HPLC resolution of diacylglycerol moieties of natural triacylglycerols on a chiral phase consisting of bonded (R)-(+)-1-(1-naphthyl)ethylamine

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Abstract Chiral phase high performance liquid chromatographic resolution of *sn*-1,2(2,3)- and X-1,3-diacylglycerols generated by partial Grignard degradation from natural triacylglycerols was carried out using a chiral column (25 cm × 4.6 mm i.d.) containing (R)-(+)-1-(1-napthyl)ethylamine polymer chemically bonded to 300A wide pore spherical silica (5 μm particles). The diacylglycerols were chromatographed as 3,5-dinitrophenylurethanes and detected at 226 or 254 nm UV. By an isocratic elution with n-hexane– 1,2-dichloroethane–ethanol 40:10:1 (v/v/v) as the mobile phase, the *sn*-1,2(2,3)-diacylglycerols from corn, linseed, and menhaden oils were resolved into two clearly distinguishable enantiomer groups, although some peak overlappings between the enantiomers were observed in the linseed and menhaden oil diacylglycerols. In addition to the excellent enantiomer resolution, each enantiomer and the X-1,3-isomers were partially resolved into several peaks, which could be tentatively identified on the basis of equivalent carbon number. It is concluded that chiral phase high performance liquid chromatography can be utilized for effective resolution, identification, and quantitation of enantiomeric diacylglycerols from complex natural mixtures.— **Itabashi, Y., A. Kuksis, L. Marai, and T. Takagi.** HPLC resolution of diacylglycerol moieties of natural triacylglycerols on a chiral phase consisting of bonded (R)-(+)-1-(1-naphthyl)ethylamine. *J. Lipid Res.* 1990. **31:** 1711–1717.

Supplementary key words *sn*-1,2-diacylglycerols • *sn*-2,3-diacylglycerols • X-1,3-diacylglycerols • molecular species • dinitrophenylurethanes • equivalent carbon number • thin-layer chromatography • corn oil • linseed oil • menhaden oil

Michelsen et al. (1) reported a partial resolution of enantiomeric *sn*-1,2- and *sn*-2,3-diacylglycerols as diastereomeric 1-(1-naphthyl)ethylurethanes by high performance liquid chromatography (HPLC) using an achiral silica column. Subsequently, Itabashi and Takagi (2) reported a complete resolution of the enantiomeric diacylglycerols as 3,5-dinitrophenylurethanes (3,5-DNPUs) by HPLC on a chiral liquid phase consisting of N-(S)-2-(4-chlorophenyl)isovaleroyl-(R)-phenylglycine. For this purpose, however, a long column (75 cm) and extremely long elution times (several hours) were required. Recently, Takagi and Itabashi (3) presented an improved separation of enantiomeric diacylglycerols as the 3,5-DNPU derivatives using another type of chiral phase, N-(R)-1-(1-naphthyl)-ethylaminocarbonyl-(S)-valine. This method permitted complete enantiomer resolution within 10 min on a 25-cm-long column.

All these studies were done with synthetic diacylglycerols, and have not been applied to the resolution of enantiomeric diacylglycerols derived from natural sources. This report describes the HPLC resolution of the enantiomeric *sn*-1,2(2,3)- and isomeric X-1,3-diacylglycerols derived from natural sources on a (R)-1-(1-naphthyl)ethylamine column, which has higher enantioselectivity for the diacylglycerols as 3,5-DNPUs than previously used chiral columns (2, 3). A highly satisfactory separation was obtained between the enantiomeric diacylglycerols derived by Grignard degradation from corn, linseed, and menhaden oil triacylglycerols.

MATERIALS AND METHODS

Acylglycerols

Trioleoylglycerol (99%) and monoacid 1,2-diacyl-*rac*-glycerols of palmitic and oleic acids, and optically active 1,2-dipalmitoyl-*sn*-glycerol were obtained from Sigma (St. Louis, MO). Corn (Mazola), linseed, and menhaden (Zapata Haynie Corp., Reedville, VA) oils were commercial products and the triacylglycerols were isolated by silicic acid thin-layer chromatography (TLC) with petroleum ether–diethyl ether 7:3 (v/v) as the developing solvent.

Abbreviations: HPLC, high performance liquid chromatography; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; LC/CIMS, liquid chromatography with chemical ionization mass spectrometry; 3,5-DNPU, 3,5-dinitrophenylurethanes; ECN, equivalent carbon number; Vr, retention volume; 16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid, 18:3, linolenic acid; 20:5, eicosapentaenoic acid; 22:6, docosahexaenoic acid.

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sn-1,2(2,3)- and X-1,3-Diacylglycerols were generated by partial Grignard degradation from the triacylglycerols (100 mg) and were purified by boric acid-TLC as described previously (4).

