A Rapid Method for Enzymatic Synthesis and Purification of the Structured Triacylglycerol, 1,3-Dilauroyl-2-oleoyl-glycerol

Susumu Miura*, Asuka Ogawa, and Hiroaki Konishi

Technology and Research Institute, Snow Brand Milk Products Co. Ltd., Saitama, Japan

ABSTRACT: A rapid method for synthesis and purification of the structured triacylglycerol (TAG), 1,3-dilauroyl-2-oleoyl-glycerol (LaOLa), has been developed. A fraction containing 70% LaOLa was obtained by enzymatic transesterification between triolein and lauric acid using Lipozyme IM, which has 1,3-regioselectivity on the glycerol moiety of TAG. The fraction was passed through a Bond Elut SI Column to remove mono- and diacylglycerols produced during the transesterification. The TAG fraction thus obtained was applied to a reversed-phase column, eluted with acetonitrile/tetrahydrofuran (8:2, vol/vol) to separate different TAG species. The LaOLa fraction obtained by this method was of greater than 99% purity. The concentrations of total fatty acids and fatty acids bound to the *sn*-2 position of this LaOLa fraction were determined by gas–liquid chromatography after hydrolysis by pancreatic lipase, to confirm the purity of stereospecific isomers in this fraction. The final purity of LaOLa was found to be greater than 95%, which was in good agreement with the result obtained by high-performance liquid chromatography using a Lichrosorb Si60 argentated column.

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KEY WORDS: Argentated column, 1,3-dilauroyl-2-oleoyl-glycerol, enzymatic transesterification, high-performance liquid chromatography, ODS column chromatography, pancreatic lipase, solid-phase extraction, structured triacylglycerol.

Transesterification of fats and oils has been widely adopted by the food industry (1–3). It is used to obtain modified fats to substitute hydrogenated fats in margarines and shortenings (4–9), since hydrogenation of fats produces *trans* isomers of unsaturated fatty acids, the nutritional consequences of which are subjects of worldwide controversy (10–12).

Enzymatic modification of fats and oils has advantages over chemical transesterification in that some lipases show regioselectivity concerning the position of fatty acids bound to the triacylglycerol (TAG) where hydrolysis is carried out (13–16). This enables the incorporation of desired fatty acids onto specific positions of the TAG molecule, while chemical transesterification leads to a randomization of fatty acids on the glyceride moiety. Lipases with 1,3-positional selectivity generate a new TAG molecule while maintaining the fatty acid at the *sn*-2-position unchanged.

*To whom correspondence should be addressed at Technology and Research Institute, Snow Brand Milk Products Co. Ltd., 1-1-2 Minamidai, Kawagoe, Saitama, Japan, 350-1165. E-mail: YRB00152@nifty.ne.jp

Nutritional values and applications of structured TAG with medium-chain fatty acids at the *sn*-1- and *sn*-3-positions and unsaturated long-chain fatty acid at the *sn*-2-position were investigated extensively (17–21). These structured TAG are readily hydrolyzed by pancreatic lipases to give two molecules of free medium-chain fatty acid and one molecule of 2-monoacylglycerol. These free medium-chain fatty acids are absorbed in intestines and are carried rapidly into the liver *via* the bloodstream to be consumed as a source of energy, while the remaining 2-monoacylglyceride becomes a source of essential fatty acids. On the other hand, TAG found in vegetable oils is known to contain unsaturated fatty acids at the *sn*-2-position and long-chain fatty acids at the *sn*-1- and *sn*-3-positions. These TAG are also hydrolyzed by pancreatic lipases and are absorbed in the intestines where free fatty acids and 2-monoacylglycerols are reincorporated into TAG molecules. These TAG molecules are carried to adipose tissues *via* the bloodstream in chylomicions. Therefore, unlike long-chain free fatty acids, the structured TAG are known as a concentrated and rapid source of calories and nutrients.

These structured TAG are difficult to prepare by chemical transesterification and therefore remain very expensive to use in the food industry. Soumanou *et al.* (22,23) reported the synthesis of structured TAG using lipase catalysis. In their report, the final product contained more than 94% medium-chain fatty acids in the *sn*-1- and *sn*-3-positions, whereas the *sn*-2-position was composed of 78% unsaturated long-chain fatty acids. This method enables the rapid synthesis of desired structured TAG to be used in foods, although the purity may not be sufficient for the study of crystallization and other physical properties of TAG molecules.

