed two bands. The lower yellow band corresponded to the excess of 3,5-dinitrophenyl isocyanate reagent and the upper band to the DG derivatives. On the plates we used the reaction mixture products were separated into more than two bands. The analysis by reversed-phase HPLC of the compounds adsorbed in each band showed that only the main band, located just above that of the remaining reagent, contained DG derivatives. This was confirmed by HPLC on the chiral column. Because of this difficulty of identification of the proper band, the previous purification step by TLC was abandoned and the mixture was directly fractionated by reversed-phase HPLC. The remaining reagent eluted with the injection solvent and, as indicated above, the DG derivatives were separated according to the nature and the positions of the constituent fatty acids.

The last problem encountered was the lack of stability of the unsaturated diacylglycerol urethane derivatives during storage when compared with the underivatized molecules. In an experiment with twelve mixtures of mixed diacylglycerols, the relative decrease in the proportion of linoleic acid was 10–30% and that of oleic acid 4–5% after 1 month of storage. This decrease was only partly due to oxidation; another additional type of degradation occurred that remains to be elucidated. It is thus recommended to proceed rapidly to stereospecific analysis of the diacylglycerol urethane derivatives after fractionation by reversed-phase HPLC in order to obtain accurate results.

Separation of diacylglycerol derivatives

A wide range of diacylglycerols were prepared by deacylation of natural triacylglycerols from peanut and cottonseed oils (see Experimental). They were separated as urethane derivatives by reversed-phase HPLC.

Figs. 2 and 3 illustrate the separation of diacylglycerol mixtures, separately (Fig. 2) or mixed (Fig. 3). Tables I and II give some observed chromatographic parameters calculated for the two series of *sn*-1,3- and *sn*-1,2(2,3)-diacylglycerols. Fig. 3 shows that all the *sn*-1,2(2,3)-diacylglycerols studied were clearly separated, even the critical pairs, *i.e.*, those presenting the same equivalent carbon number (*ECN*) in a given series of isomers (Table I), *e.g.*, *sn*-1,2(2,3)-18:1 18:1 and −16:0 18:1, peaks 9 and 10 on the chromatogram in Fig. 3B. In both series of isomers (Table I) the diacylglycerols were eluted according to increasing *ECN*, and when the *ECN* was the same the more unsaturated of the pair eluted earlier (*e.g.*, in the above example). For