Derivatives

The 3,5-DNPU derivatives of the diacylglycerols were prepared by reacting 1 mg of free diacylglycerols and about 2 mg of 3,5-dinitrophenyl isocyanate (Sumitomo Chemical Co., Ltd., Osaka, Japan) in 400 μl dry toluene in the presence of 40 μl dry pyridine for 1 h at room temperature (2). The resulting 3,5-DNPUs were purified by TLC on a silicic acid plate (20 cm × 20 cm, 0.25-mm-thick layer) containing a fluorescence indicator (Kodak, Rochester, NY). Prior to use, the plate was activated at 110°C–120°C for 1 h. The reaction mixture, dissolved in 400 μl of chloroform, was spotted on the plate and developed up to 15 cm using petroleum ether–1,2-dichloroethane–ethanol 40:10:3 (v/v/v) as the developing solvent. Bands were visualized under UV and the 3,5-DNPU fraction (R_f 0.5–0.6) was scraped off the plate. The pure 3,5-DNPUs were recovered from the absorbent by ether extraction.

Chiral phase HPLC

The resolution of enantiomeric *sn*-1,2- and *sn*-2,3-, and the isomeric X-1,3-diacylglycerols as their 3,5-DNPU derivatives was performed on a Hewlett-Packard Model 1084 liquid chromatograph equipped with a chiral column (25 cm × 4.6 mm i.d.) containing (R)-(+)-1-(1-naphthyl)ethylamine polymeric phase covalently bonded to 300A wide pore spherical silica (5 μm particles, YMC-Pack A-K03, YMC Inc., Kyoto, Japan). The analysis was done isocratically at 28°C using n-hexane–1,2-dichloroethane–ethanol 40:10:1 (v/v/v) as the mobile phase at a constant flow rate of 0.5–1 ml/min. Usually 10–20 μg of the 3,5-DNPUs dissolved in the same solvent as the mobile phase was injected on the column using an automatic sample injector. Peaks were monitored at 226 (λ_{max}) or 254 nm, which gave essentially the same chromatograms (2,5), using a variable wavelength detector.

RESULTS AND DISCUSSION

Resolution of synthetic diacylglycerols

Fig. 1 shows the chiral phase HPLC resolution of the 3,5-DNPUs of synthetic *sn*-1,2-, *sn*-2,3-, and X-1,3-dipalmitoylglycerols on the (R)-(+)-1-(1-naphthyl)- ethylamine column (A-K03). In addition to the excellent resolution of the *sn*-1,2- and *sn*-2,3-enantiomers in a short elution time, the X-1,3-isomer is eluted well ahead of the *sn*-1,2-enantiomer with complete baseline resolution. The elution order of each diacylglycerol on the A-K03 column was the same as that on the previously used chiral columns. N-(S)-2-(4-chlorophenyl)isovaleroyl-(R)-phenylglycine (OA-2100) (2) and N-(R)-1-(1-naphthyl)ethyl-aminocarbonyl-(S)-valine (OA-4100) (3). This suggests that the separation mechanism of the 3,5-DNPUs of diacylglycerols on these chiral columns is essentially the same. Generally enantiomer resolution on the chiral columns is mainly due to attractive interactions between solutes and chiral stationary phases, i.e., hydrogen bonding, π–π complexing, and dipole stacking (6). Therefore, the 3,5-DNPU group in the *sn*-1-position of diacylglycerols would interact more strongly with the chiral stationary phases than that in the *sn*-3-position. The faster elution of the X-1,3-isomer than *sn*-1,2- and *sn*-2,3-enantiomers is probably due to its lesser polarity. Similar results were obtained in the chiral phase HPLC of synthetic diacylglycerols using OA-2100 and OA-4100 columns (2,3).

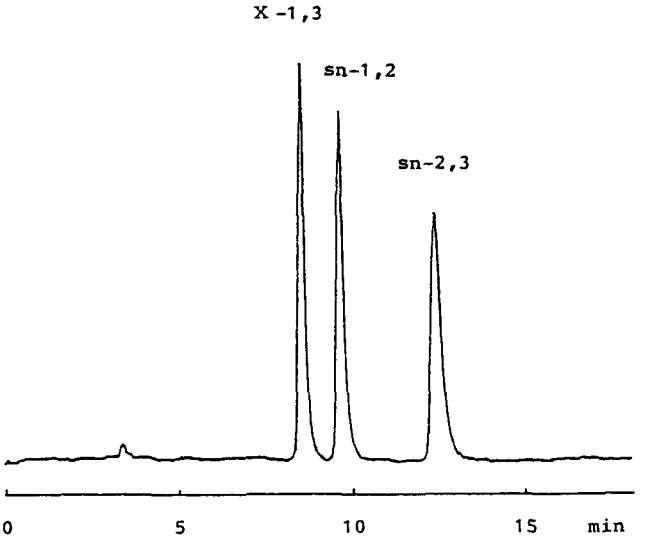

Fig. 1. Chiral phase HPLC resolution of a mixture of X-1,3-, *sn*-1,2, and *sn*-2,3-diapalmitoylglycerols as 3,5-dinitrophenylurethanes. Solvent system: n-hexane–1,2-dichlorethane–ethanol 40:10:1 (v/v/v). Flow rate, 1 ml/min. Other HPLC conditions as given in the text.

