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# High-performance liquid chromatographic resolution of reverse isomers of 1,2-diacyl-*rac*-glycerols as 3,5-dinitrophenylurethanes

Yutaka Itabashi[1], John J. Myher, Arnis Kuksis*

*Banting and Best Department of Medical Research, C.H. Best Institute, University of Toronto, 112 College Street, Toronto, Ontario M5G 1L6, Canada*

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**Abstract**

The resolution of reverse isomers remains a major unsolved problem in glycerolipid chromatography. We have investigated the separation of the reverse isomers of 1,2-diacyl-*rac*-glycerols under a variety of high-performance liquid chromatography (HPLC) conditions. The reverse isomers of diacylglycerols having various pairs of acyl groups including short and highly unsaturated chains, which were prepared by partial Grignard degradation of the corresponding triacylglycerols, were chromatographed as 3,5-dinitrophenylurethanes. Excellent resolution was achieved for the reverse isomers of very different pairs of acyl groups, such as acetate–palmitate and docosahexaenoate–palmitate, by chiral-phase HPLC on columns containing (*R*)- and (*S*)-1-(1-naphthyl)ethylamine polymeric phases, reversed-phase HPLC on a highly efficient $C_{18}$ column (4 μm particle size) and silver ion HPLC on a silver loaded cation-exchange column. The chiral-phase HPLC also permitted complete enantiomer resolution for all the reverse isomers examined. No satisfactory resolution by any of the HPLC methods, however, was obtained for the reverse isomers possessing minor differences in chain lengths and degree of unsaturation, such as laurate–palmitate and oleate–linoleate. The limitations of resolution and characteristics of elution are described. 

*Keywords:* Reverse isomers; Enantiomer separation; Diacylglycerols; 3,5-Dinitrophenylurethanes

## 1. Introduction

The discovery of the resolution of enantiomeric diacylglycerols on chiral-phase high-performance liquid chromatography (HPLC) [1] along with the earlier reported resolution of molecular species of diacylglycerols on polar capillary gas chromatography (GC) [2] and reversed-phase HPLC [3] essentially completes the resolution of natural diacylglycerols [4] except for reverse isomers (Fig. 1), which have thus far defied satisfactory resolution by chromatography.

At various times certain pairs of the reverse isomers of phospholipids and their constituent diacylglycerols have been claimed [5–7] to be resolved by HPLC, but these reports have not been documented by means of separations of pertinent standards. Only a few papers have been published for the resolution of several pairs of synthetic reverse isomers of 1,2-diacyl-X-glycerols. Takagi and Nishi-

*Corresponding author. Tel.: +1-416-978-2590; fax: +1-416-978-8528.

*E-mail address:* arnis.kuksis@utoronto.ca (A. Kuksis).

[1]Present address: Faculty of Fisheries, Hokkaido University, Hakodate 041-8611, Japan.

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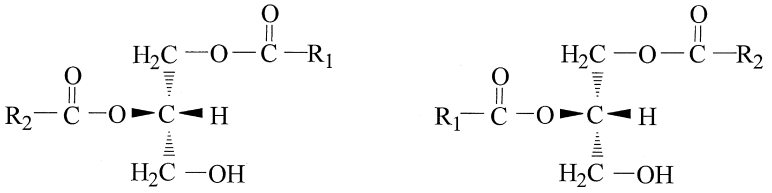

Fig. 1. Structures of the reverse isomers of 1,2-diacyl-*sn*-glycerol ($R_1 \neq R_2$).

mura [8] obtained almost complete resolution of the three pairs of reverse isomers of 1-palmitoyl-2-oleoyl-*rac*-glycerol (16:0–18:1), 1-palmitoyl-2-linoleoyl-*rac*-glycerol (16:0–18:2) and 1-palmitoyl-2-linolenoyl-*rac*-glycerol (16:0–18:3) as their 3,5-dinitrobenzoate (3,5-DNB) derivatives by silver ion HPLC with recycle elution, which required very long analysis times (120–900 min). Adlof [9] reported clear resolution of the reverse isomers of 1-acetyl-2-palmitoyl-X-glycerol (2:0–16:0) and 1-acetyl-2-oleoyl-X-glycerol (2:0–18:1) as their acetates by silver ion HPLC over a much shorter time period (up to 35 min). Satouchi et al. [10] also reported complete reverse isomer resolution of 1-acetyl-2-palmitoyl-*sn*-glycerol as *tert*.-butyldimethylsilyl (*t*-BDMS) ether within an analysis time of 10 min by gas chromatography–mass spectrometry (GC–MS). Although these reports give encouraging information on the resolution of diacylglycerol reverse isomers, systematic investigations on the limitations of the obtained resolution and characteristics of elution under other HPLC conditions have not been carried out.

Our previous work [11] on the chiral-phase HPLC of synthetic diacylglycerols containing short and long acyl chains provided an opportunity to reexamine the chromatographic resolution of reverse isomers of known composition and maximum differences in chain lengths and degree of unsaturation. In the present study we have attempted to document the extent of HPLC resolution of the reverse isomers of glycerolipids by examining the separation of diacylglycerols as 3,5-dinitrophenylurethanes (3,5-DNPU) on chiral-phase, reversed-phase and silver ion HPLC. A preliminary report on the reverse isomer resolution of the diacylglycerols having short and long acyl chains on chiral-phase HPLC has been published [12].

## 2. Experimental

### 2.1. Chemicals

Acetic (2:0) and butyric (4:0) anhydrides were obtained from Aldrich (Milwaukee, WI, USA). Caproic (6:0), caprylic (8:0), capric (10:0), lauric (12:0), palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2) and linolenic (18:3) acids, 1-oleoyl-*rac*-glycerol, 1-linoleoyl-*rac*-glycerol, 1-linolenoyl-*rac*-glycerol and 1,2-dipalmitoyl-*rac*-glycerol were obtained from Sigma (St. Louis, MO, USA). 1,2-Dipalmitoyl-*sn*-glycerol and 1- and 3-palmitoyl-*sn*-glycerols were gifts from Dr. Dmytro Buchnea. These anhydrides, fatty acids, monoacylglycerols and diacylglycerols were used without further purification. Arachidonic (20:4), eicosapentaenoic (20:5) and docosahexaenoic (22:6) acids with purities of 94, 91 and 97%, respectively, were prepared from porcine liver lipids or fish oils, as described previously [13]. Prior to use, the polyunsaturated fatty acids (as methyl esters) were purified by silicic acid column chromatography using *n*-hexane–diethyl ether (90:10, v/v) as the developing solvent. HPLC-grade solvents, acetonitrile, chloroform (alcohol-free), dichloromethane, ethanol and *n*-hexane, were supplied by Kanto (Tokyo, Japan). Prior to use, the solvents were filtered with a 0.45-μm PTFE membrane filter (Fuji Film, Tokyo, Japan). *N*,*N*′-Dicyclohexylcarbodiimide (DCC) and *N*,*N*′-dimethyl-4-aminopyridine (DMAP) were purchased from Wako (Osaka, Japan). 3,5-Dinitrophenyl isocyanate (3,5-DNPI) was obtained from Fluka (Buchs, Switzerland).

### 2.2. Synthesis

The reverse isomers of 1,2-diacyl-*rac*-glycerols

were prepared by partial Grignard degradation of the corresponding triacylglycerols [2], which were prepared by esterification of 1-monoacyl-*rac*-glycerols ($4.5 \cdot 10^{-5}$ mol) with free fatty acids ($1 \cdot 10^{-4}$ mol) in the presence of DCC and DMAP. The reverse isomers of 1-acetyl-2-palmitoyl-*rac*- and 1-butyroyl-2-palmitoyl-*rac*-glycerols were synthesized in the same manner but using excess amounts of acetic and butyric anhydrides in the presence of DMAP, respectively. The reverse isomers of enantiomeric 1,2- and 2,3-diacyl-*sn*-glycerols were made in the same manner but starting with enantiomeric 1- and 3-monoacyl-*sn*-glycerols, respectively. The resulting 1,2-diacyl-X-glycerols were purified by thin-layer chromatography (TLC) on Silica Gel G (Merck, Darmstadt, Germany) impregnated with boric acid, using chloroform–acetone (98:2, v/v) as the developing solvent [2]. After conversion to the 3,5-DNPU derivatives as described below, the desired diacylglycerol reverse isomers were isolated from the mixtures of the 1,2-diacyl-X-glycerols by preparative reversed-phase HPLC on a J'sphere ODS-H80 column (150×4.6 mm I.D., 4 μm particles, YMC, Kyoto, Japan) using acetonitrile–propan-2-ol (70:30, v/v) as the mobile phase.

The highly unsaturated monoacylglycerols, 1-arachidonoyl-, 1-eicosapentaenoyl- and 1-docosahexaenoyl-*rac*-glycerols, which were not commercially available, were prepared with 1,2-isopropylidene-*rac*-glycerol as a starting material. The acyl groups were introduced by reaction of equimolar amounts of 1,2-isopropylidene-*rac*-glycerol with free fatty acid in the presence of DCC and DMAP. The enantiomeric 1- and 3-monoacyl-*sn*-glycerols were made in the same manner but starting with enantiomeric 2,3- and 1,2-isopropylidene-*sn*-glycerols, respectively. The isopropylidene protecting group was removed by heating a solution of the reaction products in the presence of boric acid and trimethyl borate [14]. The resulting 1-monoacyl-X-glycerols were purified by TLC on Silica Gel G impregnated with boric acid using chloroform–methanol (98:2, v/v) as the developing solvent [15].

### *2.3. High-performance liquid chromatography*

For HPLC analyses the diacylglycerols were converted into the 3,5-DNPU derivatives by reacting with 3,5-DNPI in dry toluene in the presence of pyridine at room temperature as previously described [1].

Chiral-phase HPLC was performed on a Hitachi L-7000 liquid chromatograph (Hitachi, Tokyo, Japan) equipped with a Hitachi L-4100 UV detector. Two columns containing (*R*)- and (*S*)-1-(1-naphthyl)ethylamine polymers as the stationary phases (250×4.6 mm I.D., 5 μm particles, YMC-Pack A-K03 and A-L03, YMC) were used. A guard column, Sumipax Filter PG-ODS (Sumika Chemical Analysis Service, Osaka, Japan), was attached to the inlet of each column. The analysis was done isocratically at 20°C using *n*-hexane–dichloromethane–ethanol (80:20:2, v/v/v) and *n*-hexane–dichloromethane–acetonitrile (80:15:5, v/v/v) as the mobile phases at a constant flow-rate of 0.5 ml/min [1].