In this study, a rapid method for the preparation of a structured TAG with a purity of over 95% using a commercially available 1,3-positionally specific lipase, Lipozyme IM, was investigated. Lauric acid was selected in this study as a medium-chain free fatty acid. First, lauric acid was transesterified with triolein to obtain a crude reaction mixture, which contained 70% LaOLa in the TAG fraction. Secondly, LaOLa was separated from other TAG molecules by column chromatography to obtain purified LaOLa.

EXPERIMENTAL PROCEDURES

Reagents. Unless noted, all chemicals were of reagent grade and purchased from Wako Pure Chemicals (Tokyo, Japan).

Triolein was of 99% purity and was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). Trilaurin, 1,3-dipalmitoyl-2-oleoyl-glycerol (POP), 1,2-dipalmitoyl-3-oleoyl-glycerol (PPO), and lauric acid were also of 99% purity and obtained from Sigma Chemical Co. (St. Louis, MO).

Lipozyme IM, a 1,3-regiospecific lipase from *Rhizomucor miehei* immobilized on an anion exchange resin, was obtained from Novo Nordisk (Bagsvaerd, Denmark). It had an activity of 5–6 Batch Acidolysis Units Novo (BAUN/g).

Porcine pancreatic lipase, a 1,3-regiospecific lipase with an activity of 220 units/mg protein where one unit releases 1.0 μmole of 2-monoglyceride from 1,2-diglyceride per min at pH 8.1 at 37°C, and bile salts were obtained from Sigma Chemical Co. Bond Elut SI column (5 g/20 mL and 500 mg/3 mL) were obtained from Varian Sample Preparation Products (Harbor City, CA). ODS resin (ODS-AM 120-S50) was obtained from YMC Inc. (Wilmington, NC). An argentated Lichrosorb Si60 (5 μm) column (250 mm × 4.6 mm I.D.) was purchased from Shenshu Kagaku Co. Ltd. (Tokyo, Japan).

Transesterification. Transesterification between 10 g (0.011 mole) of triolein and 22.6 g (0.11 mole) of lauric acid was carried out by adding 8% (w/w total TAG) of Lipozyme IM. The reaction mixture was stirred gently by a mechanical stirrer in a 200-mL glass flask. Reaction temperature was maintained at 70°C. Progress of transesterification was monitored by following the time course of the TAG composition in the reaction mixture. For this purpose, 100 mg aliquots were taken from the reaction mixture and passed through a Bond Elut SI column to remove free fatty acids, mono-, and diacylglycerols, and to obtain the TAG fraction. The TAG molecular composition of the TAG fraction was determined by high-performance liquid chromatography (HPLC). The detailed procedure is described below.

Purification of TAG fraction from the reaction mixture by Bond Elut SI column. After transesterification, the reaction mixture containing TAG, monoacylglycerols, diacylglycerols, and free fatty acids was dissolved in hexane/diethylether (8:2, vol/vol). The mixture was then applied to a Bond Elut SI (5 g/20 mL) column, and eluted with hexane/diethylether (8:2, vol/vol) to obtain a pure TAG fraction while the other species remained adsorbed onto the column. The purity of the TAG fraction was determined by thin-layer chromatography (TLC). An aliquot of the TAG fraction was dissolved in hexane/diethylether (1:1, vol/vol) and applied to a kieselgel plate, then developed by hexane/diethylether (6:4, vol/vol). The plate was visualized by spraying with 5% sulfuric acid dissolved in methanol, followed by heating at 150°C.

Determination of composition of TAG by HPLC. The composition of the TAG in each fraction was determined by HPLC using a Lichrosorb C18 column (5 μm, 250 mm × 4 mm I.D.; Merck, Darmstadt, Germany) and an evaporative light-scattering detector (Varex II; Varex, Burtonsville, MD). The column temperature was 30°C. The elution solvent used was acetonitrile/tetrahydrofuran (7:3, vol/vol), at a flow rate of 1.0 mL/min.