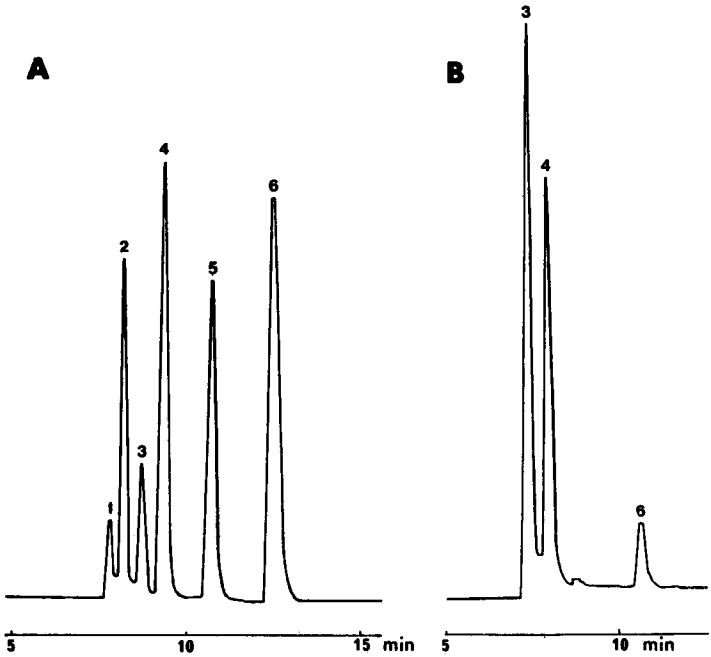

Fig. 2. RP-18 HPLC analysis of 3,5-dinitrophenyl isocyanate derivatives of natural source *sn*-1,3- and *sn*-1,2(2,3)-diacylglycerols formed by deacylation of one peanut oil triacylglycerol (16:0 18:1 18:2). 1 = *sn*-1,3-18:1 18:2; 2 = *sn*-1,3-16:0 18:2; 3 = *sn*-1,2(2,3)-18:1 18:2; 4 = *sn*-1,2(2,3)-16:0 18:2; 5 = *sn*-1,3-16:0 18:1; 6 = *sn*-1,2(2,3)-16:0 18:1. (A) Mixture of *sn*-1,3- and *sn*-1,2(2,3)-isomers; (B) only *sn*-1,2(2,3)-isomers. Analytical conditions: eluent, acetonitrile–acetone (A, 60:40, v/v; B, 55:45, v/v) at 1.2 ml min^{-1}; ambient temperature (19°C); other conditions as in Fig. 1.

diacylglycerols with the same two-component fatty acids or the same *ECN*, the *sn*-1,3-isomers eluted earlier than the corresponding *sn*-1,2(2,3)-isomers. The data reported in Table I quantify the separation observed between peaks of each series and those in Table II between adjacent peaks. The resolution factor, R_s, between two adjacent peaks was found in each instance to be at least equal to 1, confirming their good separation. In each series of isomers (Table I), the *sn*-1,2(2,3)-diacylglycerols were better separated than the corresponding *sn*-1,3-isomers, exhibiting higher separation and resolution factors between two successive peaks. This property is important, because in the stereospecific analysis of triacylglycerols the *sn*-1,2(2,3)-diacylglycerols have to be separated from the *sn*-1,3-isomers after deacylation and fractionated by reversed-phase HPLC, as derivatives, before further separation on a chiral column. The good separation observed here leads to good purity of the fractionated diacylglycerols.

If one compares the separation parameters (last two columns in Table I) for *sn*-1,3- and *sn*-1,2(2,3)-isomers of a given diacylglycerol, it can be seen that generally they increased with increasing retention times. This means that if necessary, the separation could be improved between isomers by choosing chromatographic conditions that increase the retention time, such as a lower column temperature. For a given