Reversed-phase HPLC was performed on the same instruments as those employed for chiral-phase HPLC. An ODS column (Superspher RP-18e, 250×4.6 mm I.D., 4 μm particles, Merck) with a LiChrosorb RP-18 guard column was used. The analysis was done isocratically at 18°C using acetonitrile as the mobile phase at a constant flow-rate of 0.5 ml/min, which produced a pump pressure of about 55 kg/cm$^2$. Reversed-phase HPLC with on-line electrospray ionization mass spectrometry (ESI-MS) of the reverse isomers was performed on a LCQ ion trap mass spectrometer (Thermo Separation Products, San Jose, CA, USA) using the same ODS column and mobile phase as those described above. The negative ESI-MS spectra of the DNPU derivatives, which was essentially the same for both reverse isomers, gave a prominent pseudomolecular ion $[M-1]^-$.

Silver ion HPLC was performed on a Shimadzu LC-6A instrument equipped with a Shimadzu 6A UV detector. A silver loaded cation-exchange column (Chromsphere 5 Lipids, 250×4.6 mm I.D., 5 μm particles, Chrompack, Middleburg, The Netherlands) was used with a Guard-Pac pre-column module with a silica insert (Millipore, Milford, MA, USA). The analysis was done isocratically at 10°C with chloroform (alcohol-free)–acetonitrile as the mobile phase at a constant flow-rate of 0.5 ml/min. The composition of the mobile phase is given in Table 5.

For the HPLC analyses, several μg of the 3,5-DNPU derivatives were dissolved in dichlorome-

thane or propan-2-ol and were injected into chromatograph through a Rheodyne Model 7125 injector and the effluents were monitored at 254 nm. Chromatograms were recorded on a Chromatopac C-R6A (Shimadzu).

## 3. Results and discussion

### *3.1. Chiral-phase HPLC separation*

Fig. 2 shows the chiral-phase HPLC separation of 1-palmitoyl-2-acetyl-*rac*-glycerol (16:0–2:0) and 1-acetyl-2-palmitoyl-*rac*-glycerol (2:0–16:0) obtained with two solvent systems on (*R*)-1-(1-naphthyl)ethylamine (A-K03). There is an excellent resolution of the reverse isomers within each enantiomer class when a mobile phase containing acetonitrile is used (Fig. 2A). Almost complete resolution of the reverse isomers and enantiomers was also observed in a shorter elution time (up to 30 min) with the same mobile phase but a higher flow-rate (1 ml/min) [12]. On the other hand, only shouldering of peaks was seen for the *sn*-1,2-enantiomers when the acetonitrile was replaced by ethanol (Fig. 2B). These peaks were still not resolved clearly when the elution time was increased (up to 70 min) using the same mobile phase but a lower flow-rate (0.3 ml/min). In both enantiomer classes the reverse isomers with the acetyl chains in the primary positions emerged first. The elution order was established by separate analyses of 16:0–2:0 and 2:0–16:0 or by analysis of a mixture of the reverse isomers spiked with the 2:0–16:0 species. Individual enantiomer peaks were also identified with synthetic standards as previously described [11].

Fig. 3 shows the chiral-HPLC separation of 1-palmitoyl-2-butyroyl-*rac*-glycerol (16:0–4:0) and 1-butyroyl-2-palmitoyl-*rac*-glycerol (4:0–16:0) as obtained with two solvent systems on the A-K03 column. There is barely discernible widening and shouldering of peaks within the *sn*-1,2- and *sn*-2,3-enantiomers, respectively, when a mobile phase containing acetonitrile is used (Fig. 3A). The peak of the *sn*-1,2-enantiomer was not resolved even when longer retention times were achieved using a mobile phase containing a smaller proportion of acetonitrile. On the other hand, there is a complete overlapping and a partial resolution within the *sn*-1,2- and *sn*-2,3-enantiomers, respectively, when a mobile phase containing ethanol is used (Fig. 3B). Again the reverse isomers with the shorter butyroyl chain in the primary positions emerged first. The peak shapes of the completely overlapped and partially resolved reverse isomers (Fig. 3B) were not much improved when the retention time was increased (96.4 min for the *sn*-1,2-enantiomer) by substituting *n*-hexane–dichloromethane–ethanol (80:20:1, v/v/v) at the same flow-rate (0.5 ml/min). No discernible resolution was seen for the reverse isomers of 6:0–16:0 and 16:0–6:0, and higher homologues. There was also no resolution for the enantiomeric 1-butyroyl-3-palmitoyl-*sn*- and 1-palmitoyl-3-butyroyl-*sn*-glycerols on the chiral-phase HPLC. Apparently the (*R*)-1-(1-naphthyl)ethylamine polymer retards the *sn*-2,3-enantiomer in relation to the *sn*-1,2-enantiomer by interacting more strongly with the 3,5-DNPU function in the *sn*-1 position compared to the same functional group in the *sn*-3 position.

Table 1 gives the chiral-phase HPLC data for the acetylpalmitoyl- and butyroylpalmitoylglycerols on A-K03. The A-K03 column used in this study shows better enantiomer resolution for the diacylglycerols having short acyl chains in the secondary position than those having long acyl chains in the same position. For example, the separation factors ($\alpha$) of the enantiomers of 16:0–2:0 and 2:0–16:0 were 1.45 and 1.35, respectively, when the mobile phase containing ethanol was used. Similarly the $\alpha$ values of the enantiomers of 16:0–4:0 and 4:0–16:0 were 1.517 and 1.471, respectively (Table 1). Apparently these differences reflect the resolution of each reverse isomer of 2:0–16:0 and 4:0–16:0 on the chiral stationary phase (see Figs. 2 and 3). The close $\alpha$ values between the enantiomers of 16:0–4:0 ($\alpha$= 1.52) and 4:0–16:0 ($\alpha$=1.47) when the mobile phase containing ethanol was used (Table 1), in comparison with those of 16:0–2:0 ($\alpha$=1.45) and 2:0–16:0 ($\alpha$=1.35), reflect the poorer separation of the reverse isomers of the butyroylpalmitoylglycerols than those of the acetylpalmitoylglycerols (see Figs. 2 and 3). The $\alpha$ values between the enantiomers of diacylglycerols having longer acyl chains than butyrate were almost identical and no resolution of the reverse isomers was observed.

The replacement of ethanol by acetonitrile in the

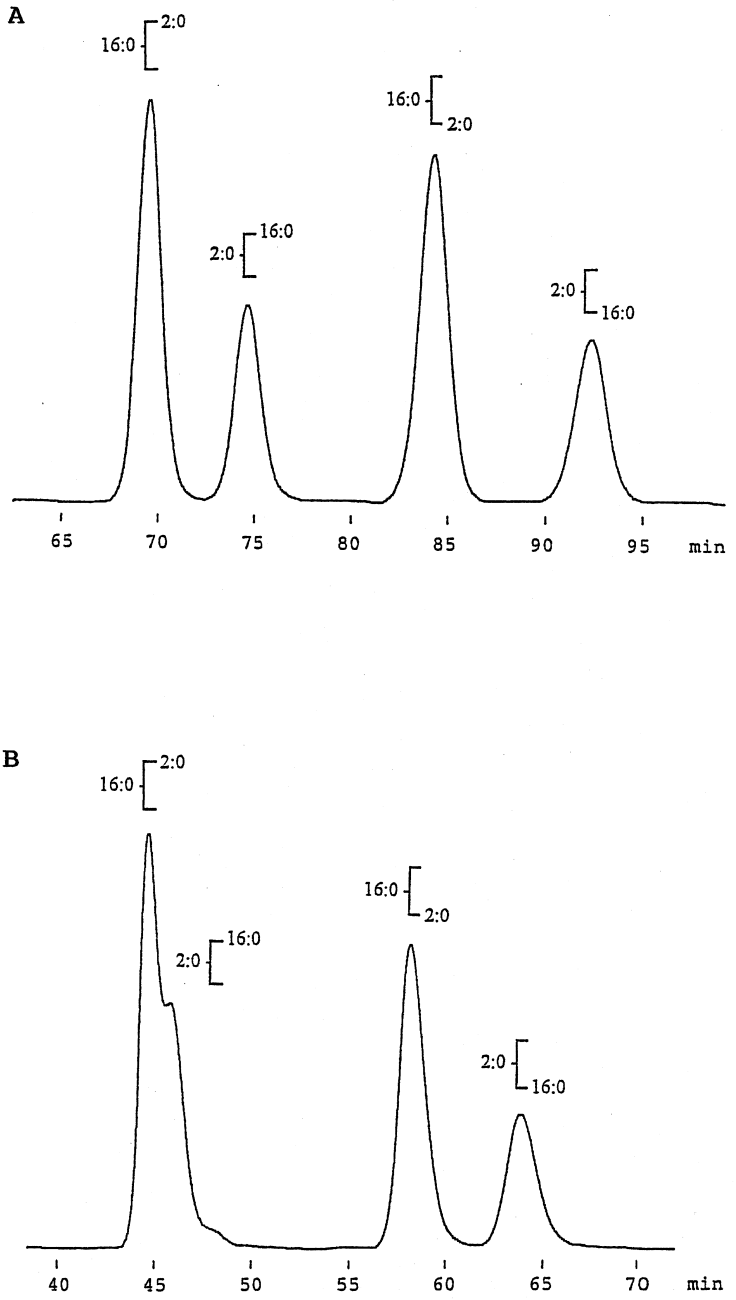


Fig. 2. Chiral-phase HPLC resolution of the reverse isomers and enantiomers of 1,2-acetylpalmitoyl-*rac*-glycerols as 3,5-dinitrophenylurethanes on (*R*)-1-(1-naphthyl)ethylamine (A-K03). Peak identification (from left to right): 1-acetyl-2-palmitoyl-*sn*-glycerol, 1-palmitoyl-2-acetyl-*sn*-glycerol, 2-palmitoyl-3-acetyl-*sn*-glycerol and 2-acetyl-3-palmitoyl-*sn*-glycerol. A mixture of 1-acetyl-2-palmitoyl-*rac*-glycerol and 1-palmitoyl-2-acetyl-*rac*-glycerol was analyzed. Mobile phase, A; *n*-hexane–dichloromethane–acetonitrile (85:10:5, v/v/v); B, *n*-hexane–dichloromethane–ethanol (80:20:2, v/v/v). Other HPLC conditions as given in text.