Separation of presumed LaOLa from the TAG fraction by ODS column. LaOLa fraction was separated from other TAG in the TAG fraction by ODS column chromatography. In order to determine optimal conditions for obtaining LaOLa of high purity, the column chromatography was done in a small scale as follows: 20 g of ODS resin was added to methanol, stirred gently to make a slurry, and poured onto the column (220 mm × 15 mm I.D.). Methanol was replaced by acetonitrile, and then finally by acetonitrile/tetrahydrofuran (8:2, vol/vol). 230 mg of the TAG fraction was dissolved in 1 mL of acetonitrile/tetrahydrofuran (6:4, vol/vol), and applied to the column. The column was eluted with acetonitrile/tetrahydrofuran (8:2, vol/vol). The eluent was collected in 5 mL/fractions by a fraction collector. Qualitative analysis of recovered fractions was performed by TLC to determine the fraction containing TAG. Each fraction containing TAG was subjected to HPLC as described above to determine the composition of TAG molecular species.

Determination of fatty acid content and stereospecific isomers of the TAG by gas–liquid chromatography (GLC). Stereospecific isomers (for example, in this study, 1,3-dilauroyl-2-oleoyl-glycerol and 1,2-dilauroyl-3-oleoyl-glycerol) of the TAG molecular species could not be distinguished by HPLC with the above conditions. Therefore, the LaOLa fraction was first subjected to hydrolysis using porcine pancreatic lipase to determine the fatty acid composition at the *sn*-2 position of TAG by GLC. At the same time, GLC was carried out to determine the total fatty acid composition of the LaOLa fraction.

In a typical reaction 3 mg of LaOLa fraction was dried under nitrogen at 40°C in a screw-capped tube (100 mm × 15 mm; Pyrex, Corning, NY) and was emulsified in 1 mL of Tris(hydroxymethyl)aminomethane (Trizma Base)-HCl buffer (1 M, pH 8.2) by sonicating for 2 min. Bile salts solution (1 g/L, 35 μL) and 60 μL of calcium chloride solution (220 g/L) were added to the emulsified mixture and mixed well. Porcine pancreatic lipase (15 mg) was added to the mixture and incubated at 37°C for 30 min with continuous shaking. After the reaction, the lipids were extracted three times with 3 mL of diethylether. The lipid extracts were dried under nitrogen at 40°C and redissolved in 300 μL of hexane. 2-Monoacylglycerols were separated by solid-phase extraction using a Bond Elut SI column (500 mg/3 mL). The lipid extracts dissolved in hexane were applied to the column, washed with 20 mL of hexane/diethylether (9:1, vol/vol), and then with 3 mL of hexane/diethylether/acetic acid (1:1:0.1, vol/vol/vol) to remove TAG, diglycerides, and fatty acids. 2-Monoacylglycerols were finally eluted with 4 mL of methanol and dried under nitrogen at 40°C. The 2-monoacylglycerols were transmethylated by conventional methods to obtain fatty acid methyl esters (FAME). FAME were separated by GLC (Hewlett-Packard 5890 Series II, with FID detector; Hewlett-Packard, Avondale, PA) using a fused silica capillary column (HP-INNOWax, 30 m × 0.25 mm I.D., Hewlett-Packard), with helium as carrier gas. The initial temperature was set at 150°C held for 1 min, incrementing at 3°C/min to 230°C and finally held at 230°C for a further minute.

Total fatty acid of the LaOLa fraction was also determined as described above, without hydrolysis by porcine pancreatic

lipase.

Determination of the positional isomers of the TAG fraction using argentation HPLC. The purity of LaOLa determined as described above was further confirmed by separating the LaOLa fraction by HPLC, using an argentation column as reported by Smith *et al.* (24). Benzene was used as the mobile phase at the flow rate of 1.0 mL/min. The detector and the column temperature were the same as described above.

RESULTS AND DISCUSSION

Transesterification. The identities of species in the TAG fraction obtained by transesterification between triolein and lauric acid is shown in Figure 1, as determined by HPLC. After 3 h of reaction, *ca.* 50% of total TAG was 1- (or 3-)lauroyl-2,3 (or 1,2-)-dioleoyl-glycerol. The proportion of 1,3-dilauroyl-2-oleoyl-glycerol reached 70% after 24 h. Figure 2 shows the HPLC chromatogram of the TAG fraction obtained from the reaction products after 24 h. Triolein with a retention time of 27 min was found to be completely consumed in this transesterification after 24 h. The peak A observed at 9.8 min was determined to be trilaurin from the retention time of standard trilaurin. Therefore, peak B and peak C were presumed to be dilaurate–monooleate (LaOLa) and monolaurate–dioleate, respectively. The fact that trilaurin was formed during this transesterification suggests that acyl migration may have occurred during the reaction. This result implies that the positional isomers of LaOLa, 1,2(or 2,3)-lauroyl-3(or 1)-oleoyl-glycerol (LaLaO) may have formed during the reaction and may be included in the single peak B.

