# Micro Method for Stereospecific Analysis of Triacyl-*sn*-Glycerols by Chiral-Phase High-Performance Liquid Chromatography[1]

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**A procedure for micro stereospecific analysis of triacyl-*sn*-glycerols (TGs) by high-performance liquid chromatography (HPLC) on a chiral column is presented. TGs were partially hydrolyzed with ethyl magnesium bromide, and total products were immediately converted to 3,5-dinitrophenylurethane derivatives. Each of the 1- and 2-monoacylglycerol (MG) derivatives was isolated by HPLC on a silica column. The 1-MGs were resolved into *sn*-1 and *sn*-3 MG fractions by HPLC on a Sumichiral OA-4100 column (Sumitomo Chemical, Osaka, Japan). Fatty acid methyl esters obtained from the *sn*-1, *sn*-2 and *sn*-3 MG fractions were analyzed by gas–liquid chromatography on a capillary column. Analyses of standard TGs showed that, even with 1 mg of sample, accuracy was comparable to that obtained with 100-mg samples. Applying this procedure to the stereospecific analysis of 5 mg of jujube pulp, TGs revealed the positional distribution of the (n-5) series of monounsaturated fatty acids they contained.**

KEY WORDS: Chiral-phase high-performance liquid chromatography, ethyl magnesium bromide, stereospecific analysis, triacyl-*sn*-glycerols.

Some efforts have been made to reduce the scale of methods for stereospecific analysis of minor amounts of triacyl-*sn*-glycerols (TGs). In 1965, the Brockerhoff enzymatic method originally required about 1 g of TGs (1). The improved method can be used with 10–40 mg or less of TGs (2). Recently, some new chemical methods have been developed for stereospecific analysis of TGs (3–6). One of them allowed the analysis of 1–2 mg of vegetable oil TGs or their main fractions (5,6).

All of the new chemical methods were based on chromatographic separation of isomeric and enantiomeric monoacylglycerols (MGs) or diacylglycerols (DGs) formed from TGs (3–6). Our chemical procedure involved partial hydrolysis of TGs to MGs, preparation of enantiomeric di-3,5-dinitrophenylurethane (di-3,5-DNPU) derivatives and resolution of them by chiral-phase high-performance liquid chromatography (HPLC) (3). Although the method required 100 mg of TGs, it has the advantage of analyzing TGs that may contain a wide range of fatty acids. Positional distribution of fatty acids in fish oil TGs were detailed by that method (7).

In the present study, we have used a modified procedure for stereospecific analysis of minor amounts of TGs. The modified procedure gave more accurate results with 1–10 mg of TGs than did the original one.

## MATERIALS AND METHODS

*TGs.* 1,3-Distearoyl-2-oleoylglycerol was obtained from Sigma Chemical Co. (St. Louis, MO). Jujube (*Zizyphus jujuba* var. *inermis*) pulp lipids were extracted with chloroform/methanol (1:1, vol/vol). Before use, TG samples were purified by preparative thin-layer chromatography (TLC) on Kiesel Gel 60G plates (Merck, Darmstadt, Germany) with *n*-hexane/diethyl ether (80:20, vol/vol) for development.

*Preparation of MG urethanes.* TGs (1–10 mg) were dissolved in dry diethyl ether (0.23 mL). Ethyl magnesium bromide (1M) in dry diethyl ether (0.1 mL) was added, and the mixture was stirred for 25 s before 0.1 mL of glacial acetic acid/diethyl ether (1:9, vol/vol) and water (2 mL) were added to stop the reaction. The products were extracted with diethyl ether, washed once with 2% aqueous sodium bicarbonate followed by water and dried over anhydrous sodium sulfate. After evaporating the solvent in a stream of nitrogen at room temperature, the total products were immediately dissolved in dry toluene (1 mL) and then reacted with 40 mg of 3,5-dinitrophenyl isocyanate (Sumitomo Chemical, Osaka, Japan) in the presence of dry pyridine (0.1 mL) overnight at room temperature.