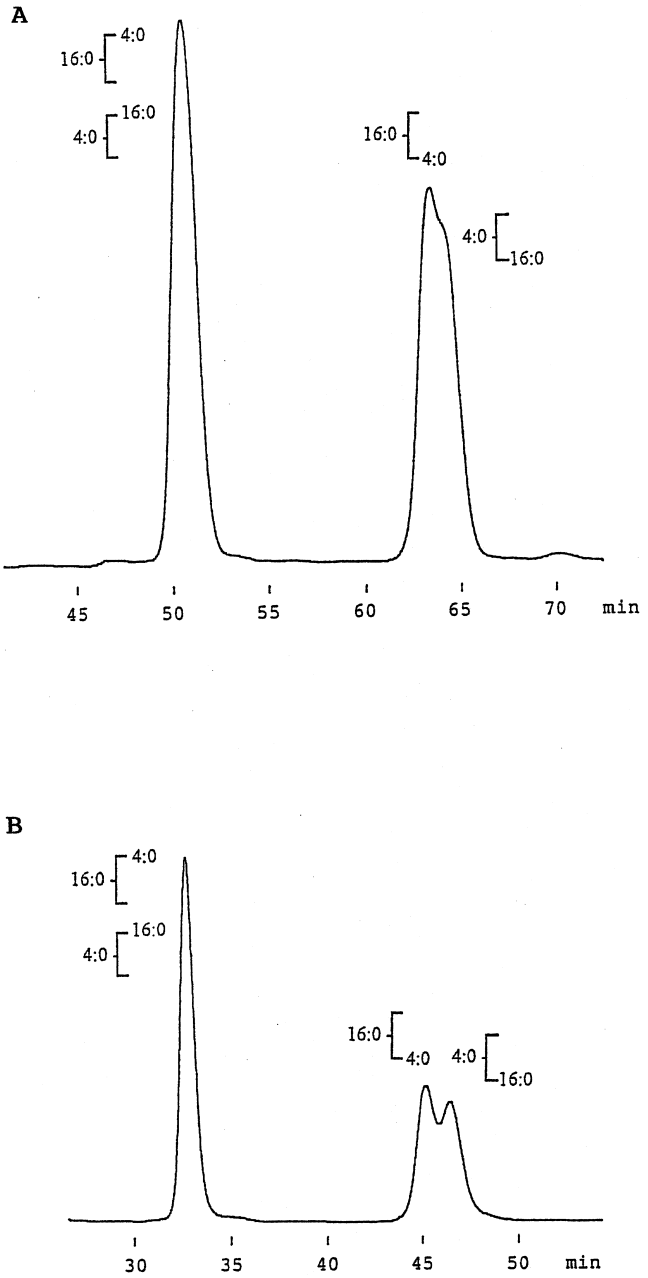


Fig. 3. Chiral-phase HPLC resolution of the reverse isomers and enantiomers of 1,2-butyroylpalmitoyl-*rac*-glycerols as 3,5-dinitrophenylurethanes on (*R*)-1-(1-naphthyl)ethylamine (A-K03). Peak identification (from left to right): 1-butyroyl-2-palmitoyl-*sn*-glycerol plus 1-palmitoyl-2-butyroyl-*sn*-glycerol, 2-palmitoyl-3-butyroyl-*sn*-glycerol and 2-butyroyl-3-palmitoyl-*sn*-glycerol. A mixture of 1-butyroyl-2-palmitoyl-*rac*-glycerol and 1-palmitoyl-2-butyroyl-*rac*-glycerol was analyzed. Mobile phase, A; *n*-hexane–dichloromethane–acetonitrile (85:10:5, v/v/v); B, *n*-hexane–dichloromethane–ethanol (80:20:2, v/v/v). Other HPLC conditions as given in text.

Table 1
Chiral-phase HPLC of the reverse isomers and enantiomers of diacylglycerols containing short and long chains as their 3,5-dinitrophenylurethanes on (*R*)-1-(1-naphthyl)ethylamine

| Diacylglycerol[b] | | | Mobile phase[a] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *sn*-1 | *sn*-2 | *sn*-3 | A | | | | | B | | | |
| | | | $k'$[c] | Reverse isomer | | Enantiomer | | $k'$ | Reverse isomer | | Enantiomer | |
| | | | | $\alpha$[d] | $R_s$[e] | $\alpha$ | $R_s$ | | $\alpha$ | $R_s$ | $\alpha$ | $R_s$ |
| 4:0 | 16:0 | | | | | | | | | | | |
| | | | 6.910 | –[f] | –[f] | | | 4.133 | –[g] | –[g] | | |
| 16:0 | 4:0 | | | | | 1.291 | 5.25 | | | | 1.471 | 7.66 |
| | 16:0 | 4:0 | 8.922 | | | 1.308 | 5.56 | 6.078 | | | 1.517 | 8.92 |
| | | | | 1.013 | –[h] | | | | 1.031 | 0.63 | | |
| | 4:0 | 16:0 | 9.039 | | | | | 6.268 | | | | |
| 2:0 | 16:0 | | 9.872 | | | | | 6.021 | | | | |
| | | | | 1.081 | 2.02 | | | | 1.028 | 0.59 | | |
| 16:0 | 2:0 | | 10.67 | | | 1.233 | 5.61 | 6.187 | | | 1.348 | 5.96 |
| | 16:0 | 2:0 | 12.17 | | | 1.259 | 6.08 | 8.114 | | | 1.453 | 7.66 |
| | | | | 1.103 | 2.68 | | | | 1.108 | 2.02 | | |
| | 2:0 | 16:0 | 13.43 | | | | | 8.991 | | | | |

[a] A, *n*-Hexane–dichloromethane–acetonitrile (85:10:5, v/v/v); B, *n*-hexane–dichloromethane–ethanol (80:20:2, v/v/v). Retention volume (ml) for 1-butyroyl-2-palmitoyl-*sn*-glycerol: A, 17.89; B, 14.37. Column void volume, 3.20 ml.
[b] 16:0, palmitoyl; 4:0, butyroyl; 2:0, acetyl.
[c] Capacity factor.
[d] Separation factor.
[e] Peak resolution. $R_s=2(t_2-t_1)/(W_1+W_2)$, where $t$=retention time (min) and $W$=peak width (min).
[f] Discernible widening of peak.
[g] Completely overlapping peak.
[h] Shoulder peak.

mobile phase reduces appreciably the enantiomer separation of diacylglycerols, but gives sharper peaks [1]. In this study we obtained 13 800 theoretical plates for the 2-palmitoyl-3-acetyl-*sn*-glycerol peak using acetonitrile as the mobile phase, but obtained only 8300 plates using ethanol as the mobile phase. This increase in column efficiency caused by acetonitrile greatly improved the reverse isomer separation of 1-acetyl-2-palmitoyl-*sn*-glycerol (Fig. 2A), although the separation of the 2-palmitoyl-3-acetyl-*sn*-glycerol isomers became somewhat decreased with a decrease of the enantiomer separation (Table 1).

The reverse isomer peaks of the 1-butyroyl-2-palmitoyl-*sn*-glycerol and 1-palmitoyl-2-butyroyl-*sn*-glycerol, which completely overlapped on A-K03 (Fig. 3B) were partially resolved on the chiral stationary phase having an opposite configuration, (*S*)-1-(1-naphthyl)ethylamine (A-L03) (chromatogram not shown). Two or three columns connected in series and yielding higher theoretical plate number might in this case be suitable for obtaining a better resolution of the partially overlapping peaks.

Fig. 4 shows the chiral-phase HPLC separation on the A-K03 column of 1,2-diacyl-*rac*-glycerols containing unsaturated acyl chains using ethanol as the mobile phase. There is reasonably good resolution of the reverse isomers of 1-docosahexaenoyl-2-palmitoyl-*rac*-glycerol (22:6–16:0), 1-docosahexaenoyl-2-oleoyl-*rac*-glycerol (22:6–18:1) and 1-eicosapentaenoyl-2-oleoyl-*rac*-glycerol (20:5–18:1) only within their *sn*-2,3-enantiomers, for which the isomers with the acyl groups of the higher degree of unsaturation are in the secondary position. There is similar resolution of the reverse isomers within *sn*-1,2-enantiomers on the chiral stationary phase having the opposite configuration (A-L03) (Fig. 5). Again the reverse isomers with the acyl groups of higher