Fig. 2 shows the chiral phase HPLC resolution of the 3,5-DNPUs of X-1,3- and *rac*-1,2-dioleoylglycerols generated by partial Grignard degradation from trioleoylglycerol on the A-KO3 column. Again excellent enantiomer resolution with a 1:1 peak area ratio was obtained for the racemate of this unsaturated diacylglycerol (Fig. 2B). As in case of dipalmitoylglycerols, the X-1,3-isomer eluted ahead of the *sn*-1,2-enantiomer with a complete baseline resolution (Fig. 2A). Fig. 2 also confirms that the X-1,3- and *rac*-1,2- dioleolyglycerols were resolved without cross-contamination and isomerization by preparative boric acid-TLC.

Table 1 compares the chromatographic data on three columns containing different chiral stationary phases. As indicated by the separation factors, the A-KO3 column used in this study showed the highest enantioselectivity for the diacylglycerols. On the other hand, the best resolu-

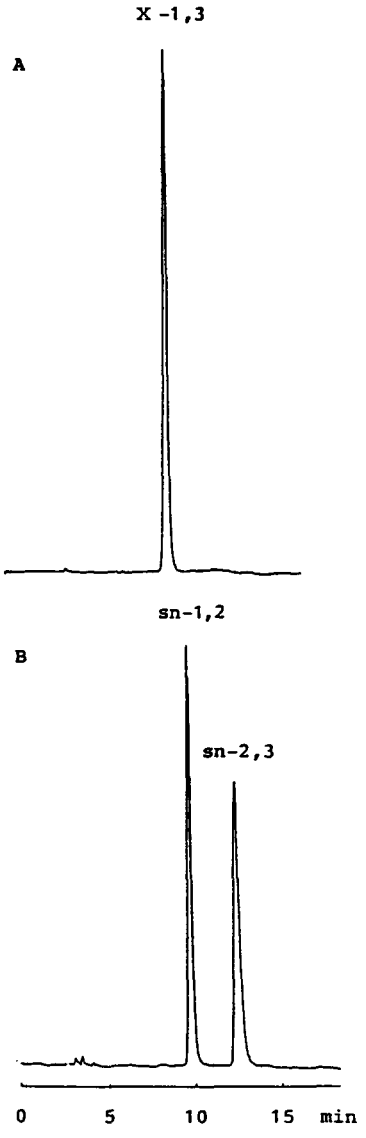

Fig. 2. Chiral phase HPLC resolution of *sn*-2,3- and *rac*-1,2-dioleoylglycerols generated by Grignard degradation from trioleoylglycerol, as 3,5-dinitrophenylurethanes. Solvent system, as in Fig. 1. Flow rate, 1 ml/min. Other HPLC conditions as given in the text.

tion between X-1,3-isomers and the enantiomers was obtained on the OA-2100 column. The dioleoyl- and dipalmitoylglycerols with the same configuration were not effectively resolved under these HPLC conditions, although small differences were seen between their retention volumes.

To resolve the critical pair (7) of diacylglycerols, 16:0–16:0 and 18:1–18:1, both possessing an equivalent carbon number (ECN) 32, we replaced 1,2-dichloroethane or ethanol in the mobile phase by acetonitrile, a common solvent in reversed phase HPLC of acylglycerols. **Fig. 3** shows the resolution of this critical pair resulting from changing the mobile phase. The increased resolution is probably caused by an increase in column efficiency. We obtained 7,200 theoretical plates for the *sn*-2,3-dioleoylglycerol peak with n-hexane–1,2-dichloroethane–ethanol 40:10:1 (v/v/v), while the use of isooctane–methyl tertiary butylether–acetonitrile–2-propanol 80:10:5:5 (v/v/v/v) or n-hexane–1,2,dichloroethane–acetonitrile 85:10:5 (v/v/v) doubled the plate value. The separation factor for the enantiomer resolution, however, decreased from 1.42 to 1.20 by the mobile phase change. To retain satisfactory enantiomer resolution for the diacylglycerols derived from natural triacylglycerols, therefore, we used the original mobile phase containing both 1,2-dichloroethane and alcohol, which had given excellent enantiomer resolutions of other alcohols on chiral phases containing amide groups (8–10).