Separation of LaOLa fraction by ODS column. The TAG fraction obtained by solid-phase extraction using a Bond Elut SI column was subjected to ODS column chromatography. TLC was done on each eluted fraction from the ODS column, and it was found that the elution of TAG started at the 24th fraction. HPLC was carried out using Lichrosorb C18 column for fraction No. 24 and onward to determine the content (%) of TAG in each fraction. In Figure 3, the content (%) of TAG is plotted against the fraction number. The presumed LaOLa fraction was eluted in fractions No. 35 to No. 47. These fractions were collected, and the solvent was removed by rotary

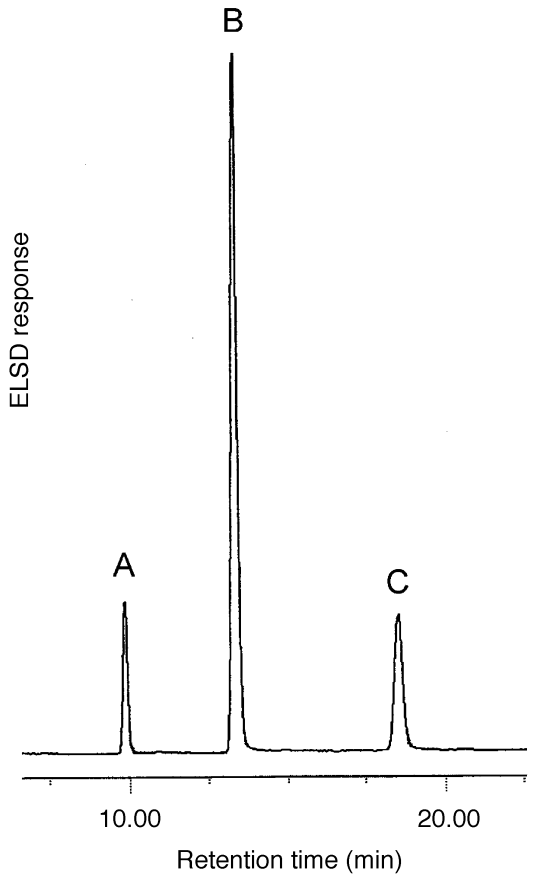

FIG. 2. High-performance liquid chromatography (HPLC) chromatograms of TAG species obtained from transesterification products after 24 h. TAG species were separated on Lichrosorb C18 (5 µm, 250 mm × 4 mm I.D.) column. See Figure 1 for other abbreviation.

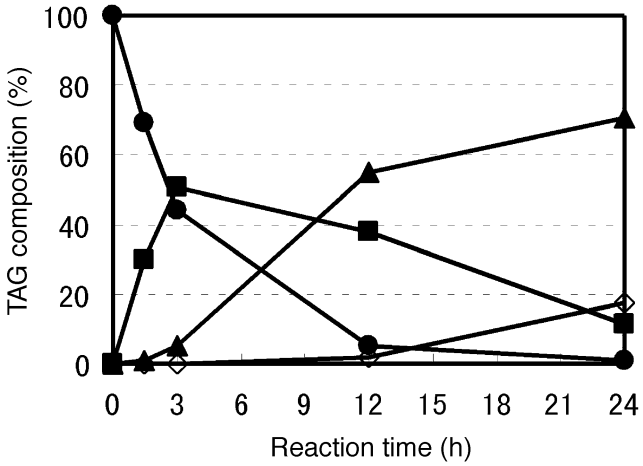

FIG. 1. Time course of triacylglycerol (TAG) composition. ●, Triolein; ■, 1-lauroyl-2,3-dioleoyl-glycerol; ▲, 1,3-dilauroyl-2-oleoyl-glycerol; ◇, trilaurin.