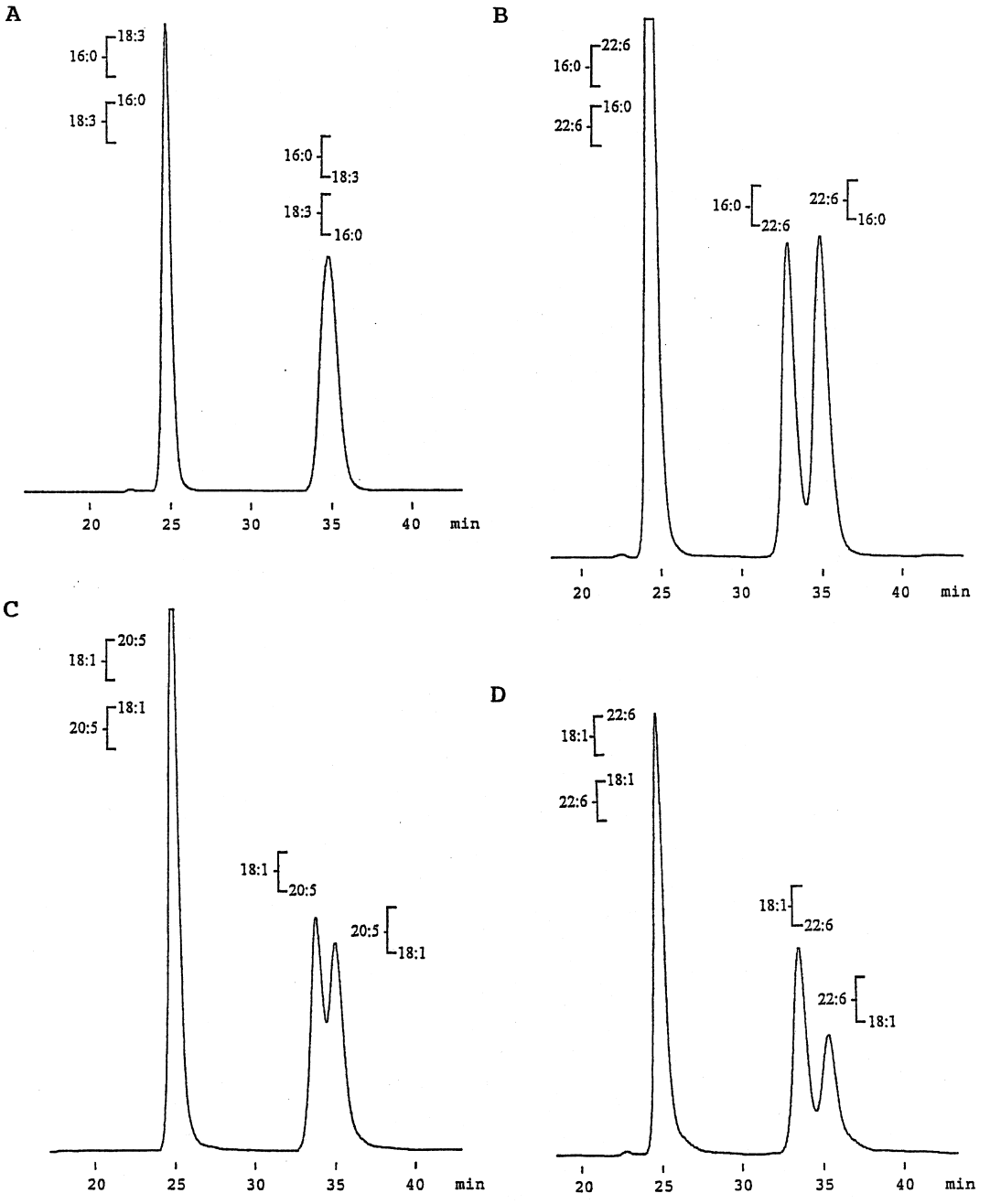


Fig. 4. Chiral-phase HPLC resolution of the reverse isomers and enantiomers of 1,2-diacyl-*rac*-glycerols containing polyunsaturated acyl chains as 3,5-dinitrophenylurethanes on (*R*)-1-(1-naphthyl)ethylamine (A-K03). Peak identification (from left to right): A, 1-linolenoyl-2-palmitoyl-*sn*-glycerol plus 1-palmitoyl-2-linolenoyl-*sn*-glycerol and 2-palmitoyl-3-linolenoyl-*sn*-glycerol plus 2-linolenoyl-3-palmitoyl-*sn*-glycerol; B, 1-docosahexaenoyl-2-palmitoyl-*sn*-glycerol plus 1-palmitoyl-2-docosahexaenoyl-*sn*-glycerol, 2-palmitoyl-3-docosahexaenoyl-*sn*-glycerol and 2-docosahexaenoyl-3-palmitoyl-*sn*-glycerol; C, 1-eicosapentaenoyl-2-oleoyl-*sn*-glycerol plus 1-oleoyl-2-eicosapentaenoyl-*sn*-glycerol, 2-oleoyl-3-eicosapentaenoyl-*sn*-glycerol and 2-eicosapentaenoyl-3-oleoyl-*sn*-glycerol; D, 1-docosahexaenoyl-2-oleoyl-*sn*-glycerol plus 1-oleoyl-2-docosahexaenoyl-*sn*-glycerol, 2-oleoyl-3-docosahexaenoyl-*sn*-glycerol and 2-docosahexaenoyl-3-oleoyl-*sn*-glycerol. A mixture of the reverse isomers of 1,2-diacyl-*rac*-glycerol was analyzed to obtain each chromatogram. Mobile phase, *n*-hexane–dichloromethane–ethanol (80:20:2, v/v/v). Other HPLC conditions as given in text.

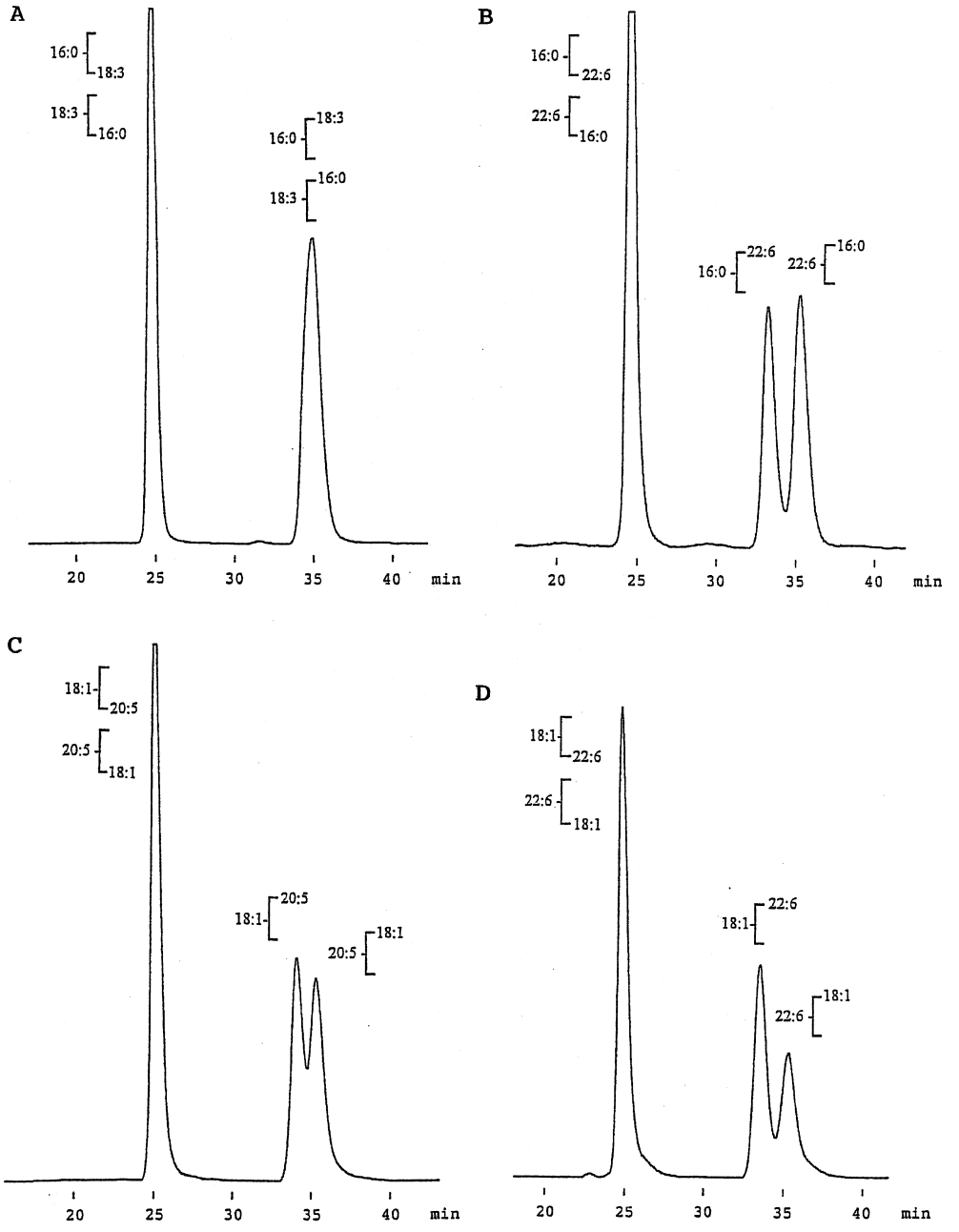


Fig. 5. Chiral-phase HPLC resolution of the reverse isomers and enantiomers of 1,2-diacyl-*rac*-glycerols containing polyunsaturated acyl chains as 3,5-dinitrophenylurethanes on (*S*)-1-(1-naphthyl)ethylamine (A-L03). Peak identification (from left to right): A, 2-palmitoyl-3-linolenoyl-*sn*-glycerol plus 2-linolenoyl-3-palmitoyl-*sn*-glycerol and 1-linolenoyl-2-palmitoyl-*sn*-glycerol plus 1-palmitoyl-2-linolenoyl-*sn*-glycerol; B, 2-palmitoyl-3-docosahexaenoyl-*sn*-glycerol plus 2-docosahexaenoyl-3-palmitoyl-*sn*-glycerol, 1-docosahexaenoyl-2-palmitoyl-*sn*-glycerol and 1-palmitoyl-2-docosahexaenoyl-*sn*-glycerol; C, 2-oleoyl-3-eicosapentaenoyl-*sn*-glycerol plus 2-eicosapentaenoyl-3-oleoyl-*sn*-glycerol, 1-eicosapentaenoyl-2-oleoyl-*sn*-glycerol and 1-oleoyl-2-eicosapentaenoyl-*sn*-glycerol; D, 2-oleoyl-3-docosahexaenoyl-*sn*-glycerol plus 2-docosahexaenoyl-3-oleoyl-*sn*-glycerol, 1-docosahexaenoyl-2-oleoyl-*sn*-glycerol and 1-oleoyl-2-docosahexaenoyl-*sn*-glycerol. A mixture of the reverse isomers of 1,2-diacyl-*rac*-glycerol was analyzed to obtain each chromatogram. HPLC conditions are the same as those in Fig. 4, except for the chiral stationary phase.

degree of unsaturation in the secondary position are retained more strongly. The isomer elution orders on both columns were established with synthetic standards as described for the acetylpalmitoyglycerols. The presence of long chains of saturated and monounsaturated fatty acids in the secondary position, which are close to 3,5-DNPU group, apparently interfered with diastereomeric hydrogen-bonding and π–π donor–acceptor interaction between the 3,5-DNPU and (*R*)- or (*S*)-1-(1-naphthyl)ethylamine stationary phases [1], and resulted in impaired separation of diacylglycerols having the long acyl chains in the secondary position. This steric hindrance may also contribute to the earlier elution of the reverse isomers having long acyl chains in the secondary position. No discernible resolution was observed for the reverse isomers of 1-linolenoyl-2-palmitoyl-*rac*-glycerol (18:3–16:0) (Figs. 4A and 5A), 1-linoleoyl-2-oleoyl-*rac*-glycerol (18:2–18:1) and 1-linolenoyl-2-oleoyl-*rac*-glycerol (18:3–18:1). These completely overlapping reverse isomers within each *sn*-1,2- and 2,3-enantiomer remained unresolved when run under longer retention times (up to 60 min) obtained with the same mobile phase but a lower flow-rate (0.3 ml/min).

Table 2 gives the chiral-phase HPLC data for the diacylglycerols containing polyunsaturated acyl chains on two columns having chiral phases of opposite configuration, (*R*)- and (*S*)-1-(1-naphthyl)ethylamine (A-K03 and A-L03). The best resolution of the reverse isomers on both columns was obtained for the pair of 16:0–22:6 and 22:6–16:0, which differed most in both carbon number and degree of unsaturation of the acyl groups. On the other hand, no resolution was obtained for the pair of 16:0–18:3 and 18:3–16:0, which differed least in both carbon number and degree of unsaturation of the acyl groups. As observed with diacylglycerols of short and long acyl chains (Table 1), better enantiomer separation was obtained for the diacylglycerols having polyunsaturated acyl chain in the secondary position rather than a saturated or a monounsaturated one. For example, the $\alpha$ values for each enantiomer of 16:0–22:6 and 22:6–16:0 were 1.58 and 1.47 on both columns, respectively, when the mobile phase was ethanol (Table 2). This difference in the enantiomer separation reflects an effective resolution of the reverse isomers of 22:6–16:0 on both chiral stationary phases (Figs. 4B and 5B). Under the same HPLC conditions, the $\alpha$ and $R_s$ values of 2-oleoyl-3-arachidonoyl-*sn*-glycerol and 2-arachidonoyl-3-oleoyl-*sn*-glycerol on A-K03 were 1.038 and 0.68, respectively (Table 2), which were almost the same as those for 2-palmitoyl-3-butyroyl-*sn*-glycerol and 2-butyroyl-3-palmitoyl-*sn*-glycerol (1.031 and 0.63, respectively) (Table 1). Thus, in the reverse isomer separation, the species 18:1 and 20:4 are equivalent to 16:0 and 4:0, respectively, in their acyl chain lengths.

