Enzymatic Synthesis of Symmetrical 1,3-Diacylglycerols by Direct Esterification of Glycerol in Solvent-Free System

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ABSTRACT: 1,3-Diacylglycerols were synthesized by direct esterification of glycerol with free fatty acids in a solvent-free system. Free fatty acids with relatively low melting points (<45°C) such as unsaturated and medium-chain saturated fatty acids were used. With stoichiometric ratios of the reactants and water removal by evaporation at 3 mm Hg vacuum applied at 1 h and thereafter, the maximal 1,3-diacylglycerol content in the reaction mixture was: 84.6% for 1,3-dicaprylin, 84.4% for 1,3-dicaprin, 74.3% for 1,3-dilinolein, 71.7% for 1,3-dieicosapentaenoin, 67.4% for 1,3-dilaurin, and 61.1% for 1,3-diolein. Some of the system's parameters (temperature, water removal, and molar ratio of the reactants) were optimized for the production of 1,3-dicaprylin, and the maximal yield reached 98%. The product was used for the chemical synthesis of 1,3-dicapryloyl-2-eicosapentaenoylglycerol. The yield after purification was 42%, and the purity of the triacylglycerol was 98% (both 1,3-dicapryloyl-2-eicosapentaenoylglycerol and 1,2-dicapryloyl-3-eicosapentaenoylglycerol included) by gas chromatographic analysis, of which 90% was the desired structured triacylglycerol (1,3-dicapryloyl-2-eicosapentaenoylglycerol) as determined by silver ion high-performance liquid chromatographic analysis.

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KEY WORDS: 1,3-Diacylglycerol, esterification, *Rhizomucor miehei* lipase, structured lipid synthesis.

Besides their use as nonionic surfactants in food, cosmetics, and pharmaceutical industries, 1,3-diacylglycerols (1,3-DAG) are intermediates for synthesis of various chemical compounds with pharmaceutical applications (1,2). Diglyceride prodrugs can be obtained when the second position is esterified with a drug bearing a carboxylic group such as the antiinflammatory niflumic acid (3), by covalently binding an alcohol, for example the β-blocker bupranolol (4), or phenytoin (5) to 2-chlorosuccinyl-1,3-diacylglycerol, etc. These forms are better adsorbed and have fewer side effects than the original drugs (1,4). In a similar manner, structured glycerides with a polyunsaturated fatty acid such as docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and the like in the second position and medium-chain acyl groups in 1- and 3-positions can be used as carriers of these therapeutically and nutritionally valuable acids (6).

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High yields of 1,3-DAG cannot be obtained by direct chemical methods because they lack positional selectivity. Multistep reaction sequences are necessary, and large quantities of solvents are used as reaction media and for purification (7,8). The classical synthesis requires starting from the 1-monoacylglycerol (1-MAG), which is acylated with the acid chloride in the presence of pyridine and chloroform. Yields of 50–70% are obtained after purification (9).

Better 1,3-DAG yields (85%) can be obtained by solid-phase enzymatic glycerolysis of triglycerides (TAG), but this method, based on the selective crystallization of 1,3-DAG, is limited to the glycerides of saturated fatty acids (FA) with relatively high melting points (10). The process is economically advantageous for natural fats, but use of the products is limited to applications where acyl species homogeneity is not important. For obtaining 1,3-DAG of the purity necessary for drug synthesis, pure homogeneous TAG should be synthesized first.

In another study, esterification of glycerol adsorbed beforehand onto a solid support was performed in organic solvents with 1,3-specific lipases (11,12). The synthesis of liquid 1,3-DAG (medium-chain and unsaturated 1,3-DAG) was done *via* irreversible acyl transfer using vinyl esters as acyl donors, adding an extra step to the reaction scheme plus the requisite purification of the vinyl ester. The yields obtained with stoichiometric ratios of reactants ranged between 52 and 76% for unsaturated fatty acid vinyl esters and between 80 and 85% for saturated acid vinyl esters.

In this work, a simple one-step procedure for synthesis of 1,3-DAG in solvent-free system using free fatty acids (FA) in liquid phase is described. Glycerol was esterified directly with a variety of FA by immobilized 1,3-specific *Rhizomucor miehei* lipase (Lipozyme). The equilibrium was shifted to the synthetic side by removing the water formed during the reaction by evaporating at reduced pressure or by blowing dry nitrogen gas or by adding molecular sieves. 1,3-Dicapryloyl-2-eicosapentaenoylglycerol, a structured glyceride with high pharmaceutical potential, was chemically synthesized from 1,3-dicapryloylglycerol obtained by this method.