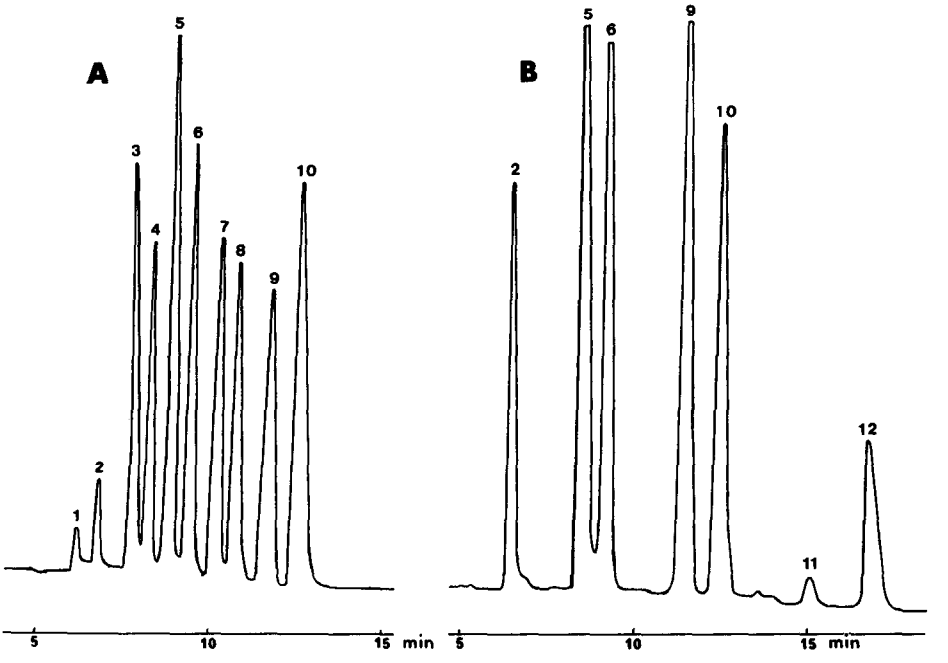

Fig. 3. RP-18 HPLC analysis of 3,5-dinitrophenyl isocyanate derivatives of natural source *sn*-1,3 and *sn*-1,2(2,3)-diacylglycerols formed by deacylation of several peanut oil triacylglycerols. 1 = *sn*-1,3-18:2 18:2; 2 = *sn*-1,2(2,3)-18:2 18:2; 3 = *sn*-1,3-18:1 18:2; 4 = *sn*-1,3-16:0 18:2; 5 = *sn*-1,2(2,3)-18:1 18:2; 6 = *sn*-1,2(2,3)-16:0 18:2; 7 = *sn*-1,3-18:1 18:1; 8 = *sn*-1,3-16:0 18:1; 9 = *sn*-1,2(2,3)-18:1 18:1; 10 = *sn*-1,2(2,3)-16:0 18:1; 11 = *sn*-1,2(2,3)-18:1 20:1; 12 = *sn*-1,2(2,3)-18:0 18:1. (A) Mixture of *sn*-1,3- and *sn*-1,2(2,3)-isomers; (B) only *sn*-1,2(2,3)-isomers. Analytical conditions: eluent, acetonitrile–acetone (60:40, v/v) at 1.2 ml min^{-1}; temperature, (A) 20°C and (B) 19°C; other conditions as in Fig. 1.

series of isomers, a decrease in the analysis temperature considerably improves the separation of the different diacylglycerols. This is illustrated in Fig. 4A and B, which show chromatograms registered in the separation of two mixtures of *sn*-1,2(2,3)-isomers of oleoyllinoleoylglycerol (18:1 18:2), palmitoyllinoleoylglycerol (16:0 18:2) and palmitoyloleoylglycerol (16:0 18:1) at 19 and 10°C, respectively. Both mixtures resulted from hydrolysis of the triacylglycerol 16:0 18:1 18:2, but isolated from two different oils: peanut oil for the first mixture and cottonseed oil for the second. Table III gives some chromatographic parameters for these analyses. As can clearly be seen in Fig. 4 and Table III, when the analysis temperature was decreased from 19 to 10°C, with the other conditions remaining unchanged, the corrected retention times were increased by 37% for the more unsaturated and by 53% for the more saturated diacylglycerols. The separation factor was increased by *ca.* 6% for the two pairs of adjacent peaks. The better separation of the first two peaks at 10°C leads to less contamination of the second fraction by the first, after collection of the three fractions for further stereoisomerism HPLC analysis on a chiral column. The resolution factor for the first two adjacent peaks increased by 34% from 19 to 10°C whereas it did not increase or even decreased for the second pair of adjacent peaks (2 and 3) because of

TABLE I

SOME CHEMICAL AND CHROMATOGRAPHIC PARAMETERS OF DIFFERENT *sn*-1,3- AND *sn*-1,2(2,3)-DIACYLGLYCEROLS SEPARATED BY REVERSED-PHASE HPLC AS URETHANE DERIVATIVES

Isomers	Peak No.	Diacylglycerols	*CN*[a]	*DB*[b]	*ECN*[c]	t_R (min)[d]	A[e]		B[f]	
							α[g]	R_s[h]	α[g]	R_s[h]
sn-1,3-	1	18:2 18:2	36	4	28	4.3				
	2	18:1 18:2	36	3	30	5.9	1.37	5.88		
	3	16:0 18:2	34	2	30	6.3	1.07	1.19		
	4	18:1 18:1	36	2	32	8.3	1.31	5.16		
	5	16:0 18:1	34	1	32	8.8	1.06	1.00		
sn-1,2(2,3)-	6	18:2 18:2	36	4	28	4.9			1.14	2.42
	7	18:1 18:2	36	3	30	6.8	1.40	6.62	1.15	2.78
	8	16:0 18:2	34	2	30	7.5	1.10	1.60	1.19	3.29
	9	18:1 18:1	36	2	32	9.7	1.30	5.30	1.17	3.10
	10	16:0 18:1	34	1	32	10.5	1.08	1.43	1.19	3.61