### *3.2. Reversed-phase HPLC separation*

Fig. 6 shows the reversed-phase HPLC separation of the reverse isomers of 1,2-diacyl-*rac*-glycerols having short and long acyl chains. There are clear resolutions for the reverse isomers of 1-acetyl-2-palmitoyl-*rac*-glycerol (2:0–16:0), 1-butyroyl-2-palmitoyl-*rac*-glycerol (4:0–16:0) and 1-caprooyl-2-palmitoyl-*rac*-glycerol (6:0–16:0) within appropriate elution times using only acetonitrile as the mobile phase, but no resolution was observed for the reverse isomers containing the longer chain capric and lauric acids (10:0–16:0 and 12:0–16:0). The overlapping reverse isomer peaks of 10:0–16:0 and 12:0–16:0 (Fig. 6E and F) remained unresolved, even when longer elution times made possible by using mobile phases containing water, were applied. It would have been anticipated that the non-polar $C_{18}$ reversed-phase column would have retained longer the less polar isomers with the longer chains in the primary positions. Again, the isomers with the shorter chains in the primary positions emerged first consistent with the elution order seen on chiral-phase HPLC.

Table 3 gives the reversed-phase HPLC data of the reverse isomers of 1,2-diacyl-*rac*-glycerols of short and long acyl chains. The reverse isomer resolution, however, is seen to decrease with decreasing differences between the two acyl chains as indicated by the $\alpha$ and $R_s$ values. The data and the chromatograms (Fig. 4) show that at least a difference of 10 acyl carbons is necessary to obtain a clear-cut resolution between the reverse isomers under the conditions employed. Under identical HPLC conditions, the $\alpha$ and $R_s$ values for 22:6–18:1 and 18:1–22:6 were 1.038 and 1.261, respectively (Table 4), which were almost the same as those for 6:0–16:0 and 16:0–6:0 (1.042 and 1.282, respectively)

Table 2
Chiral-phase HPLC of the reverse isomers and enantiomers of diacylglycerols containing polyunsaturated acyl chains as their 3,5-dinitrophenylurethanes on (*R*)- and (*S*)-1-(1-naphthyl)ethylamine

| Diacylglycerol[b] | | | ECN[c] | (*R*)-1-(1-Naphthyl)ethylamine (A-K03)[a] | | | | | (*S*)-1-(1-Naphthyl)ethylamine (A-L03)[a] | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *sn*-1 | *sn*-2 | *sn*-3 | | $k'$[d] | Reverse isomer | | Enantiomer | | $k'$ | Reverse isomer | | Enantiomer | |
| | | | | | $\alpha$[d] | $R_s$[d] | $\alpha$ | $R_s$ | | $\alpha$ | $R_s$ | $\alpha$ | $R_s$ |
| 18:3 | 16:0 | | 28 | | | | | | | | | | |
| | | | | 2.884 | –[e] | –[e] | | | 4.466 | –[f] | –[f] | | |
| 16:0 | 18:3 | | | | | | 1.545 | 6.51 | | | | 1.549 | 6.64 |
| | 16:0 | 18:3 | | | | | 1.545 | 6.51 | | | | 1.549 | 6.64 |
| | | | | 4.456 | –[f] | –[f] | | | 2.884 | –[e] | –[e] | | |
| | 18:3 | 16:0 | | | | | | | | | | | |
| 20:4 | 18:1 | | 28 | | | | | | 4.034 | | | | |
| | | | | 2.759 | –[e] | –[e] | | | | 1.040 | 0.68 | | |
| 18:1 | 20:4 | | | | | | 1.482 | 7.54 | 4.194 | | | 1.485 | 7.00 |
| | 18:1 | 20:4 | | 4.088 | | | 1.538 | 7.17 | | | | 1.544 | 6.94 |
| | | | | | 1.038 | 0.68 | | | 2.716 | –[e] | –[e] | | |
| | 20:4 | 18:1 | | 4.244 | | | | | | | | | |
| 22:6 | 16:0 | | 26 | | | | | | 4.222 | | | | |
| | | | | 2.847 | –[e] | –[e] | | | | 1.074 | 1.27 | | |
| 16:0 | 22:6 | | | | | | 1.466 | 6.15 | 4.534 | | | 1.470 | 6.54 |
| | 16:0 | 22:6 | | 4.175 | | | 1.575 | 7.00 | | | | 1.579 | 7.50 |
| | | | | | 1.074 | 1.20 | | | 2.872 | –[e] | –[e] | | |
| | 22:6 | 16:0 | | 4.484 | | | | | | | | | |
| 22:6 | 18:1 | | 26 | | | | | | 4.231 | | | | |
| | | | | 2.897 | –[e] | –[e] | | | | 1.065 | 1.05 | | |
| 18:1 | 22:6 | | | | | | 1.466 | 6.43 | 4.506 | | | 1.470 | 6.41 |
| | 18:1 | 22:6 | | 4.247 | | | 1.563 | 6.95 | | | | 1.566 | 6.72 |
| | | | | | 1.067 | 1.08 | | | 2.878 | –[e] | –[e] | | |
| | 22:6 | 18:1 | | 4.528 | | | | | | | | | |
| 20:5 | 18:1 | | 26 | | | | | | 4.344 | | | | |
| | | | | 2.922 | –[e] | –[e] | | | | 1.044 | 0.78 | | |
| 18:1 | 20:5 | | | | | | 1.474 | 6.83 | 4.534 | | | 1.474 | 6.99 |
| | 18:1 | 20:5 | | 4.306 | | | 1.536 | 7.04 | | | | 1.539 | 7.13 |
| | | | | | 1.042 | 0.74 | | | 2.947 | –[e] | –[e] | | |
| | 20:5 | 18:1 | | 4.488 | | | | | | | | | |

[a] Mobile phase, *n*-Hexane–dichloromethane–acetonitrile (80:20:2, v/v/v); column void volume, 3.20 ml. Retention volume (ml) for 1-palmitoyl-2-docosahexaenoyl-*sn*-glycerol: A-K03, 12.31; A-L03, 17.71.

[b] 16:0, palmitoyl; 18:0, stearoyl; 18:1, oleoyl; 18:2, linoleoyl; 18:3, linolenoyl; 20:4, arachidonoyl; 20:5, eicosapentaenoyl; 22:6, docosahexaenoyl.

[c] Equivalent carbon number. ECN=total number of acyl carbons−2×total number of double bonds.

[d] For $k'$, $\alpha$ and $R_s$, see Table 1.

[e] Completely overlapping peak.

[f] Discernible widening of peak.

(Table 3). Thus, in reverse isomer separation on reversed-phase HPLC, the species 18:1 and 22:6 are equivalent to 16:0 and 6:0, respectively, in their effective acyl chain lengths.

Earlier, we had observed that the reverse isomers of 2:0–16:0 as the trimethylsilyl (TMS) ethers are fully resolved by GC on polar (SP-2380, 30 m length) and non-polar (SE-54, 8 m length) capillary columns [20]. 2:0–16:0 emerged well ahead of 16:0–2:0 from both polar and non-polar capillary columns. Complete baseline resolution and identical order of elution were also achieved for the reverse isomers of 2:0–18:1 on both capillary columns. Satouchi et al. [10] had reported a complete resolution of 2:0–16:0 and 16:0–2:0 as *t*-BDMS ethers in this order, within an analysis time of 10 min by GC–MS. However, in