EXPERIMENTAL PROCEDURES

Materials. Commercially available Lipozyme (*R. miehei* lipase immobilized on an anion exchange resin) was a gift from Novo Nordisk A/S, Bagsvaerd, Denmark. The chemicals used

were purchased as follows: caprylic acid (min. 99%), butyric acid (min. 98%), caproic acid (min. 99%), capric acid (min. 99%), linoleic acid (min. 88%), glycerol (min. 99%), dicyclohexylcarbodiimide (DCC) (min. 95%), and 4-dimethylaminopyridine (4-DMAP) (min. 99%) from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); oleic acid (min. 90%) from Nippon Oil & Fats Co., Ltd. (Amagasaki, Japan); lauric acid (min. 98%) from Tokyo Kasei Industries, Ltd. (Tokyo, Japan). Eicosapentaenoic acid ethyl ester (min. 97%) was a gift from Nippon Suisan Co., Ltd. (Tokyo, Japan). EPA was obtained by hydrolysis of eicosapentaenoic acid ethyl ester (13).

Typical esterification reaction. Esterification was performed in a solvent-free system which contained 25 mmol FA, 12.5 mmol glycerol (at the stoichiometric ratio of 2:1), and 4% Lipozyme based on the total reaction mixture. The reaction temperature depended on the melting point of the FA used and ranged between 25–45°C. The reaction was carried out at normal pressure for 1 h and then at 3 mm Hg vacuum or under a stream of dry nitrogen gas (0.7 L/min). When butyric and caproic acid were used, instead of vacuum, 1.5 g molecular sieves powder 4 A (Nacalai Tesque, Ltd., Kyoto, Japan) was added to the reaction mixture for water removal.

Analysis of the reaction mixture. During the enzymatic reaction, 2-μL samples were withdrawn from the reaction vessel and dissolved in 0.1 mL chloroform/methanol (2:1). The immobilized enzyme was separated by centrifugation at 20,000 × *g* for 2 min. The removal of glycerol from the supernatant solution was achieved by extraction with water (0.05 mL). For effective extraction of partial glycerides (soluble in water to some extent), the water layer was extracted twice with 0.1 mL chloroform each time. The total chloroform extract (approx. 0.3 mL) was analyzed by thin-layer chromatography–flame-ionization detection (TLC–FID; Iatroscan MK-5, Iatron Laboratories, Tokyo, Japan) using Chromarod S III quartz rods. The rods were developed in benzene/chloroform/acetic acid (70:30:2) solvent for 10 cm at room temperature. The rods were scanned under the following conditions: hydrogen flow rate, 0.16 L min^{-1}; air flow rate, 2.0 L min^{-1}; scanning speed, 30 s per scan. The results were expressed as percentage of peak areas of the reaction mixture's components on a glycerol-free basis and may vary slightly from the actual weight percentages (14).

The water content of the reaction mixture was determined with a Karl Fischer moisture meter (MKS-1; Kyoto Electronics, Ltd., Kyoto, Japan).

Synthesis of 1,3-dicapryloyl-2-eicosapentaenoylglycerol. A solution of 1,3-dicaprylin (95%, 0.172 g, 0.5 mmol), EPA (97%, 0.227 g, 0.75 mmol), DCC (0.244 g, 2 mmol), and 4-DMAP (0.244 g, 2 mmol) in 10 mL ethanol-free dry chloroform was stirred at room temperature overnight. The liberated urea was filtered and the filtrate was concentrated. The crude product was purified by silica gel column chromatography with hexane/ethyl acetate (9:1), to give 0.15 g 1,3-dicapryloyl-2-eicosapentaenoylglycerol in 42% yield (15,16). The analyses of the final product were performed by gas chromatography (GC) and silver ion high-performance liquid chromatography (HPLC) (17). Triacylglycerol species were separated by high-temperature GC according to their molecular weight with an SGE-HT5 aluminum-clad fused-silica capillary column (6 m length, 0.53 mm internal diameter, and 0.1 μm film thickness) from Supelco Inc., (Bellefonte, PA). The triacylglycerol positional isomers were separated by HPLC with a ChromSpher 5 Lipids silver ion chromatography column (250 × 4.6 mm × 1/4″) from Chrompack (Middleburg, The Netherlands).