Resolution of natural diacylglycerols

The natural oils were selected to yield diacylglycerols with a wide range of ECN to provide maximum opportunity for overlap among enantiomeric species. **Fig. 4** shows the chiral phase HPLC resolution of the 3,5-DNPUs of the X-1,3- and *sn*-1,2(2,3)-diacylglycerols, generated by partial Grignard degradation of corn oil triacylglycerols, on the A-KO3 column. The *sn*-1,3-diacylglycerols were split into three major peaks due to the pairing of different fatty acids in the molecules (Fig. 4A). The *sn*-1,2(2,3)-diacylglycerols were clearly resolved into two groups with equal total peak areas, representing the *sn*-1,2- and *sn*-2,3-enantiomers (Fig. 4B). The elution profiles of each *sn*-1,2- and *sn*-2,3-enantiomer, which were also split into three major peaks each, are quite similar to that of the X-1,3-diacylglycerols. This is due to the nearly random distribution of the major fatty acids and the symmetrical positional placement of the minor fatty acids in corn oil triacylglycerols (11). This similarity in the elution profiles confirms the clear-cut resolution between *sn*-1,2- and *sn*-2,3-enantiomers. The enantiomeric X-1,3-diacylglycerols were not resolved under the present working conditions, but Takagi et al. (12) have demonstrated chiral phase HPLC resolution of 1-hexadecyl-3-hexadecanoyl-*rac*-glycerols as the 3,5-DNPU derivatives.

Since the chiral stationary phase used in this study has a polar functional group, 1-(1-naphthyl)ethylamine, covalently bonded to silica gel, the diacylglycerols as 3,5-DNPUs elute from the column in order of increasing double bond and decreasing carbon number. This phenomenon has been observed in the HPLC of mono- and diacylglycerols as their 3,5-DNPUs on the chiral columns used previously (2, 3, 5) and in normal phase HPLC of fatty acids (7) and triacylglycerols (13) on silica columns. Therefore, palmitoyl (16:0) and oleoyl (18:1) residues are equivalent in their retention volume, and one double bond increases the retention time by two methylene units on the chiral column when using n-hexane-1,2-dichloroethane–ethanol 40:10:1 as the mobile phase. Accordingly, the diacylglycerols as 3,5-DNPUs elute from the chiral column in order of decreasing ECN

TABLE 1. Chiral phase HPLC of diacylglycerols as 3,5-dinitrophenylurethanes

Molecular Species	Chiral Column								
	A-KO3[a]			OA-4100[b]			OA-2100[c]		
	Vr[d]	k'[e]	a[f]	Vr	k'	a	Vr	k'	a
Dipalmitoylglycerol									
X-1,3-	5.10	1.50		5.81	2.17		51.9	6.99	
			1.23			1.07			1.35
sn-1,2-	6.24	1.84		6.22	2.32		70.2	9.46	
			1.44			1.15			1.03
sn-2,3-	9.00	2.65		7.15	2.67		72.4	9.75	
Dioleoylglycerol									
X-1,3-	5.14	1.51		5.99	2.24		51.9	6.99	
			1.23			1.04			1.33
sn-1,2-	6.28	1.85		6.24	2.33		68.2	9.27	
			1.44			1.15			1.03
sn-2,3-	9.03	2.67		7.18	2.68		71.0	9.56	

[a]A 25 cm × 4.6 mm i.d. column containing (R)-(+)-1-(1-naphthyl)ethylamine; mobile phase, n-hexane–1,2-dichloroethane–ethanol 40:10:1 (v/v/v) (this study).

[b]A 25 cm × 4 mm i.d. column containing N-(R)-1-(1-naphthyl)ethylaminocarbonyl-(S)-valine; mobile phase, n-hexane–1,2-dichloroethane- ethanol 80:20:1 (v/v/v); data from Takagi and Itabashi (3).

[c]Three 25 cm × 4 mm i.d. columns connected in series containing N-(S)-2-(4-chlorophenyl)isovaleroyl-(R)-phenylglycine; mobile phase, n-hexane–ethanol 125:1 (v/v); data from Itabashi and Takagi (2).

[d]Vr, retention volume (ml) corrected by subtracting the column void volume (3.40 ml).

[e]k', Capacity ratio.

[f]a, Separation factors of *sn*-1,2-/*sn*-1,3- and *sn*-2,3-/*sn*-1,2-diacylglycerols.

values. On the basis of this relationship and a comparison of the molecular species resolution obtained by gas–liquid chromatography (GLC) on polar cyanosiloxanes (14, 15), the numbered peaks in Fig. 4 were tentatively identified as follows: peak 1 (ECN = 32), palmitoyloleoyl- (16:0–18:1) and dioleoylglycerols (18:1–18:1); peak 2 (ECN = 30), palmitoyllinoleoyl- (16:0–18:2) and oleoyllinoleoylglycerols (18:1–18:2), and peak 3 (ECN = 28), dilinoleoylglycerols (18:2–18:2). No reverse isomer resolution was observed on the chiral column under these conditions. While the X-1,3- and *sn*-1,2-diacylglycerols with the same fatty acids are completely resolved on the chiral column (Fig. 1), mixtures of molecular species made up of different fatty acids give some peak overlap between the X-1,3-diacylglycerols with a high degree of unsaturation and *sn*-1,2-diacylglycerols with a low degree of unsaturation (see also Fig. 6). Therefore, a preliminary separation of the X-1,3- and the *sn*-1,2(2,3)-diacylglycerols may be necessary before chiral phase HPLC. The most extensive TLC separation is obtained with the free diacylglycerols on boric acid-treated silica gel (12), but the 3,5-DNPU derivatives are also well resolved on plain silicic acid (results not shown).