[a] *CN* = carbon number = total acyl carbon number of the two component fatty acids.
[b] *DB* = total double-bond number.
[c] Equivalent carbon number $ECN = CN - 2DB$.
[d] Retention time corrected for the column void volume.
[e] Between two successive diacylglycerols as listed in column 2.
[f] Between *sn*-1,3- and *sn*-1,2(2,3)-isomers of diacylglycerols with the same two component fatty acids.
[g] Separation factor between two peaks or ratio of the corrected retention times.
[h] Resolution factor between two peaks 1 and 2 calculated from the retention times (t_R) and the peak widths (w) according to the equation $R_s = 2(t_{R2} - t_{R1})/(w_2 + w_1)$.

TABLE II

SOME CHROMATOGRAPHIC PARAMETERS OF DIACYLGLYCEROL ISOMERS SEPARATED BY REVERSED-PHASE HPLC AS URETHANE DERIVATIVES AND LISTED ACCORDING TO ELUTION ORDER

Parameter	Diacylglycerol isomers[a]				
	sn-1,3- (18:2 18:2)	*sn*-1,2(2,3)- (18:2 18:2)	*sn*-1,3- (18:1 18:2)	*sn*-1,3- (16:0 18:2)	*sn*-1,2(2,3)- (18:1 18:2)
Equivalent carbon number, *ECN*[b]	36	36	36	34	36
Separation factor, α[c]		1.14	1.20	1.07	1.08
Resolution factor, R_s[d]		2.42	3.92	1.19	1.61
	sn-1,2(2,3)- (16:0 18:2)	*sn*-1,3- (18:1 18:1)	*sn*-1,3- (16:0 18:1)	*sn*-1,2(2,3)- (18:1 18:1)	*sn*-1,2(2,3)- (16:0 18:1)
Equivalent carbon number, *ECN*[b]	34	36	34	36	34
Separation factor, α[c]	1.10	1.11	1.06	1.10	1.08
Resolution factor, R_s[d]	1.60	2.06	1.00	2.15	1.43

[a] *sn*-1,3- and *sn*-1,2(2,3)-diacylglycerol isomers are listed according to their elution order (see Fig. 4).
[b–d] Definitions as in Table I.