RESULTS AND DISCUSSION

Synthesis of 1,3-DAG. The synthesis of 1,3-DAG by direct esterification is based on the 1,3-positional specificity of immobilized *R. miehei* lipase (Lipozyme) and on the critical feature of the support to retain the water essential for the catalytic function of the lipase (18). Esterification is a reversible reaction, and the effect of water mass action is used to minimize the hydrolytic side reactions and to allow the lipase to catalyze the synthetic reactions. Lipase speeds up the reaction and equilibrium is reached faster, but the yields of the esterification products are not in the practical range at stoichiometric molar ratios of the reactants. The equilibrium composition at constant temperature can be modified in two ways: one is the use of excess acid and the other is the removal of water formed as by-product.

The synthesis of 1,3-dicapryloylglycerol, which can be an intermediate for synthesis of structured triacylglycerols with a polyunsaturated fatty acid in the 2-position, was used as a model for studying the effect of water removal and some other parameters of the system.

When the reaction was performed at normal pressure in a closed vessel, equilibrium was reached after 3 h at only 60% conversion of the acid (Fig. 1). Adding extra water to the ini-

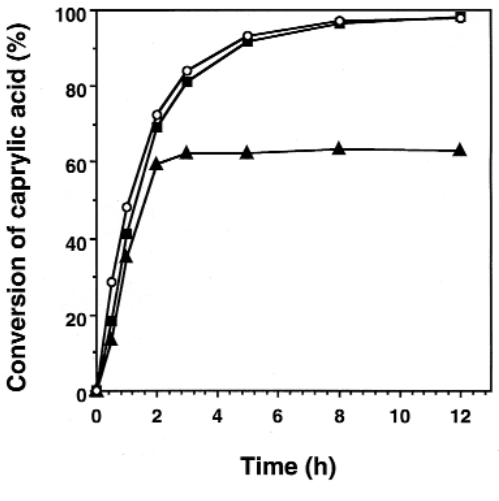

FIG. 1. Effect of water removal on conversion. The reaction was performed with 1.150 g (12.5 mmol) glycerol, 3.605 g (25 mmol) caprylic acid, and 0.200 g Lipozyme (Novo Nordisk A/S, Bagsvaerd, Denmark) at 25°C and 300 rpm stirring speed. Reaction performed without water removal at normal pressure in a closed vessel (▲), reaction with water removal by evaporation under a stream of nitrogen gas (0.7 L/min) applied at 1 h (■), reaction with water removal by evaporation under 3 mm Hg vacuum applied at 1 h (○).

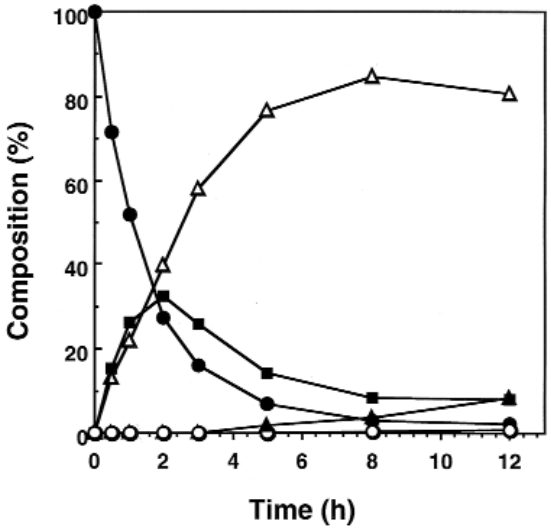

FIG. 2. Time course of esterification of glycerol with caprylic acid. The reaction was performed with 1.150 g (12.5 mmol) glycerol, 3.605 g (25 mmol) caprylic acid, and 0.200 g Lipozyme at 25°C and 300 rpm stirring speed. Water removal was started at 1 h by evaporation under 3 mm Hg vacuum. Caprylic acid (●), 1,3-dicapryloylglycerol (△), 1,2-dicapryloylglycerol (○),1-monocapryloylglycerol (■), and tricapryloylglycerol (▲). For manufacturer see Figure 1.