Fig. 5 shows the chiral phase HPLC resolution of the 3,5-DNPUs of the X-1,3- and *sn*-1,2(2,3)-diacylglycerols generated by partial Grignard degradation from linseed oil triacylglycerols on the A-KO3 column. The X-1,3-diacylglycerols of linseed oil are split into more peaks than those derived from corn oil (Fig. 5A). This complexity is

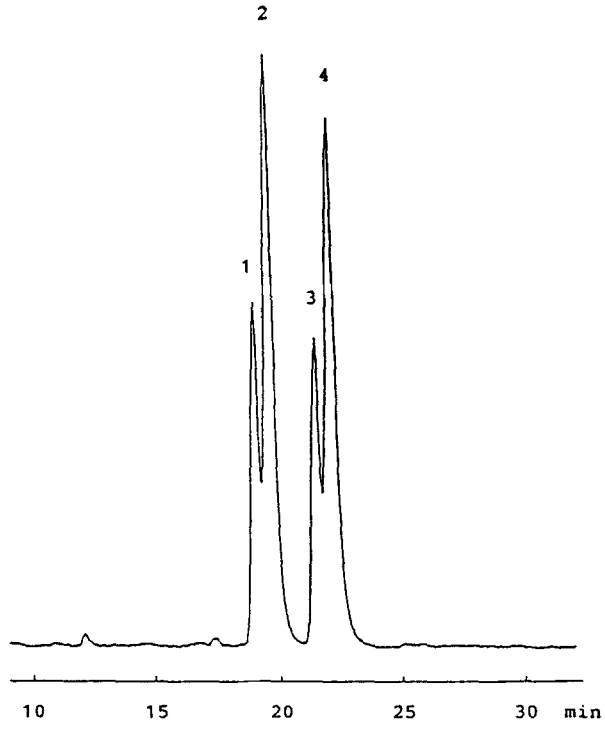

Fig. 3. Chiral phase HPLC resolution of a mixture of racemic dipalmitoyl- and dioleoylglycerols as 3,5-dinitrophenylurethanes. 1, *sn*-1,2-dipalmitoylglycerol; 2, *sn*-1,2-dioleoylglycerol; 3, *sn*-2,3-dipalmitoylglycerol; 4, *sn*-2,3-dioleoylglycerol. Mobile phase: isooctane–methyl tertiary butyl ether–acetonitrile–isopropanol 80:15:5:5 (v/v/v/v); Flow rate, 0.5 ml/min. Other HPLC conditions as given in the text.

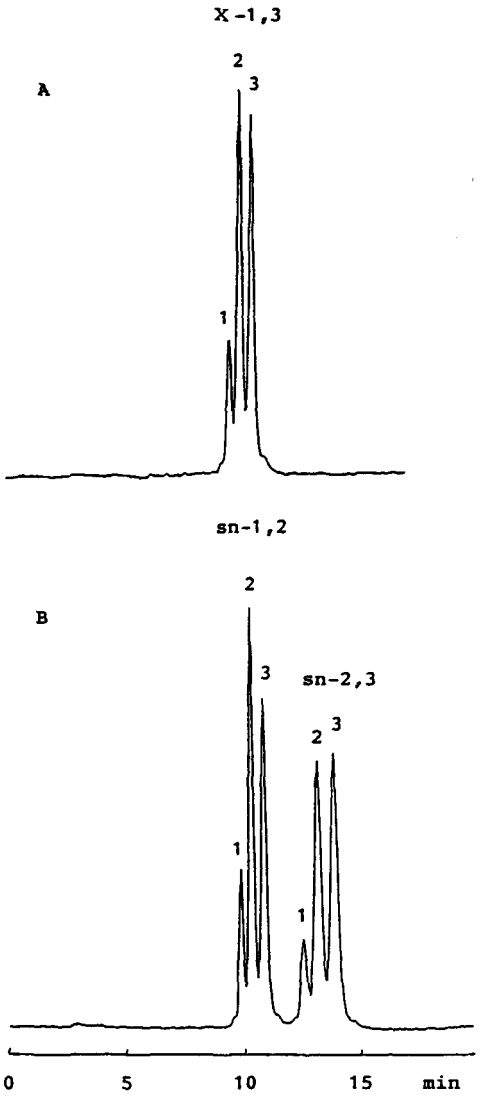

Fig. 4. Chiral phase HPLC resolution of the diacylglycerol moieties of corn oil triacylglycerols, as 3,5-dinitrophenylurethanes. A, X-1,3-diacylglycerols; B, *sn*-1,2(2,3)-diacylglycerols. Peaks were tentatively identified as described in the text. 1, 16:0–18:1 + 18:1–18:1; 2, 16:0–18:2 + 18:1–18:2; 3, 18:2–18:2. Solvent system, as in Fig. 1. Flow rate, 1 ml/min. Other HPLC conditions as given in the text.