*HPLC on silicic acid.* Each of the 1- and 2-MG di-3,5-DNPUs was isolated from the products by micro-preparative HPLC on silicic acid. The isolation was carried out with a Shimadzu LC-6A isocratic pump (Kyoto, Japan) with a Shimadzu SPD-6A UV-detector and a Shimadzu C-R6A integrator. For separation, a column of silica gel (Hypersil 3 μm, 25 cm × 4.6 mm i.d.; HiChrom, Reading, United Kingdom) was utilized with *n*-hexane/2-propanol (200:5, vol/vol) as mobile phase at a flow rate of 1.0 mL/min. Detection was at 254 nm.

*Chiral-phase HPLC.* The 1-MG di-3,5-DNPUs were resolved into *sn*-1 and *sn*-3 MG fractions by HPLC on a chiral column (Sumichiral OA-4100, 25 or 50 cm × 4 mm i.d.; Sumitomo Chemical) with *n*-hexane/1,2-dichloroethane/ethanol (40:12:3, vol/vol/vol) as mobile phase at a flow rate of 1.0 mL/min. They were detected with a Jasco 875-UV detector (Japan Spectroscopic, Tokyo, Japan) at 254 nm.

*Gas–liquid chromatography (GLC).* The MG di-3,5-DNPUs were converted to fatty acid methyl esters by the procedure of Christie and co-workers (5). The di-3,5-DNPUs were dissolved in 1,2-dichloroethane (0.6 mL) and methyl acetate (25 μL), and a solution of 1M sodium methoxide in methanol (25 μL) was added. The mixture was heated for 2 h at 50°C. Acetic acid (7 μL) was added, and the solvents were evaporated in a stream of nitrogen. Original TGs were also methylated similarly. The products were taken up in *n*-hexane. A Shimadzu GC-14A gas chromatograph, equipped with a split/splitless injection system, was used for fatty acid analysis with a fused-silica column coated with Omegawax 320 (30 m × 0.32 mm i.d., 0.25 μm film thickness; Supelco, Bellefonte, PA). The temperature of the oven was maintained at 75°C for 6 min, then it was raised to 195°C at 8°C/min. Hydrogen was the carrier gas.

## RESULTS AND DISCUSSION

*Modified procedure.* To minimize the opportunity for acyl migration to occur, 1- and 2-MGs produced from TGs

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by partial hydrolysis must be immediately converted to stable di-3,5-DNPU derivatives. The MG products were originally isolated by TLC on boric acid-impregnated plates prior to the conversion (3). In this study, the hydrolysis mixture was converted directly to the di-3,5-DNPU derivatives, and then the 1- and 2-MG derivatives were isolated by HPLC on silicic acid. The approach of derivatizing directly prior to isolation of the products was utilized by Christie *et al.* (5) in the stereospecific analysis *via* HPLC separation of diastereomeric DG derivatives. Our previous paper also presented such methodology and application to the analysis of 100 mg of cacao butter TGs (8). TLC on silicic acid could also be used for the isolation of 1- and 2-MG derivatives with chloroform/acetone (96:4, vol/vol) as developing solvent. The 1-MG derivatives collected were resolved into *sn*-1 and *sn*-3 MGs by chiral-phase HPLC. Fatty acids of *sn*-1, *sn*-2 and *sn*-3 MG fractions were analyzed by GLC after conversion to methyl esters.

The yields of 1- and 2-MGs were less than 5% of the products of partial hydrolysis of TGs, and they could not be improved, even if the conditions were altered. With 1–10 mg of TG sample, the amounts of resulting 1- and 2-MGs were sufficient for ultraviolet detection in HPLC of their di-3,5-DNPUs. However, the amounts of fatty acid methyl esters obtained at the end seemed to be insufficient for GLC analysis on a capillary column with an ordinary split injection system. In this study, the splitless injection procedure was introduced at the stage of GLC analysis of the methyl esters.

*Stereospecific analysis of synthetic TGs.* The analytical procedure was applied to 1 and 10 mg of the synthetic standard, 1,3-distearoyl-2-oleoylglycerol (Table 1). With 10 mg of the sample, *sn*-1 and *sn*-3 positions were contaminated with about 1.7–1.8 mole % of 18:1, which had migrated from the *sn*-2 position, and the *sn*-2 position was contaminated with about 3.7 mole % of 18:0, which had migrated from the *sn*-1 and *sn*-3 positions. These values were lower than those obtained with 100 mg of samples by the original method, where the primary and secondary positions contained about 2.4–3.0 mole % and 4.2–6.1 mole % of migrated fatty acids, respectively (3). With the 1-mg sample, *sn*-1 and *sn*-3 positions showed compositions similar to those found with the 10-mg sample (Table 1). The contaminant in the *sn*-2 position (18:0) showed somewhat higher values of the proportion (4.7 mole %) and standard deviation (1.0 mole %). However, the accuracy was in no way inferior to that of the original method. These results show that the procedure used in this study can give more accurate results than the original one even with a tenth to one-hundredth of the sample amount. Immediate conversion of the hydrolysis products to the di-3,5-DNPU derivatives is an effective procedure for the analysis of minor amounts of sample.