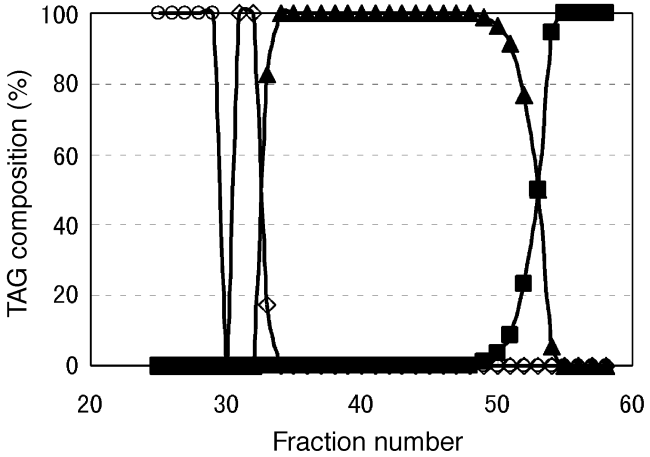

FIG. 3. ODS column chromatography. ○, Unknown; ◇, trilaurin; ▲, 1,3-dilauroyl-2-oleoyl-glycerol; ■, 1-lauroyl-2,3-dioleoyl-glycerol. Fraction numbers 35 to 47 show the elution of pure LaOLa.

evaporation under vacuum. Figure 4 shows the HPLC chromatogram of this fraction obtained by using Lichrosorb C18 column. The fraction indeed showed a single peak corresponding to peak B in Figure 2. The total weight of LaOLa obtained was 76 mg from 230 mg of the TAG fraction applied onto the column. The fraction thus collected was further subjected to the identification of fatty acid content and stereospecific isomers to verify the identity and final purity of LaOLa.

Verification of the identity and determination of the positional isomers of the LaOLa fraction by GLC. Total fatty acid composition and *sn*-2-fatty acid composition of the LaOLa fraction obtained by GLC are shown in Table 1. Approximately two-thirds of the total fatty acids in the LaOLa fraction were lauric acid, and the remaining one-third was oleic acid. At the same time, over 95% of the fatty acids bound to the *sn*-2- position were found to be oleic acid. The results suggest that LaOLa of over 95% purity was obtained in this fraction.

TABLE 1
Total and *sn*-2-Fatty Acid Compositions (wt%) of the LaOLa Fraction

	Fatty acid	
	12:0	18:1
Total	65.3	34.7
sn-2-	3.8	96.2

Determination of the positional isomers of the LaOLa fraction by HPLC using argentated silica column. To support the GLC results described above, the LaOLa fraction was subjected to HPLC using the Lichrosorb Si60 argentated silica column. As shown in Figure 5, two peaks A and B were detected. The area ratio of A to B was shown to be 93:7, which nearly corresponds to the value obtained by GLC. Therefore, peak A was estimated to be LaOLa, and the purity of LaOLa in this fraction was determined to be over 93%, which showed