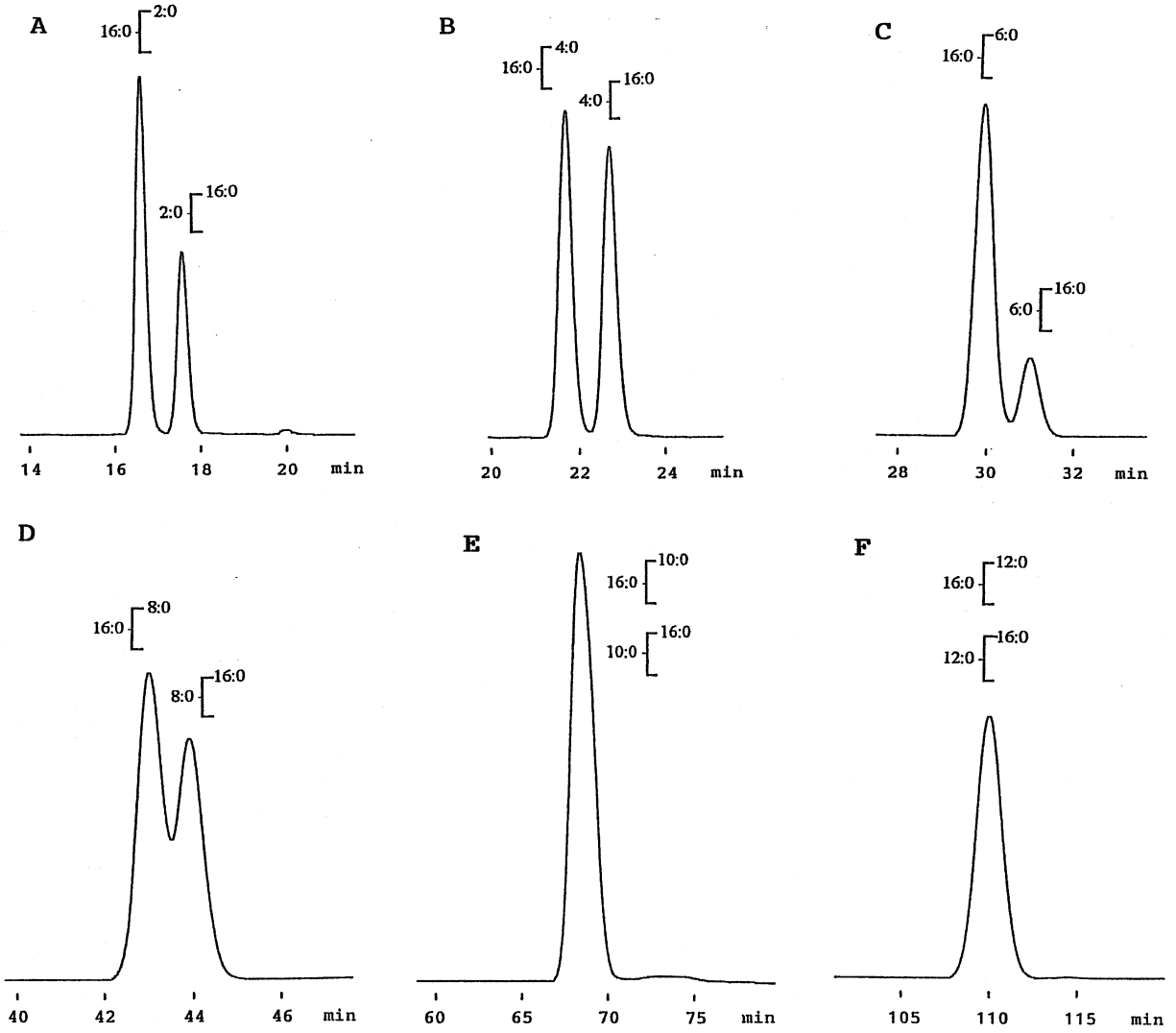


Fig. 6. Reversed-phase HPLC resolution of the reverse isomers of 1,2-diacyl-*rac*-glycerols of saturated short and long acyl chains as 3,5-dinitrophenylurethanes. Peak identification (from left to right): A, 1-acetyl-2-palmitoyl-*rac*-glycerol and 1-palmitoyl-2-acetyl-*rac*-glycerol; B, 1-butyroyl-2-palmitoyl-*rac*-glycerol and 1-palmitoyl-2-butyroyl-*rac*-glycerol; C, 1-caprooyl-2-palmitoyl-*rac*-glycerol and 1-palmitoyl-2-caprooyl-*rac*-glycerol; D, 1-capryloyl-2-palmitoyl-*rac*-glycerol and 1-palmitoyl-2-capryloyl-*rac*-glycerol; E, 1-caproyl-2-palmitoyl-*rac*-glycerol plus 1-palmitoyl-2-caproyl-*rac*-glycerol; F, 1-lauroyl-2-palmitoyl-*rac*-glycerol plus 1-palmitoyl-2-lauroyl-*rac*-glycerol. A mixture of the reverse isomers of 1,2-diacyl-*rac*-glycerol was analyzed to obtain each chromatogram. Column temperature, 18°C. Other HPLC conditions as given in text.

this case the exact nature of the obtained resolution remains in doubt, because these authors had prepared the derivatives by heating with the silylating reagent at 110°C for 15 min, which would isomerize the acetyl group at the second position to X-1,3-position. The reverse isomers of 4:0–16:0 as the TMS ethers gave a more limited resolution between the isomers on either polar and non-polar capillary columns, but the 4:0–16:0 isomer could be clearly seen to emerge ahead of the 16:0–4:0 isomer from both columns [20]. The splitting of the peaks was increased by using longer capillary columns. However, there was

Table 3
Reversed-phase HPLC of the reverse isomers of diacylglycerols containing short and long chains as their 3,5-dinitrophenylurethanes

| Diacylglycerol[a] | | $V_r$[b] | $k'$[c] | $\alpha$[c] | $R_s$[c] |
|---|---|---|---|---|---|
| *sn*-1(3) | *sn*-2 | (ml) | | | |
| 2:0 | 16:0 | 6.03 | 2.637 | | |
| | | | | 1.080 | 2.05 |
| 16:0 | 2:0 | 6.61 | 2.847 | | |
| 4:0 | 16:0 | 8.59 | 3.757 | | |
| | | | | 1.060 | 1.69 |
| 16:0 | 4:0 | 9.10 | 3.983 | | |
| 6:0 | 16:0 | 12.69 | 5.553 | | |
| | | | | 1.042 | 1.28 |
| 16:0 | 6:0 | 13.23 | 5.786 | | |
| 8:0 | 16:0 | 19.25 | 8.423 | | |
| | | | | 1.024 | 0.82 |
| 16:0 | 8:0 | 19.71 | 8.621 | | |
| 10:0 | 16:0 | | | | |
| | | 32.01 | 14.00 | –[d] | –[d] |
| 16:0 | 10:0 | | | | |
| 12:0 | 16:0 | | | | |
| | | 52.71 | 23.06 | –[e] | –[e] |
| 16:0 | 12:0 | | | | |

[a] 2:0, acetyl; 4:0, butyroyl; 6:0, caprooyl; 8, capryloyl; 10:0, caproyl; 12:0, lauroyl; 16:0, palmitoyl.
[b] Retention volume corrected for column void volume (2.29 ml).
[c] For $k'$, $\alpha$ and $R_s$, see Table 1.
[d] Discernible widening of peak.
[e] Completely overlapping peaks.

Table 4
Reversed-phase HPLC of the reverse isomers of diacylglycerols containing polyunsaturated acyl chains as their 3,5-dinitrophenylurethanes

| Diacylglycerol[a] | | | | $V_r$[b] | $k'$[b] | $\alpha$[b] | $R_s$[b] |
|---|---|---|---|---|---|---|---|
| *sn*-1(3) | *sn*-2 | | ECN[b] | (ml) | | | |
| 20:5 | 18:1 | 38:6 | 26 | 40.42 | 17.68 | | |
| | | | | | | 1.029 | 1.08 |
| 18:1 | 20:5 | 38:6 | 26 | 41.61 | 18.20 | | |
| 22:6 | 18:1 | 40:7 | 26 | 45.51 | 19.91 | | |
| | | | | | | 1.038 | 1.26 |
| 18:1 | 22:6 | 40:7 | 26 | 47.22 | 20.66 | | |
| 18:3 | 18:1 | 36:4 | 28 | | | | |
| | | | | 50.05 | 21.89 | –[c] | –[c] |
| 18:1 | 18:3 | 36:4 | 28 | | | | |
| 22:6 | 16:0 | 38:6 | 26 | 49.78 | 21.78 | | |
| | | | | | | 1.052 | 1.73 |
| 16:0 | 22:6 | 38:6 | 26 | 52.39 | 22.92 | | |
| 18:3 | 16:0 | 34:3 | 28 | 54.73 | 23.94 | | |
| | | | | | | 1.023 | 0.85 |
| 16:0 | 18:3 | 34:3 | 28 | 55.97 | 24.49 | | |
| 18:1 | 20:4 | 38:5 | 28 | 58.89 | 25.76 | | |
| | | | | | | 1.027 | 0.94 |
| 20:4 | 18:1 | 38:5 | 28 | 60.46 | 26.45 | | |
| 20:5 | 18:0 | 38:5 | 28 | 72.80 | 31.85 | | |
| | | | | | | 1.042 | 1.41 |
| 18:0 | 20:5 | 38:5 | 28 | 75.82 | 33.17 | | |

[a] 16:0, palmitoyl; 18:0, stearoyl; 18:1, oleoyl; 18:3, linolenoyl; 20:4, arachidonoyl; 20:5, eicosapentaenoyl; 22:6, docosahexaenoyl.
[b] For ECN, $V_r$, $k'$, $\alpha$ and $R_s$, see Table 2.
[c] Discernible widening of peak.

no resolution of the reverse isomers of 6:0–16:0 and higher homologues of diacylglycerols [20].

Fig. 7 shows the reversed-phase HPLC separation of the reverse isomers of 1,2-diacyl-*rac*-glycerols containing polyunsaturated acyl chains. Clear resolutions were obtained for the reverse isomers having highly unsaturated acyl chains under somewhat longer elution times (up to 130 min) using only acetonitrile as the mobile phase, but no resolution was seen for the reverse isomers of 18:1–18:3 (Fig. 7C), 18:1–18:2 and 18:2–18:3. As on chiral-phase HPLC, the best resolution of the unsaturated reverse isomers was observed for the 16:0–22:6 species. It would have been anticipated that the non-polar $C_{18}$ reversed-phase would have retained longer the less polar isomers with the lower degree of unsaturation in the primary positions. The reverse isomers with the acyl chains of the higher degree of unsaturation in the primary position emerged first in keeping with the elution order seen on chiral-phase HPLC.

In order to improve the resolution of the overlapping or partially resolved peaks (Figs. 6 and 7), we employed lower column temperatures, which increased greatly the elution times. Typical chromatograms are presented in Fig. 8, which shows much improved resolution for some of the reverse isomer species. The reverse isomers of 10:0–16:0, which had given a barely discernible peak broadening at 18°C (Fig. 6E), were partially resolved at 10°C ($\alpha$=1.019, $R_s$=0.38) (Fig. 8B). However, the reverse isomers of 12:0–16:0 and 18:1–18:3, which had completely overlapped at 18°C (Figs. 6F and 7C), were eluted without resolution in 184.5 min and 143.8 min at 10°C, respectively. We examined several commercially available reversed-phase columns