caused by the presence of linolenic acid as a main component of linseed oil. The *sn*-1,2(2,3)-diacylglycerols are resolved into two clearly distinguishable groups, which represent the *sn*-1,2- and *sn*-2,3-enantiomers, although some peak overlapping between *sn*-1,2-enantiomers of high degree of unsaturation and *sn*-2,3-enantiomers of low degree of unsaturation is also observed (Fig. 5B). The elution profiles of the X-1,3-, *sn*-1,2-, and *sn*-2,3-diacylglycerols of linseed oil are quite similar, which again is due to a nearly random positional placement of the major fatty acids (11). The elution profile of the X-1,3-diacylglycerols has considerable similarity to that obtained by GLC on polar cyanosiloxanes (14), although the GLC system yielded more peaks. From a knowledge of the molecular species resolution on polar GLC columns and the ECN concept, the numbered peaks obtained on chiral phase HPLC in Fig. 5 were tentatively identified as follows: peak 1 (ECN = 34), stearoyloleoylglycerol (18:0–18:1); peak 2 (ECN = 32), palmitoyloleoyl- (16:0–18:1), dioleoyl- (18:1–18:1), and stearoyllinoleoylglycerols (18:0–18:2); peak 3 (ECN = 30), palmitoyllinoleoyl- (16:0–18:2), stearoyllinolenoyl- (18:0–18:3), and oleoyllinoleoylglycerols (18:1–18:2); peak 4 (ECN = 28), palmitoyllinolenoyl- (16:0–18:3), oleoyllinolenoyl- (18:1–18:3), and dilinoleoylglycerols (18:2–18:2); peak 5 (ECN = 26), linoleoyllinolenoylglycerol (18:2–18:3), and peak 6 (ECN = 24), dilinolenoylglycerols (18:3–18:3). **Fig. 6** shows plots of log retention volumes (Vr) versus the ECN values for the X-1,3-, *sn*-1,2-, and *sn*-2,3-diacylglycerols. Linear relationships are observed for the respective diacylglycerols. This

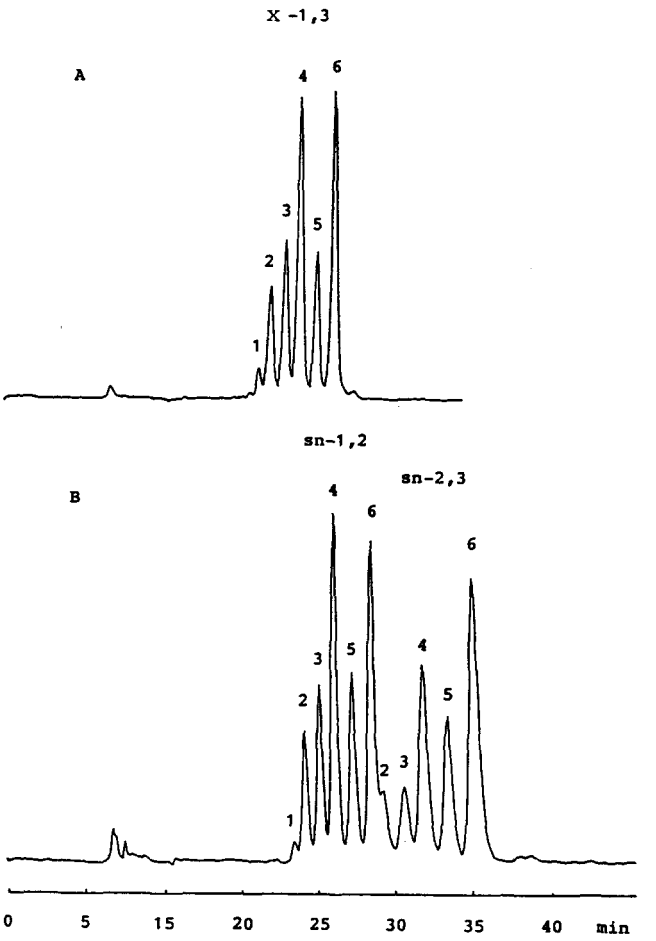

Fig. 5. Chiral phase HPLC resolution of the diacylglycerol moieties of linseed oil triacylglycerols, as 3,5-dinitrophenylurethanes. A, X-1,3-diacylglycerols; B, *sn*-1,2(2,3)-diacylglycerols. Peaks were tentatively identified as described in the text. 1, 18:0–18:1; 2, 16:0–18:1 + 18:1–18:1 + 18:0–18:2; 3, 16:0–18:2 + 18:0–18:3 + 18:1–18:2; 4, 16:0–18:3 + 18:1–18:3 + 18:2–18:2; 5, 18:2–18:3; 6, 18:3–18:3. Solvent system, as in Fig. 1. Flow rate, 0.5 ml/min. Other HPLC conditions as given in the text.