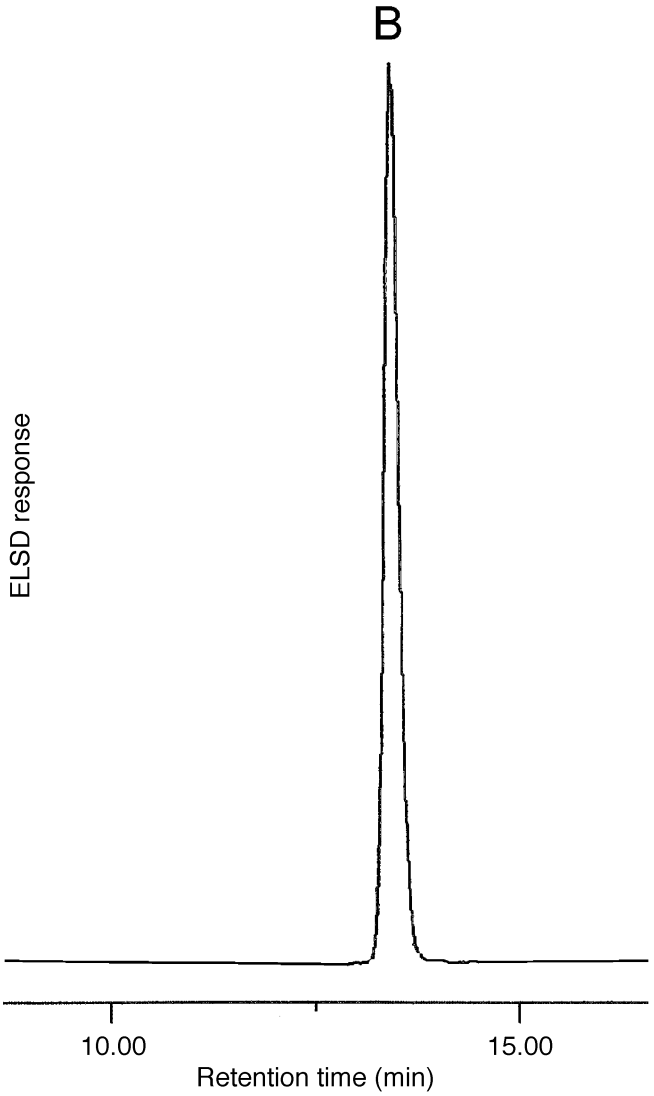

FIG. 4. HPLC chromatograms of the LaOLa fraction obtained from ODS column chromatography. TAG species were separated on Lichrosorb C18 (5 µm, 250 mm × 4 mm I.D.) column. See Figures 1 and 2 for abbreviations.

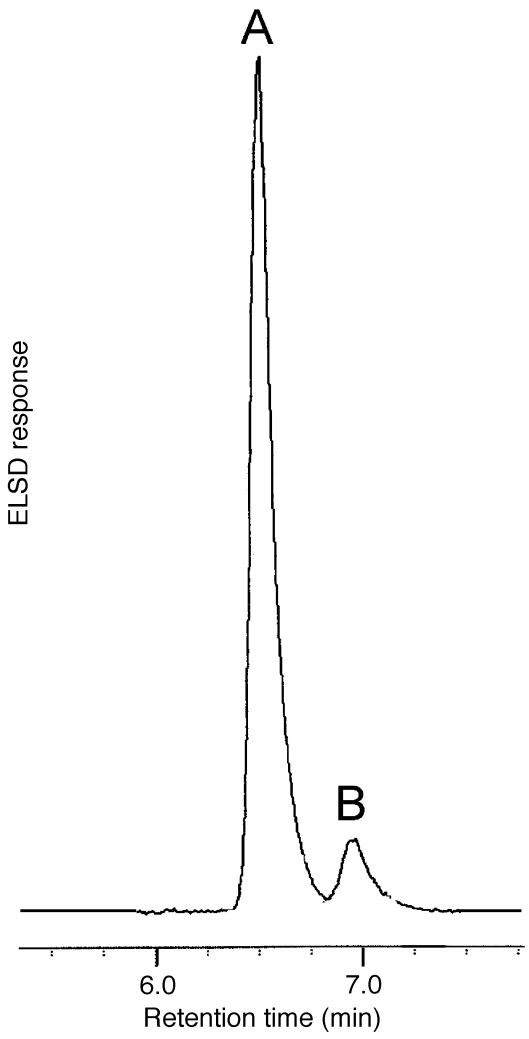

FIG. 5. HPLC chromatograms of the LaOLa fraction. TAG species were separated on Lichrosorb SI60 (5 µm, 250 mm × 4.6 mm) column impregnated with 5% silver nitrate. See Figures 1 and 2 for abbreviations.

a good agreement with the purity determined by GLC. The remaining 7% was estimated to be 1,2-(or 2,3-)dilauroyl-3-(or 1-)oleoyl-glycerol (LaLaO), which may have formed as a result of acyl migration during the transesterification. This identification was made by comparing the elution orders of the peak and of LaOLa with the elution orders of POP and PPO.

The ODS column could be reutilized after washing with acetonitrile/tetrahydrofuran (1:9, vol/vol). By using a larger ODS column of similar dimensions, rapid purification of LaOLa is possible at a larger scale.

In this report, a rapid method for the preparation of a structured TAG, LaOLa, was introduced. LaOLa of over 95% purity was obtained rapidly by this method. By applying this method, similarly structured TAG with medium-chain fatty acids incorporated onto the *sn*-1- and *sn*-3-positions, and unsaturated fatty acids on the *sn*-2-position, may be easily obtained at a purity of over 90%, for use in studies of the physical properties of TAG as well as in foods.

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