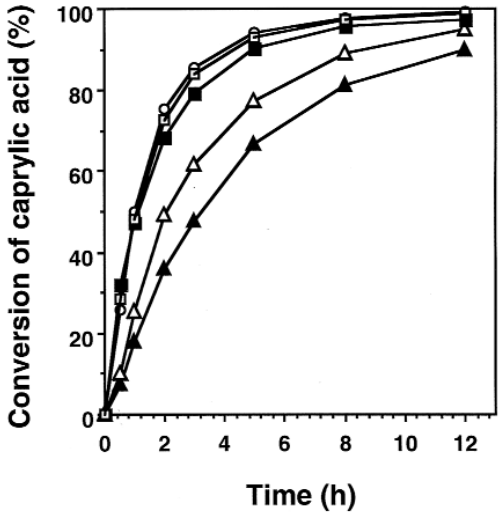

FIG. 3. Effect of temperature on reaction rate for the esterification of glycerol with caprylic acid. Reaction performed at 15 (▲), 20 (△), 25 (■), 35 (○), and 50°C (■). For the rest of the reaction conditions see Figure 2.

tial reaction mixture to enhance the enzyme activity was unnecessary because the commercially available enzyme preparation (Lipozyme) already contained 4.20% water. Water formed in the reaction accumulated in the reaction mixture, and increased from 0.24% initially to 4.50% at equilibrium. The removal of water formed during the reaction is very important for shifting the reaction equilibrium toward high conversions of the acid and thereby high yields of 1,3-dicapryloylglycerol. Water, the most volatile component in this reaction system, was removed efficiently by a stream of nitrogen gas (0.7 L/min) or by a 3-mm Hg vacuum, bringing the conversion at 12 h to 98% for both cases (Fig. 1). The water content of the reaction mixture at 12 h was reduced from 4.5% for the reaction at normal pressure in a closed vessel to 0.45% when evaporation under nitrogen was used, and to 0.33% for the reaction at 3 mm Hg. Water removal was started at 1 h to maintain the high initial reaction rates. A study of water removal under a stream of nitrogen gas showed that applying nitrogen from the beginning lowered the initial rate of the acid conversion to 8.1%/h from 25.1%/h for the reaction without water removal. Perhaps the water formed in the initial stage of the reaction helps the enzyme reach maximal activity. The extension of the initial stage to 2 h did not bring any further improvement of the reaction rates for the two methods. For the rest of the experiments, 3 mm Hg vacuum applied after 1 h was used for the water removal.

Figure 2 is an example of the time course of the reaction. The high reaction rate ensured a consumption of more than 95% of caprylic acid in 8 h. The content of 1,3-dicapryloylglycerol was maximal at 8 h (84.6%), but decreased later owing to tricapryloylglycerol formation. 1,2-Dicapryloylglycerol content remained low throughout the reaction (less than 1%). 2-Monocapryloylglycerol was not detected in the reaction mixture.

The reaction temperature influenced the reaction rate (Fig. 3) and the yield of 1,3-dicapryloylglycerol (Fig. 4). From 15 to 25°C, the reaction accelerated, but still higher temperatures did not improve the reaction rate significantly (Fig. 3). The content of 1,3-dicapryloylglycerol in the reaction mixture was maximal at 8 h for the reaction performed at 25, 35, and 50°C (Fig. 4). At 15 and 20°C, the reaction was slow, and maximal 1,3-dicapryloylglycerol content could not be attained in 8 h. Maximal 1,3-dicapryloylglycerol content (approximately

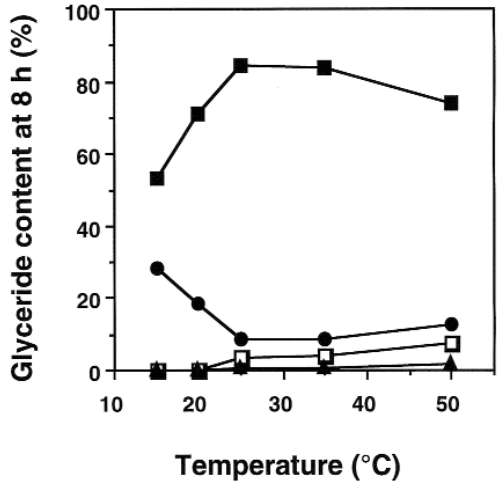

FIG. 4. Effect of temperature on glyceride content for the esterification of glycerol with caprylic acid at 8 h. The content of 1,3-dicapryloylglycerol (■), 1,2-dicapryloylglycerol (▲), tricapryloylglycerol (□), and 1-monocapryloylglycerol (●). For the rest of the reaction conditions see Figure 2.