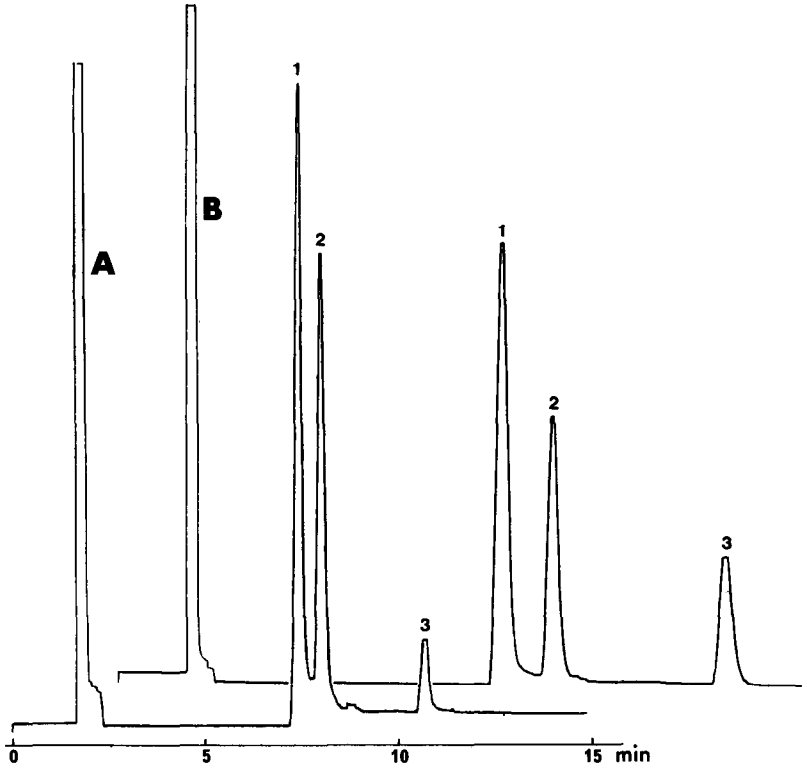

Fig. 4. RP-HPLC separations of 3,5-dinitrophenyl isocyanate derivatives of 1,2(2,3)-diacylglycerols originating from Grignard degradation of (A) peanut oil palmitoyloleoyllinoleoylglycerol (16:0 18:1 18:2) and (B) the same triacylglycerol of cottonseed oil at (A) 19°C and (B) 10°C. Other conditions as in Fig. 2B. Peaks: 1 = *sn*-1,2(2,3)-18:1 18:2; 2 = *sn*-1,2(2,3)-16:0 18:2; 3 = *sn*-1,2(2,3)-16:0 18:1.

TABLE III

SOME CHEMICAL AND CHROMATOGRAPHIC PARAMETERS OF *sn*-1,2(2,3)-DIACYLGLYCEROL URETHANE DERIVATIVES ANALYZED BY HPLC AT TWO DIFFERENT TEMPERATURES

sn-1,2(2,3)-Diacylglycerol	*ECN*[a]	19°C			10°C			t_R(10°C)/t_R(19°C)
		t_R[b]	α[c]	R_s[d]	t_R[b]	α[c]	R_s[d]	
18:1 18:2	30	6.0			8.2			1.37
16:0 18:2	30	6.6	1.10	0.92	9.6	1.17	1.23	1.45
16:0 18:1	32	9.2	1.39	4.08	14.1	1.47	3.91	1.53

[a-d] Definitions as in Table I.

rapid peak broadening with decreasing temperature. However, when lowering the analysis temperature to improve the resolution, the solubility in the eluting solvent of certain saturated diacylglycerols of long chain length can become a problem, in spite of the higher solubility of derivatized acylglycerols when compared with the underivatized compounds. In this instance, the use of longer columns at ordinary temperature would possibly represent a better alternative.

Quantitative analysis of diacylglycerol derivatives

In the stereospecific analysis of triacylglycerols, several quantitative aspects should be considered. First, deacylation with a Grignard reagent must generate representative diacylglycerols, *i.e.*, without any selective hydrolysis according to the nature or the positioning of fatty acids in the glycerol moiety. Second, the derivatization procedure must not modify the proportion and the fatty acid composition of the original diacylglycerols. Third, the derivatized diacylglycerols must be quantitatively detected by HPLC so that their proportion in a mixture can be easily calculated from the peak areas. These different aspects were examined in the following three series of experiments.

To check the absence of selectivity or of side-reactions in the derivatization procedure, twelve mixtures of natural source diacylglycerols derived from five oil triacylglycerols (Tables IV and V) were analysed in two ways. After isolation and before derivatization, an aliquot of the *sn*-1,2(2,3)-diacylglycerol mixtures was analysed by GC for fatty acid composition. The remainder was derivatized with 3,5-dinitrophenyl isocyanate and the derivatives were fractionated by HPLC. The collected HPLC fractions were analysed by GC in the presence of a known amount of heptadecanoic acid for determination of the fatty acid composition and fraction proportion. The overall fatty acid composition of the cumulated HPLC fractions was calculated from these data.

The results are reported in Table IV. The fatty acid composition of the fractionated diacylglycerol fractions easily allows their identification. Some contamination occurred between adjacent fractions, which explains some unexpected figures. For example, in the first sample, 16:0 18:1 was eluted immediately after 18:1 18:1 (they form a critical pair) and was contaminated by peak tailing. This explains the too high percentage of 18:1 as compared with 16:0 in this diacylglycerol. This type of contamination was taken into account in the calculation of the reconstituted total diacylglycerol fatty acid composition. This recalculated fatty acid composition can be compared in Table IV with the experimental values initially determined on the aliquot. There is great similarity between the two series of data, the average difference being only 2% with a maximum of 5.5%. We can therefore conclude that probably the derivatization procedure and HPLC separation did not induce any modification of the initial diacylglycerols (composition and proportion).