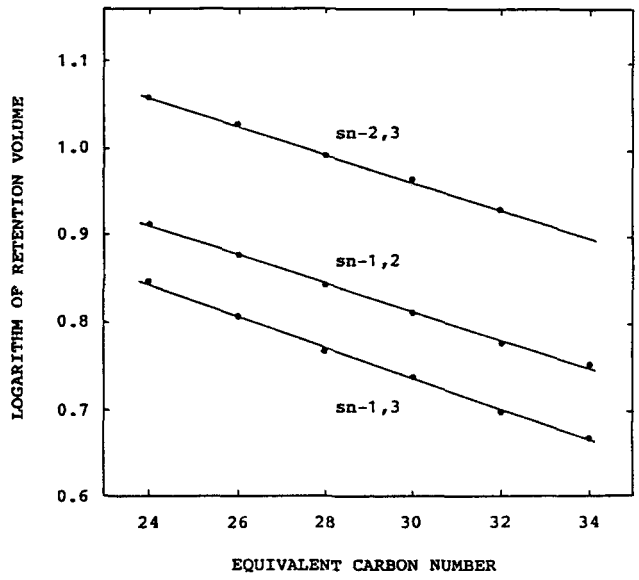

Fig. 6. Plots of log retention volume versus equivalent carbon number for *sn*-1,3-, *sn*-1,2, and *sn*-2,3-diacylglycerols resolved as 3,5-dinitrophenylurethanes by chiral phase HPLC. HPLC conditions, as in Fig. 5.

supports the hypothesis that the diacylglycerols are resolved according to their ECN values on the chiral column. The differences in log Vr between enantiomers having the same ECN values were almost equal. Therefore, the following relationship is applicable

$$\log Vr(sn\text{-}2,3) = \log Vr(sn\text{-}1,2) + 0.15 \qquad Eq.\ 1)$$

where Vr(*sn*-2,3) and Vr(*sn*-1,2) indicate the retention volumes of the *sn*-2,3- and *sn*-1,2-enantiomers, respectively, having the same ECN values. On the other hand, the differences between the enantiomers and the X-1,3-diacylglycerols increased slightly with increasing ECN value. This nonparallel relationship suggests that the resolution between X-1,3- and *sn*-1,2-diacylglycerols having the same ECL value is better for diacylglycerols having a low degree of unsaturation than for those having a higher degree of unsaturation. For the X-1,3- and *sn*-1,2-diacylglycerols, the following relationship is applicable

$$\log Vr(sn\text{-}1,2) = \log Vr(X\text{-}1,3) + F \qquad Eq.\ 2)$$

where Vr(X-1,3) indicates the retention volumes of the X-1,3-diacylglycerols having the same ECN value as *sn*-1,2-diacylglycerols, and F indicates a constant (0.07 for ECN = 24, 26, and 28; 0.08 for ECL = 30 and 32; 0.09 for ECL = 34). Fig. 6 also shows that the Vr of a X-1,3-diacylglycerol is approximately equal to that of an *sn*-1,2-diacylglycerol having an ECL value higher by 4, e.g., X-1,3-dilinolenoyl- (18:3–18:3) and *sn*-1,2-dilinoleoyl-glycerols (18:2–18:2). Such pairs may be very difficult to resolve on the chiral column.

Fig. 7 shows the chiral phase HPLC of the 3,5-DNPUs of the X-1,3- and *sn*-1,2(2,3)-diacylglycerols generated by partial Grignard degradation from menhaden oil triacylglycerols on the A-KO3 column. The chromatogram of the X-1,3-diacylglycerols is relatively simple despite the presence of very complex fatty acid pairings (Fig. 7A). Individual peaks, however, are broad when compared to those of corn and linseed oil diacylglycerols, and no clear-cut resolution among the peaks is obtained. The peak broadening and asymmetry is due to extensive overlapping of molecular species, which is caused by the occurrence of a wide variety of diacylglycerol species containing different polyunsaturated fatty acids. Nevertheless, the *sn*-1,2(2,3)-diacylglycerols from the fish oil are effectively resolved into two clearly distinguishable enantiomer groups. Some peak overlapping is observed between the more polar *sn*-1,2- and the less polar *sn*-2,3-enantiomers (Fig. 7B). The similarity in the elution profiles seen for the pure X-1,3-diacylglycerols and the *sn*-1,2-diacylglycer-