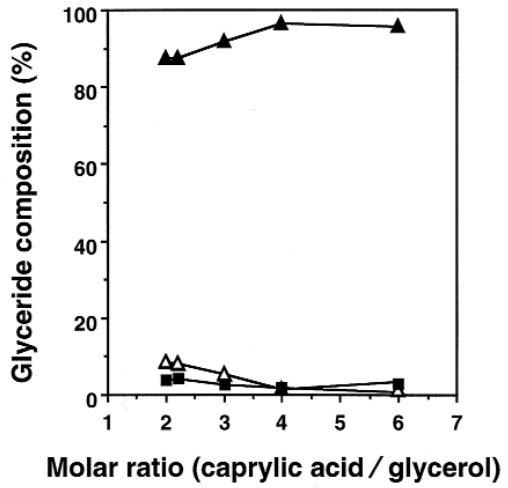

FIG. 5. Effect of molar ratio of reactants on glyceride composition for the esterification of glycerol with caprylic acid at 8 h: 1,3-dicapryloylglycerol (▲), 1-monocapryloylglycerol (■), and tricapryloylglycerol (△). For the rest of the reaction conditions see Figure 2.

85%) was achieved at temperatures between 25 and 35°C. At higher temperatures, the maximal content of 1,3-dicapryloylglycerol decreased because of the faster tricapryloylglycerol formation. The low content of 1,2-DAG at higher temperatures suggests that the 1,2-DAG formed by the spontaneous acyl migration of 1,3-DAG were esterified at a higher rate to TAG and thus could not accumulate in the reaction mixture.

The effect of molar ratio of caprylic acid to glycerol on glyceride composition is shown in Figure 5. The optimum was found at 4:1 molar ratio, for which 1,3-DAG content was maximal (98%) and 1-MAG and TAG contents were minimal (1% each). 1,2-DAG content was under the detection limit of the TLC–FID method. The relatively low boiling point of caprylic acid (240°C at normal pressure) makes possible its removal from the product by evaporation under reduced pressure at lower temperatures (molecular distillation). The boiling point of caprylic acid at 10 mm Hg is 124°C, but at a pressure under 1 mm Hg, its boiling temperature should be much lower, thus avoiding the acyl migration of 1,3- to 1,2-dicapryloylglycerol during the removal of acid from the reaction mixture. The caprylic acid recovered can be recycled, and the purified product can be used directly as an intermediate for another synthesis.

Various FA were used in this system, and the results are shown in Table 1. The reaction did not work at all for butyric and caproic acids. The best results were obtained for syntheses of 1,3-dicaprylin and 1,3-dicaprin (their maximal contents were 84.6 and 84.4%, respectively). The content of 1,3-diolein was only 61.1%, but the reaction was slow and incomplete in 12 h. The content of 1,2-DAG was very low in all syntheses, making the product purification by silica gel chromatography easier.

The lipase specificity for the FA species can be investigated using the initial reaction rates (Table 1). The initial reaction rate was defined as mmol fatty acid consumed per minute per milligram Lipozyme. The reaction rate was high for caprylic and eicosapentaenoic acids, low for linoleic and oleic acids, and zero for butyric and caproic acids. These reactions were performed at the same temperature (25°C) and therefore the initial reaction rates can be compared directly. The reaction temperature was 35°C for capric acid and 45°C for lauric acid because they have higher melting points. The higher temperatures accelerate the acid conversion, as well as the triacylglycerol formation owing to the faster spontaneous acyl migration. For capric acid at 35°C, the content of triacylglycerol was 6.62% in the reaction mixture at 12 h at approximately 98% consumption of the acid. The initial reaction rate was one-third of that for caprylic acid at 25°C, and the same maximal content of 1,3-DAG was obtained, but 4 h later. Approximately the same content of triacylglycerol (6.68%) was formed in the case of lauric acid at 45°C, but only after 3 h at 90% consumption of the acid.