*Application to the analysis of jujube pulp TGs.* The procedure was applied to the stereospecific analysis of jujube pulp TGs. Extractable lipids from jujube pulp accounted for 0.8 wt% (wet basis) of the fresh fruit, and the TG content was only about 1 wt% of the lipids. In this study, 5 mg of the TGs was subjected to the stereospecific analysis.

Figure 1 shows the separation of 1- and 2-MGs from the partially hydrolyzed TGs, as their di-3,5-DNPUs, by HPLC on silicic acid. The peaks of 1- and 2-MG derivatives were separated from other peaks (Fig. 1A). There was no separation of MG molecular species. The purities of the collected 1- and 2-MG fractions were checked by rechromatography. Figure 1 (B and C) show no cross-contamination during peak collection.

The 1-MG derivatives were resolved into *sn*-1 and *sn*-3 MGs by chiral-phase HPLC (Fig. 2). On Sumichiral OA-4100, 1-MGs could be separated into *sn*-1 and *sn*-3 MG groups (Fig. 2A). Rechromatography of the collected fractions showed no cross-contamination during peak collection (Fig. 2, B and C). Figure 2D shows the chromatogram of the 2-MG fractions collected by HPLC on silicic acid.

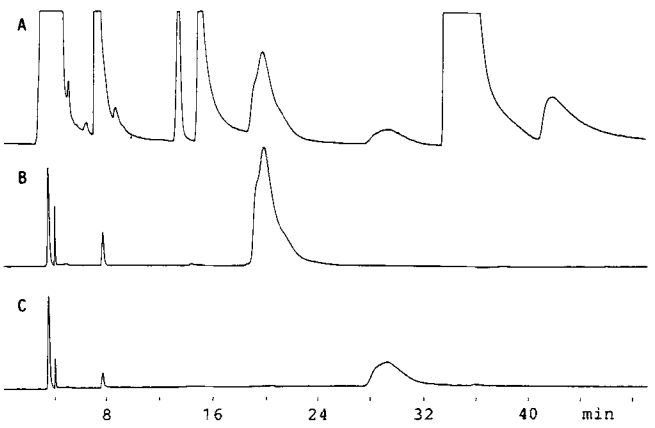


**FIG. 1. Isolation of 1- and 2-monoglyceride (MG) as their di-3,5-dinitrophenylurethane by high-performance liquid chromatography on a silica column, Hypersil 3 μm (25 cm × 4.6 mm i.d.). A, Total products of partial hydrolysis of jujube pulp triacyl-*sn*-glycerols; B, 1-MG fraction collected; C, 2-MG fraction collected. Mobile phase, *n*-hexane/2-propanol (200:5, vol/vol). Flow rate, 1.0 mL/min. Detection, 254 nm ultraviolet.**

TABLE 1

**Stereospecific Analysis of Synthetic 1,3-Distearoyl-2-Oleoylglycerol by Micro Method (mol %)**

| Triacyl-*sn*-glycerol sample | Fatty acid | Total | Position | | |
|---|---|---|---|---|---|
| | | | *sn*-1 | *sn*-2 | *sn*-2 |
| 10 mg | 18:0 | 66.7 | 98.2[a] ± 0.7[b] | 3.7 ± 0.5 | 98.3 ± 0.7 |
| | 18:1 | 33.3 | 1.8 ± 0.7 | 96.3 ± 0.5 | 1.7 ± 0.7 |
| 1 mg | 18:0 | 66.7 | 98.4 ± 0.7 | 4.7 ± 1.0 | 98.2 ± 0.6 |
| | 18:1 | 33.3 | 1.6 ± 0.7 | 95.3 ± 1.0 | 1.8 ± 0.6 |

[a]Mean value of triplicate analyses.
[b]Standard deviation.