To check the quantitative detection of the diacylglycerol derivatives by differential refractometry in HPLC analysis, the proportions, as percentages, of the different fractions were calculated from the peak areas and from the fatty acid compositions of the collected fractions as above. The two series of data reported in Table V (third and fourth columns) can be compared and are found to be similar, the average difference not exceeding 3.8%. The greatest differences were observed for diacylglycerols 16:0 18:1 formed from the 2nd and 5th triacylglycerols (16:0 18:1 18:2) as

TABLE IV

PERCENTAGES AND FATTY ACID COMPOSITIONS OF THE *sn*-1,2(2,3)-DIACYLGLYCEROLS FORMED BY DEACYLATION OF NATURAL TRIACYLGLYCEROLS

	Peanut oil								
Triacylglycerols[a]	16:0	18:1	18:1		16:0	18:1	18:2		
Diacylglycerols[b]	18:1	18:1	16:0 18:1	Total	18:1	18:2	16:0 18:2	16:0 18:1	Total
(mol%)[c]	(41.7)	(58.3)	Calc.[d]	Exp.[e]	(46.4)	(42.4)	(11.2)	Calc.[d]	Exp.[e]
Fatty acids (mol%)									
16:0	1.5	45.7	27.3	27.2	1.1	41.6	47.1	23.2	22.2
18:1	98.4	53.9	72.3	72.1	49.3	4.0	48.1	29.9	29.5
18:2					48.9	53.7	1.8	45.6	44.6

	Peanut oil								
Triacylglycerols[a]	18:1	18:1	18:2		18:1	18:2	18:2		
Diacylglycerols[b]	18:1	18:2	18:1	18:1 Total	18:2	18:2	18:1	18:2 Total	
(mol%)[c]	(77.3)	(22.7)	Calc.[d]	Exp.[e]	(43.8)	(56.2)	Calc.[d]	Exp.[e]	
Fatty acids[f] (mol%)									
16:0									
18:1	49.6	97.2	60.4	60.0	2.2	50.4	29.3	28.1	
18:2	49.6	0.8	38.5	39.2	96.5	48.2	69.4	71.1	

	Cottonseed oil					
Triacylglycerols[a]	16:0	18:1	18:2			
Diacylglycerols[b]	18:1	18:2	16:0 18:2	16:0 18:1	Total	
(mol%)[c]	(48.1)	(30.3)	(21.6)	Calc.[d]	Exp.[e]	
Fatty acids[f] (mol%)						
16:0	1.1	47.7	50.0	25.8	25.9	
18:1	49.4	2.8	49.2	35.2	34.0	
18:2	49.3	48.9		38.5	39.6	

[a] Triacylglycerols isolated from peanut (the first four) and cottonseed (the last one) oils by combined argentation TLC and reversed-phase HPLC and deacylated by Grignard reaction.

[b] *sn*-1,2(2,3)-Diacylglycerols formed by deacylation of natural triacylglycerols, isolated on borate-impregnated silica TLC plates, derivatized with 3,5-dinitrophenyl isocyanate and fractionated by reversed-phase HPLC.

[c] Percentages of the diacylglycerol derivative fractions calculated from the fatty acid composition of the collected fractions after addition of a known amount of heptadecanoic acid. Contamination between peaks was not corrected for.

[d] Fatty acid composition of total diacylglycerol derivatives calculated from the percentages and the fatty acid compositions of the fractions isolated by HPLC.

[e] Fatty acid composition of the total diacylglycerols before derivatization.

[f] Traces ($< 1.5\%$) of 18:0 and 16:1 are not reported but were taken into account in the percentage calculations.