From the data shown in Figure 6 for caprylic acid, it is evident that the reaction rate for a 4% Lipozyme content in the reaction mixture was much lower than the rates obtained for higher contents. It can be expected that for the other acid species also a higher content of enzyme in the reaction mixture would make the reaction faster with higher yields in shorter time because the acyl migration would be avoided partially.

TABLE 1
Syntheses of Some 1,3-Diacylglycerols

Fatty acid species	Temperature (°C)	Time (h)	Initial reaction rate[a] (mmol/min mg × 10^{-4})	Composition of reaction mixture[b] (%)				
				TAG	FA	1,3-DAG	1,2-DAG	1-MAG
Butyric acid (4:0)	25	12	0	0	0	0	0	0
Caproic acid (6:0)	25	12	0	0	0	0	0	0
Caprylic acid (8:0)	25	8	9.53	3.60	2.88	84.63	0.45	8.44
Capric acid (10:0)	35	12	3.45	6.62	2.03	84.37	0.83	6.15
Lauric acid (12:0)	45	3	8.66	6.68	10.15	67.35	1.75	14.07
Oleic acid (18:1)	25	12	0.41	1.34	20.63	61.10	0.85	16.08
Linoleic acid (18:2)	25	12	1.03	2.29	12.19	74.25	0.91	9.65
Eicosapentaenoic acid (20:5)	25	12	8.00	7.83	7.86	71.74	2.52	10.05

[a]Initial reaction rate was defined as mmol fatty acid consumed per minute per mg Lipozyme (Novo Nordisk A/S, Bagsvaerd, Denmark).
[b]Composition of the reaction mixture at the time when the contents of 1,3-diacylglycerol were maximum. For the reaction conditions see the Experimental Procedures section. TAG, triacylglycerols; FA, fatty acids; 1,3- and 1,2-DAG, 1,3- and 1,2-diacylglycerols; 1-MAG, 1-monoacylglycerol.

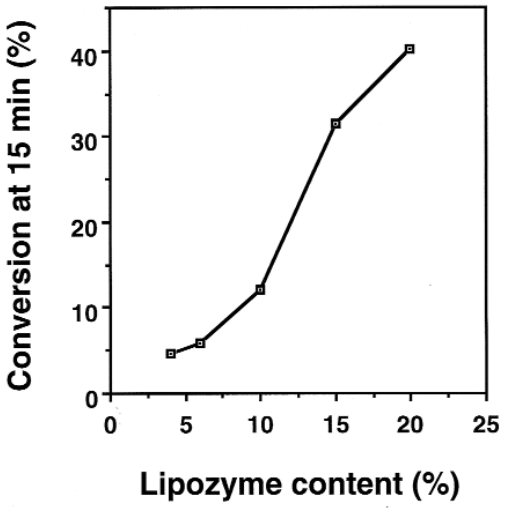

FIG. 6. Effect of Lipozyme content on the initial rate of esterification of glycerol with caprylic acid. The initial reaction rate was defined by the conversion of caprylic acid at 15 min. For reaction conditions see Figure 2. For manufacturer see Figure 1.

The yields of 1,3-DAG synthesis were comparable to those of the other enzymatic methods (10–12). Higher yields can be expected if the method is optimized for each FA employed. This direct method has the advantage of starting from the cheapest reactants without employing organic solvents as reaction media.

Chemical synthesis of 1,3-dicapryloyl-2-eicosapentaenoylglycerol. Purified 1,3-dicapryloylglycerol (95%) was used for the chemical synthesis of the structured glyceride with an eicosapentaenoyl group in position 2. The yield of 1,3-dicapryloyl-2-eicosapentaenoylglycerol after purification was 42%. The purity of the product was checked by GC and HPLC analysis. GC analysis could differentiate triacylglycerols according to their molecular weights only, giving 98% purity for the product, but two positional isomers, 1,3-dicapryloyl-2-eicosapentaenoylglycerol and 1,2-dicapryloyl-3-eicosapentaenoylglycerol, were included. Silver ion HPLC analysis was used to determine the ratio of the two isomers. The ratio of 1,3-dicapryloyl-2-eicosapentaenoylglycerol to 1,2-dicapryloyl-3-eicosapentaenoylglycerol (and/or 1-eicosapentaenoyl-2,3-dicapryloylglycerol) was 9:1.

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