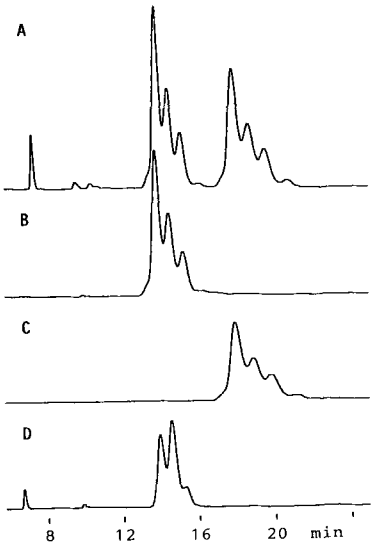


**FIG. 2. Chiral-phase high-performance liquid chromatography of monoglycerides (MGs), formed by partial hydrolysis of jujube pulp triacyl-*sn*-glycerols with ethyl magnesium bromide, as their di-3,5-dinitrophenylurethanes, on Sumichiral OA-4100 column (25 cm × 4 mm i.d., Sumitomo Chemicals, Osaka, Japan). A, 1-MGs; B, *sn*-1 MG fraction collected; C, *sn*-3 MG fraction collected; D, 2-MGs. Mobile phase, *n*-hexane/1,2-dichloroethane/ethanol (40:12:3, vol/vol/vol). Flow rate, 1.0 mL/min. Detection, 254 nm ultraviolet.**

The peaks appeared with retention times close to those of *sn*-1 MGs, and thus it is necessary to preliminarily separate 1- and 2-MG derivatives by HPLC on silicic acid. There was no peak of *sn*-3 MGs in the chromatogram. This supports the fact that the 2-MG fraction was free from 1-MGs.

Fatty acids were analyzed by GLC on a capillary column with a split/splitless injection system after preparation of methyl esters by transesterification. Table 2 shows the positional distribution of fatty acids in the jujube pulp TGs. Jujube pulp lipids are characterized by the occurrence of a (n-5) series of monounsaturated fatty acids (9). There seems to be consistency in the distribution pattern of 16:1(n-5), 18:1(n-7) and 18:1(n-5), which were abundant in *sn*-1 and *sn*-3 positions, and low in the *sn*-2 position. In the fatty acids, the olefinic bond is located at a position far from carboxyl, *i.e.*, at position 11 in 16:1(n-5) and 18:1(n-7), and at position 13 in 18:1(n-5). The distribution pattern was similar to that of 18:0. Similar tendencies were also observed for macadamia nut and high-erucic acid rapeseed TGs analyzed by the original method (3).

The procedure presented in this study can be used for stereospecific analysis of minor amounts of TGs, and will be useful for the analysis of TGs in small amounts of tissues and organs in an organism, which are generally heterogeneous.

**TABLE 2**

**Stereospecific Analysis of Jujube Pulp Triacyl-*sn*-Glycerol (TGs) by Micro Method[a] (mole%)**

| Fatty acid | Total | Position | | |
|---|---|---|---|---|
| | | *sn*-1 | *sn*-2 | *sn*-3 |
| 12:0 | 3.9 | 2.1 | 2.6 | 7.3 |
| 14:0 | 2.6 | 2.3 | 1.3 | 4.4 |
| 14:1(n-5) | 6.6 | 11.2 | 3.3 | 5.3 |
| 16:0 | 15.8 | 26.9 | 4.5 | 16.3 |
| 16:1(n-7) | 9.7 | 8.9 | 12.9 | 7.1 |
| 16:1(n-5) | 9.8 | 10.3 | 8.6 | 10.6 |
| 18:0 | 2.1 | 2.6 | 0.8 | 2.8 |
| 18:1(n-9) | 24.4 | 10.9 | 34.4 | 27.9 |
| 18:1(n-7) | 4.2 | 6.7 | 1.8 | 4.1 |
| 18:1(n-5) | 2.1 | 2.8 | 0.4 | 3.3 |
| 18:2(n-6) | 15.5 | 12.0 | 25.0 | 8.9 |
| 18:3(n-3) | 3.4 | 3.3 | 4.6 | 2.1 |

[a]TG sample, 5 mg.

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