TABLE V

PERCENTAGES OF THE *sn*-1,2(2,3)-DIACYLGLYCEROLS FORMED BY DEACYLATION OF TRIACYLGLYCEROLS AND FRACTIONATED AS URETHANE DERIVATIVES BY REVERSED-PHASE HPLC

Triacylglycerols[a]	Diacylglycerols[b]	Concentration (mol%)		
		Peak areas[c]	Fatty acids[d]	*sn*-2-MG[e]
16:0 18:1 18:1	18:1 18:1	46.9	46.1	47.9
	16:0 18:1	53.1	53.9	52.0
16:0 18:1 18:2	18:1 18:2	49.2	48.1	49.1
	16:0 18:2	42.2	41.4	40.1
	16:0 18:1	8.6	10.5	10.8
18:1 18:1 18:2	18:1 18:2	77.9	77.6	78.1
	18:1 18:1	22.1	22.4	21.9
18:1 18:2 18:2	18:2 18:2	44.8	43.3	44.0
	18:1 18:2	55.2	56.7	56.0
16:0 18:1 18:2	18:1 18:2	50.5	48.4	48.5
	16:0 18:2	29.4	29.8	30.4
	16:0 18:1	20.1	21.8	21.1

[a] Triacylglycerols isolated from peanut oil (the first four) and cottonseed oil (the last) by combined argentation TLC and reversed-phase HPLC.

[b] *sn*-1,2(2,3)-Diacylglycerol fractions separated as derivatives and collected by reversed-phase HPLC.

[c] Percentages calculated from peak areas registered in HPLC analysis of the mixture of diacylglycerol derivatives with refractometric detection (without any area correction according to fatty acids). Results are means of 3–5 different analyses.

[d] Percentages calculated from the fatty acid composition of the collected fractions after addition of a known amount of heptadecanoic acid.

[e] Percentages calculated from the percentages of *sn*-2-monoacylglycerols, as reported in the text.

listed in the table (18% and 8%, respectively). For better separation of the first two fractions, the HPLC analysis was carried out at 12°C. The last fraction, 16:0 18:1, was eluted late and the peak area was probably underestimated. However, under identical analytical conditions and with the range of fatty acids used in this work, we can conclude that the proportion of the different fractions of diacylglycerols separated by HPLC as urethane derivatives and detected by their refractive indices can be calculated from peak areas. Application of correction factors to peak areas according to the constituent fatty acids did not appear to be necessary for the samples analysed.

To check whether the *sn*-1,2(2,3)-diacylglycerols formed by hydrolysis were representative of the original triacylglycerols, their theoretical percentages were calculated from the *sn*-2-monoacylglycerol composition after enzymatic (pancreatic lipase) hydrolysis, assumed to generate representative *sn*-2-monoacylglycerols. An example is as follows: after hydrolysis of the triacylglycerol 16:0 18:1 18:1 by rat pancreatic lipase, the composition of the *sn*-2-monoacylglycerol was 16:0 4.1% and 18:1 95:9%. The two *sn*-1,2(2,3)-diacylglycerols theoretically formed in equal proportions from the *sn*-2-triacylglycerol 16:0 18:1 18:1 (18:1 at internal position) would be 16:0 18:1 47.95% and 18:1 18:1 (47.95%). The unique diacylglycerol formed from 18:1 16:0 18:1 would be 16:0 18:1 4.1%. The theoretical proportions of *sn*-1,2(2,3)-diacylglycerols would be 16:0 18:1 52.05% and 18:1 18:1 47.95%.

The results obtained are reported in Table V (last column). They can be compared with the results given in the fourth column. Both series of figures are very similar, the average difference being only 2.1% with a maximum of 3.7%. If it is considered that pancreatic lipase forms representative *sn*-2-monoacylglycerols, the *sn*-1,2(2,3)-diacylglycerols formed by deacylation also appear to be representative of the hydrolysed triacylglycerols.

In conclusion, the results reported in this section clearly show that representative *sn*-1,2(2,3)-diacylglycerols are generated by deacylation of triacylglycerols, that they can be accurately analysed by reversed-phase HPLC using differential refractometric detection and that they can be fractionated by HPLC for further stereospecific analysis on a chiral column.

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