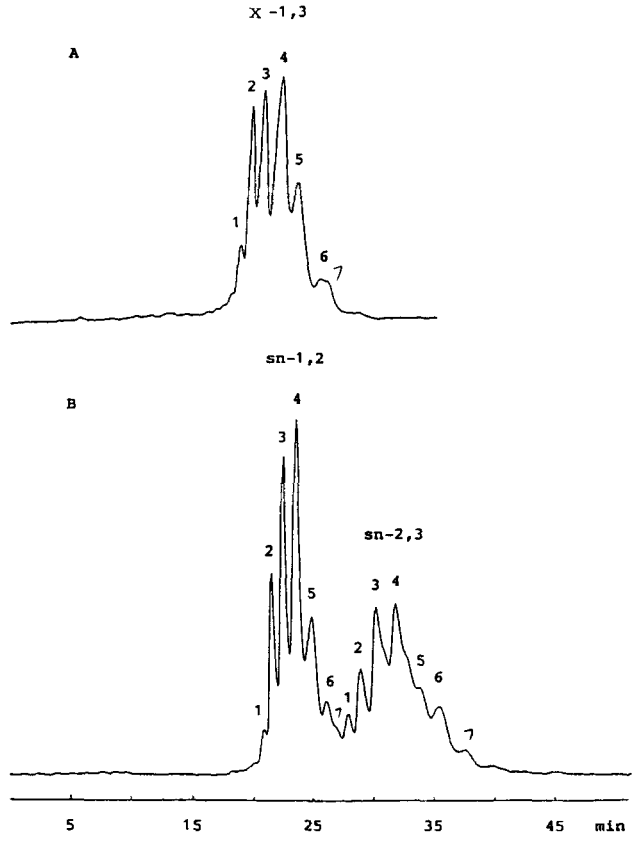

Fig. 7. Chiral phase HPLC resolution of the diacylglycerol moieties of menhaden oil triacylglycerols, as dinitrophenylurethanes. A, X-1,3-diacylglycerols; B, *sn*-1,2(2,3)-diacylglycerols. The peak identification was confirmed by chiral phase LC/NCIMS (16) as follows: 1, ECN = 32; 2, ECN = 30; 3, ECN = 28; 4, ECN = 26; 5, ECN = 24; 6, ECN = 22; 7, ECN = 20. Solvent system, as in Fig. 1. Flow rate, 0.8 ml/min. Other HPLC conditions as given in the text.

ols resolved from a mixture of enantiomers suggests that a satisfactory resolution was obtained also between the *sn*-1,2- and *sn*-2,3-enantiomers. Thus, the peaks with the same numbers in Fig. 7 would be anticipated to contain molecular species of the same fatty acid pairing. The relationships indicated by equations 1 and 2 were also observed for peaks 1, 2, and 3 in the X-1,3-diacylglycerols and peaks 1, 2, 3, and 4 in the *sn*-1,2- and *sn*-2,3-enantiomers. The F value in equation 2 was 0.05, which is slightly smaller in comparison with that observed for the polyunsaturated linseed oil diacylglycerols. Therefore, the chiral HPLC columns do not appear to retain the polyunsaturated fatty acid-containing diacylglycerols as strongly as would be anticipated from their ECN values. Nevertheless, the diacylglycerols containing two eicosapentaenoyl- (20:5–20:5), eicosapentaenoyl and docosahexaenoyl- (20:5–22:6) and two docosahexaenoyl- (22:6–22:6) fatty acids, which have the same ECN value of 20, were eluted together and after the diacylglycerols with ECN of 22 (e.g., 18:4–18:3). The elution order of the polyunsaturated diacylglycerols as the 3,5-DNPU derivatives was confirmed by chiral phase LC/MS with chloride-attachment negative chemical ionization (16). The observations suggest that the elution order of the diacylglycerols containing 20:5 and 22:6 acids from the chiral column under the present conditions also depends on their ECN values.

The present study demonstrates that useful elution profiles are obtained by chiral phase HPLC for the X-1,3-*sn*-1,2-, and *sn*-2,3-diacylglycerols derived from natural triacylglycerols. As shown in Fig. 4B, the *sn*-1,2-, and *sn*-2,3-diacylglycerols containing only 16:0, 18:0, 18:1, and 18:2 acids can be resolved without any overlapping of enantiomers. The peaks can be collected and the fatty acid composition of each of the enantiomers can be determined by GLC to establish their identity. Since the *sn*-1,2(2,3)-diacylglycerols containing fatty acids more highly unsaturated than 18:2 are not completely resolved, peak collection may not be practical, unless much longer columns or liquid phases with still higher enantioselectivity are utilized. Therefore, the exact identification and quantitation of individual molecular species in these enantiomers must be obtained by others means, such as chemical ionization mass spectrometry coupled with the chiral column HPLC (LC/CIMS) (16) or polar capillary GLC of the diacylglycerols (17) retrieved from the 3,5-DNPUs. The results of these studies are currently being prepared for publication.

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