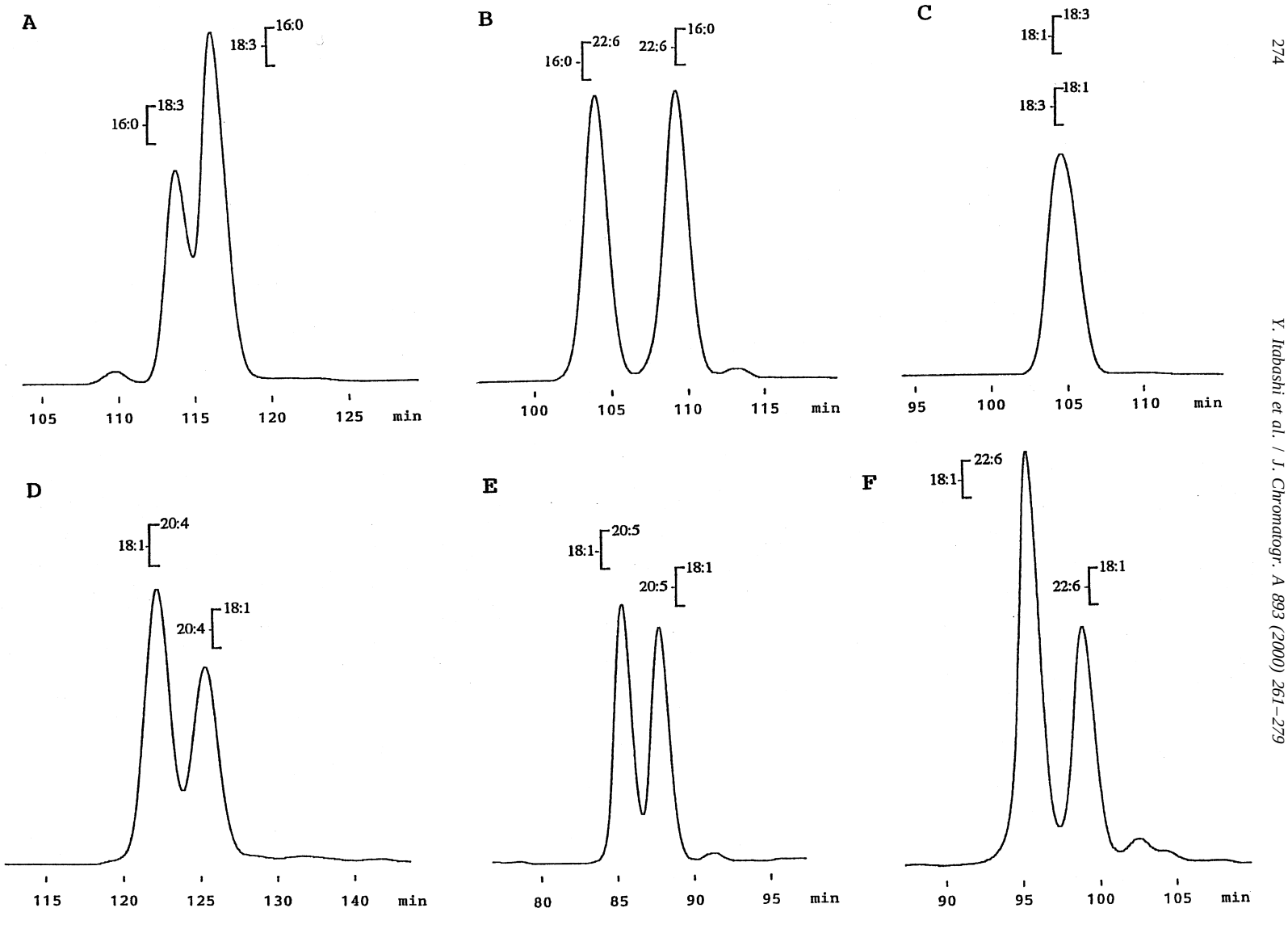


Fig. 7. Reversed-phase HPLC resolution of the reverse isomers of 1,2-diacyl-*rac*-glycerols containing polyunsaturated acyl chains as 3,5-dinitrophenylurethanes. Peak identification: A, 1-linolenoyl-2-palmitoyl-*rac*-glycerol and 1-palmitoyl-2-linolenoyl-*rac*-glycerol; B, 1-docosahexaenoyl-2-palmitoyl-*rac*-glycerol and 1-palmitoyl-2-docosahexaenoyl-*rac*-glycerol; C, 1-linolenoyl-2-oleoyl-*rac*-glycerol and 1-oleoyl-2- linolenoyl-*rac*-glycerol; D, 1-arachidonoyl-2-oleoyl-*rac*-glycerol and 1-oleoyl-2-arachidonoyl-*rac*-glycerol; E, 1-eicosapentaenoyl-2-oleoyl-*rac*-glycerol and 1-oleoyl-2-eicosapentaenoyl-*rac*-glycerol; F, 1-docosahexaenoyl-2-oleoyl-*rac*-glycerol and 1-oleoyl-2-docosahexaenoyl-*rac*-glycerol. A mixture of the reverse isomers of 1,2-diacyl-*rac*-glycerol was analyzed to obtain each chromatogram. HPLC conditions are the same as those in Fig. 6.

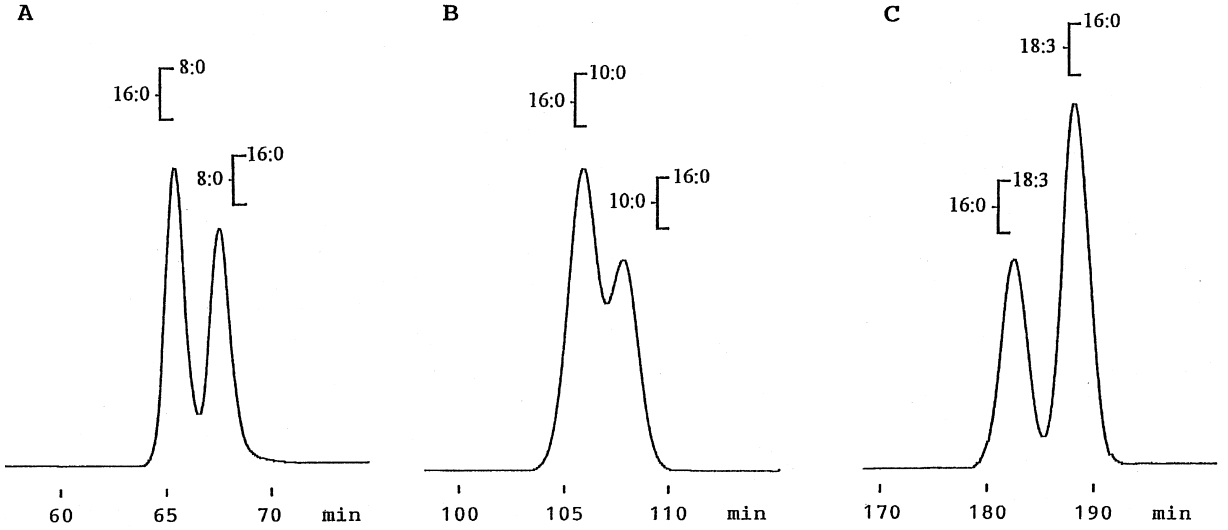


Fig. 8. Improved resolution of the reverse isomers of 1,2-diacyl-*rac*-glycerols as 3,5-dinitrophenylurethanes by reversed-phase HPLC at a lower temperature. For peak identification, see Figs. 6 and 7. Column temperature, 10°C. Other HPLC conditions as given in text.

packed with 5 μm particles having the same column length as the $C_{18}$ column (4 μm particles) used in this study, but no better resolution was obtained on these columns (chromatograms not shown). Our observations suggest that the column efficiency is one of the most important factors for resolving reverse isomers on reversed-phase HPLC. We obtained 20 900 theoretical plates for the 2:0–16:0 peak, which were higher than those obtained by chiral-phase HPLC (see Section 3.1).

Norman and St. John [5] reported a clear separation by reversed-phase HPLC of the reverse isomers of non-derivatized 1,2-linoleoyllinolenoyl (18:2–18:3) phosphoglycerocholine, which was identified by compositional analysis of fatty acids. In the present study, the synthetic reverse isomers of 18:2–18:3 diacylglycerol as 3,5-DNPU gave no resolution (chromatogram not shown). In a recent review, Bell [7] reported that the 3,5-DNB derivatives of 16:0–20:5 and 20:5–16:0 were separable and emerged in this order from a reversed-phase HPLC using acetonitrile–propan-2-ol (4:1, v/v) as the mobile phase. The present work shows that the 3,5-DNPU derivatives of this species elute in an order opposite to that noted for the 3,5-DNB derivatives. When no discrimination is made for reverse isomers, the elution order of the 3,5-DNPU derivatives of the enantiomeric diacylglycerols (Table 4) is the same as that of the 3,5-DNB [7]. We can offer no explanation for the differences observed in the elution order of these reverse isomers.

### *3.3. Silver ion HPLC separation*

Fig. 9 shows the silver ion HPLC separation of the reverse isomers of 1,2-diacyl-*rac*-glycerols containing unsaturated acyl chains on a Chromspher 5 Lipids column. The chromatographic data are given in Table 5. Excellent resolutions are obtained for the reverse isomers of saturated (or monounsaturated) and highly unsaturated fatty acid pairings, that is, 16:0–22:6, 18:1–20:4, 18:1–20:5 and 18:1–22:6, within appropriate analytical times by isocratic elution with chloroform containing small amounts (4–6%, v/v) of acetonitrile as the mobile phase, although the peak shapes are not as good as those observed on chiral- and reversed-phase HPLC. We obtained only 4500 theoretical plates for the 22:6–16:0 peak using chloroform–acetonitrile (100:5, v/v) as the mobile phase, which is considerably lower than the numbers obtained on chiral- and reversed-phase HPLC. With *n*-hexane–dichloromethane–acetonitrile (85:12:3, v/v/v) as the mobile phase, the plate number increased to 18 200 for the same species, but no clear resolution was obtained ($R_s$ = 0.93, $\alpha$ = 1.029). Thus, successful resolutions of the

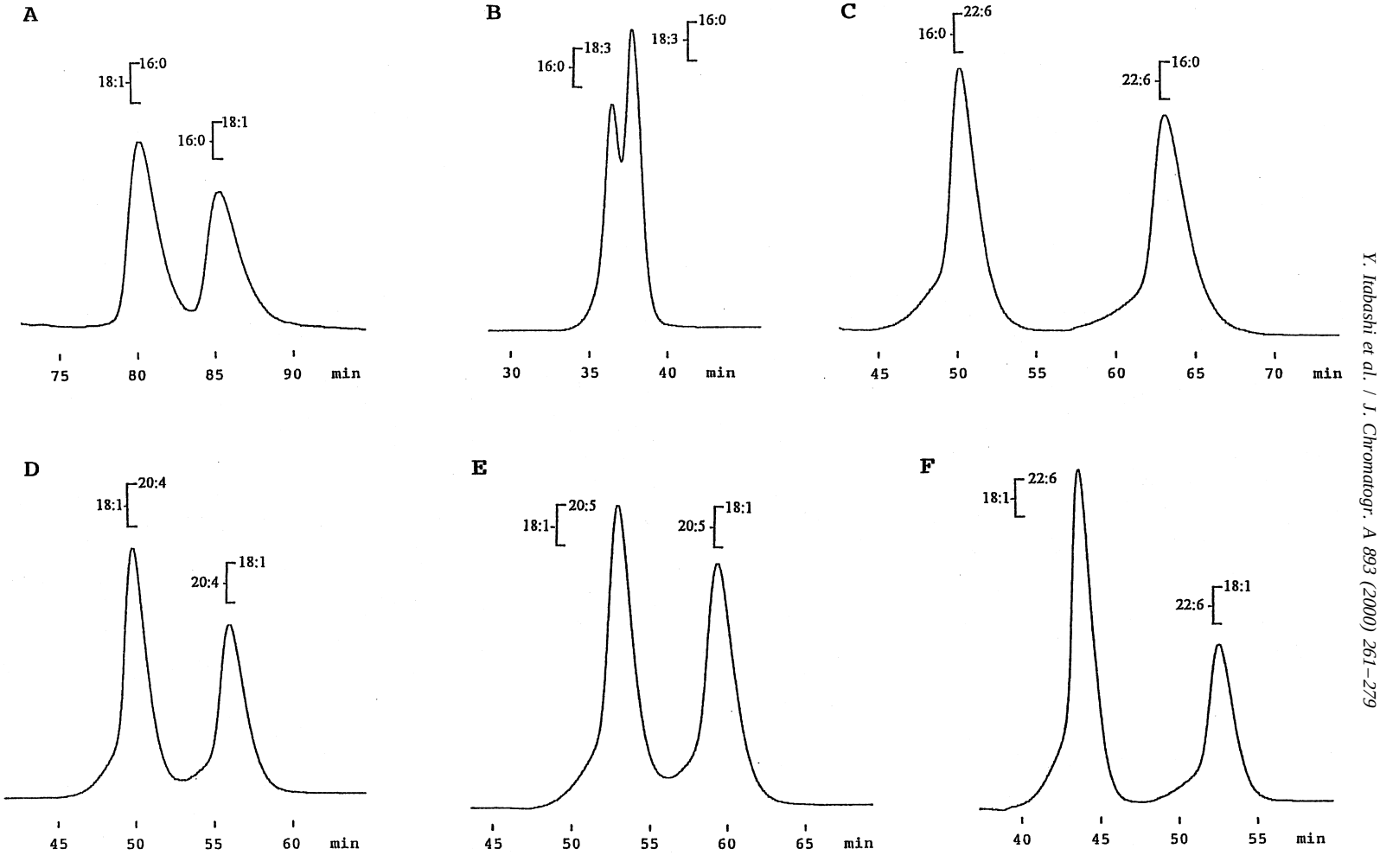


Fig. 9. Silver ion HPLC resolution of the reverse isomers of 1,2-diacyl-*rac*-glycerols containing unsaturated acyl chains as 3,5-dinitrophenylurethanes. A, 1-Palmitoyl-2-oleoyl-*rac*-glycerol and 1-oleoyl-2-palmitoyl-*rac*-glycerol; B–F; see Fig. 7. A mixture of the reverse isomers of 1,2-diacyl-*rac*-glycerol was analyzed to obtain each chromatogram. HPLC conditions as given in text.

Table 5
Silver ion HPLC of the reverse isomers of diacylglycerols containing unsaturated acyl chains as 3,5-dinitrophenylurethanes

| Diacylglycerol[a] | | | | Mobile phase[c] | $V_r$[d] | $k'$[b] | $\alpha$[b] | $R_s$[b] |
|---|---|---|---|---|---|---|---|---|
| *sn*-1(3) | *sn*-2 | | ECN[b] | | (ml) | | | |
| 18:1 | 16:0 | 34:1 | 32 | | 37.30 | 13.42 | | |
| | | | | 200:1 | | | 1.066 | 1.24 |
| 16:0 | 18:1 | 34:1 | 32 | | 39.76 | 14.30 | | |
| 18:2 | 18:1 | 36:3 | 30 | | | | | |
| | | | | 100:2 | 32.82 | 11.81 | –[e] | –[e] |
| 18:1 | 18:2 | 36:3 | 30 | | | | | |
| 18:3 | 16:0 | 34:3 | 28 | | 15.38 | 5.532 | | |
| | | | | 100:3 | | | 1.042 | 0.54 |
| 16:0 | 18:3 | 34:3 | 28 | | 16.02 | 5.764 | | |
| 18:3 | 18:1 | 36:4 | 28 | | 36.85 | 13.25 | | |
| | | | | 100:3 | | | 1.029 | 0.37 |
| 18:1 | 18:3 | 36:4 | 28 | | 37.90 | 13.63 | | |
| 20:4 | 18:1 | 38:5 | 28 | | 22.87 | 8.226 | | |
| | | | | 100:4 | | | 1.140 | 1.98 |
| 18:1 | 20:4 | 38.5 | 28 | | 26.06 | 9.376 | | |
| 20:5 | 18:1 | 38.6 | 26 | | 23.98 | 8.626 | | |
| | | | | 100:5 | | | 1.135 | 1.89 |
| 18:1 | 20:5 | 38:6 | 26 | | 27.22 | 9.793 | | |
| 22:6 | 16:0 | 38:6 | 26 | | 22.39 | 8.052 | | |
| | | | | 100:5 | | | 1.289 | 3.38 |
| 16:0 | 22:6 | 38:6 | 26 | | 28.84 | 10.38 | | |
| 22:6 | 18:1 | 40:7 | 26 | | 20.17 | 7.256 | | |
| | | | | 100:6 | | | 1.229 | 2.95 |
| 18:1 | 22:6 | 40:7 | 26 | | 24.78 | 8.915 | | |

[a] 16:0, palmitoyl; 18:0, stearoyl; 18:1, oleoyl; 18:2, linoleoyl; 18:3, linolenoyl; 20:4, arachidonoyl; 20:5, eicosapentaenoyl; 22:6, docosahexaenoyl.
[b] For ECN, $V_r$, $k'$, $\alpha$ and $R_s$, see Table 2.
[c] Chloroform–acetonitrile (v/v).
[d] Retention volume corrected for column void volume (2.78 ml).
[e] Completely overlapping peaks.

reverse isomers of 1,2-diacyl-*rac*-glycerols with up to seven double bonds were achieved by increasing the proportion of acetonitrile in chloroform. The importance of solvent selection in silver ion HPLC is described elsewhere [16].

As in chiral- and reversed-phase HPLC, there was no resolution for the reverse isomers of 18:1–18:2 and 18:2–18:3 species on the silver ion HPLC. Successful resolution, however, was obtained for the reverse isomers of 16:0–18:1 (Fig. 9A), which were not resolved on chiral- and reversed-phase HPLC, by the use of chloroform containing 0.5% (v/v) of acetonitrile as the mobile phase. This resolution, however, decreased rapidly after approximately 50 runs of the diacylglycerols including polyunsaturated ones ($R_s$=0.35, $\alpha$=1.054) and effective resolution was no longer obtainable, although satisfactory separation was still obtained for the polyunsaturated isomers (Fig. 9C–F). These observations suggest that a loss of column performance, which is probably caused by the acetonitrile in the mobile phase, affected the resolution of the reverse isomers of the saturate–monoenoate pair more than the saturate (or monoenoate)–polyenoate pairs.

Takagi and Nishimura [8] reported the reverse isomer resolution for 16:0–18:1, 16:0–18:2 and 16:0–18:3 diacylglycerols as their 3,5-DNB derivatives under very long retention times (100–900 min) by silver ion HPLC with recycling using a ternary solvent system made up of *n*-hexane, 1,2-dichloroethane and ethanol. The reverse isomers with the unsaturated acyl chains at the secondary position eluted faster than those with unsaturated ones in the primary positions. The silver ion HPLC of triacyl-

glycerols also shows earlier elution of the reverse isomers having unsaturated acyl chains at the secondary position [16,17]. These results suggest that the sterically more hindered isomers having unsaturated acyl chains in the secondary position are retained less strongly than those with unsaturated chains in the primary positions [8,18]. The elution order of the reverse isomers of the 3,5-DNPU of 1,2-diacyl-*rac*-glycerols, except for 16:0–18:1, observed in this study is opposite to that of the 3,5-DNB [8] and triacylglycerols [16,17] and this reversal of elution order does not support the separation mechanism based on the steric hindrance in the secondary position. The 3,5-DNPU group, which interacts with silver ion, would therefore appear to influence the elution order. The elution of the 16:0–18:1 and 18:1–16:0 species is unique. When chloroform–acetonitrile (200:1, v/v) was used as the mobile phase, 16:0–18:1 was eluted faster than 18:1–16:0 (Fig. 9A), while the reverse was observed when the acetonitrile was replaced by methanol (chromatogram not shown). Acetonitrile complexes more strongly with silver ion displacing the unsaturated solutes and its addition to the mobile phase results in a reduction in the contribution of olefin–$Ag^+$ interaction to the retention time [16,19]. Thus, an isomer having a polyunsaturated acyl chain in the primary position, which is more polar than that at the *sn*-2 position, would be retained more strongly. The reversal of elution order was caused by an increase in the degree of interaction between olefinic bonds (and/or 3,5-DNPU group) and silver ion in the stationary phase [19]. The faster elution of 16:0–18:1 suggests that the concentration of acetonitrile in the mobile phase is not sufficient to complex silver ion.

A successful resolution of the reverse isomers could not be obtained on silver ion HPLC using the ternary solvent system of *n*-hexane–dichloromethane–acetonitrile, which gave effective resolution for both enantiomeric and reverse isomers on chiral-phase HPLC. For example, the pair of 16:0–18:1 and 18:1–16:0 overlapped completely when run in *n*-hexane–dichloromethane–acetonitrile (85:12:3, v/v/v) and showed closely similar retention times with chloroform–acetonitrile (200:1, v/v), although the column had higher theoretical plate numbers with the former (15 200) than with the latter mobile phase (7700). The resolution of the pair of 16:0–22:6 and 22:6–16:0 was also poorer when the former mobile phase was used ($\alpha=1.029$, $R_s=0.93$, elution time= 96.9 min for 22:6–16:0), although high theoretical plate numbers (18 000) comparable to those on reversed-phase HPLC were obtained.

## 4. Conclusion

The earlier elution of the reverse isomers with the shorter acyl chain or the chain of higher degree of unsaturation in the primary positions from all the chromatographic systems is surprising. It would have been anticipated that the diacylglycerols with the shorter acyl chain or the chain of higher degree of unsaturation in the primary positions would be slightly more polar than the corresponding ones with the shorter or highly unsaturated acyl chain in the secondary position. Hence, the isomers with the short or highly unsaturated acyl chain in the primary positions would have been expected to be eluted later from the polar and earlier from the non-polar stationary phases. Since from all columns, where separations were achieved, the isomers with the short acyl chain or highly unsaturated chain in the primary positions emerged first, it must be concluded that the shape of the molecule is more important than the polarity in determining the order of elution of the reverse isomers.

The present results contradict earlier claims of chromatographic resolution of reverse isomers of 1,2-diacyl-X-glycerols containing common fatty acids. On the basis of the present study such resolutions, which are not substantiated with chromatograms showing the resolution of diacylglycerols of known reverse isomer content, are highly suspect and must be attributed to the presence of unidentified components emerging in the vicinity of the anticipated peaks. The current work demonstrates that chiral-phase, reversed-phase and silver ion HPLC all give effective resolution, identification and quantitation of the reverse isomers of such natural diacylglycerols, as those of milk fat [11], algal glycolipids [7] and fish phospholipids [6,7], which contain pairs of fatty acids of very different chain length and unsaturation, i.e., 2:0–16:0, 16:0–20:5 and 16:0–22:6. However, the present study does not give much

hope for practical resolutions of reverse isomers having minor differences in chain lengths and degree of unsaturation between the two acyl groups in any of the presently available chromatographic systems. The study suggests that higher efficiency columns or new types of stationary phases are necessary for a clear separation of reverse isomers of each pair of natural fatty acids. The apparent importance of the shape of the diacylglycerol molecule in the reverse isomer resolution suggests that new stationary phases exhibiting a more intimate interaction with the hydrophobic moiety of the molecule might give better resolution. The possibility of reverse isomer resolution based on the principle of affinity chromatography must also be considered, although this approach may necessitate the design of a special chromatographic system for each pair of fatty acids making up the molecular species. The diacylglycerol reverse isomers have been differentiated previously by GC–MS [21] and LC–chemical ionization MS [3] of the *t*-BDMS ethers. Recently it has been reported that GC–MS [22] and LC–MS [23] of diacylglycerol nicotinate derivatives can also distinguish between the reverse isomers. If the diacylglycerol reverse isomers are separable as the nicotinate derivatives by GC or HPLC, then combinations with MS methods would provide a powerful tool for the identification of the reverse isomer mixtures.

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