Study of TAG Ethanolysis to 2-MAG by Immobilized *Candida antarctica* Lipase and Synthesis of Symmetrically Structured TAG

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ABSTRACT: Regiospecific ethanolysis of homogenous TAG with immobilized *Candida antarctica* lipase (Novozym 435) was studied using trioleoylglycerol (TO) as a model substrate. Optimization of the reactant weight ratio revealed that the 2-MAG reaction yield increased when a larger amount of ethanol was used. These results suggested that Novozym 435 showed strict regiospecificity in an excess amount of ethanol. The process optimization (reaction temperature and reactant molar ratio) and a study of lipase specificity for various substrates were performed. Under the optimized conditions (ethanol/TO molar ratio = 77:1 and 25°C), 2-monooleoylglycerol (2-MO) was obtained in more than 98% content among glycerides of the reaction mixture and approximately 88% reaction yield in 4 h. The above reaction conditions were applied for ethanolysis of tridocosahexaenoylglycerol, trieicosapentaenoylglycerol, triarachidonoylglycerol, tri-α-linolenoylglycerol, and trilinoleoylglycerol. Reaction yields ranging from 71.9 to 93.7% were obtained in short reaction times (2.5 to 8 h). Purified (>98%) 2-MO and 2-monodocosahexaenoylglycerol (2-MD) were reesterified with caprylic acid by immobilized *Rhizomucor miehei* lipase (Lipozyme IM) to afford symmetrical structured TAG. At a stoichiometric ratio of 2-MAG/caprylic acid, 25°C and 2–5 mm Hg vacuum, the glyceride composition of the esterification mixture was approximately 95% 1,3-dicapryloyl-2-oleoylglycerol (COC) at 4 h, and 96% 1,3-dicapryloyl-2-docosahexaenoylglycerol (CDC) at 8 h. The regioisomeric purity of both COC and CDC was 100%.

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KEY WORDS: Ethanolysis, immobilized *Candida antarctica* lipase, 2-monoacylglycerols, polyunsaturated triacylglycerols, symmetrically structured triacylglycerols.

Although 2-monoacylglycerols (2-MAG) have very good emulsifying properties and are physiologically essential molecules involved in lipid adsorption, they have limited industrial applications owing to the difficulty of their synthesis. Chemical synthetic methods have many reaction steps that require tedious purification, and the final yields are often very low (1). Enzymatic hydrolysis of triacylglycerols (TAG) with 1,3-specific lipases has limited success as a result of high acyl migration rates, which affect the reaction yield and final product purity. This method was improved by engineering the reaction media, i.e., working in a microemulsion system (2) or in a mixture of organic solvents containing a low amount of water (3). Although the rates are higher, they involve the use of surfactants such as sodium bis(2-ethylhexyl)sulfosuccinate (AOT), which complicate the product recovery (2), and unusual solvents, such as trichlorotrifluoroethane (3).

Another enzymatic method using 1,3-specific lipases is the alcoholysis (usually ethanolysis) of TAG (4–6). The reaction was performed in an organic solvent and required a strict control of the water activity in the system (5,6). All the preceding enzymatic methods gave low reaction yields for polyunsaturated 2-MAG because of the low activity of available 1,3-specific lipases on polyunsaturated TAG.

2-MAG are convenient synthetic blocks for the synthesis of symmetrical structured TAG (SSTG) by enzymatic esterification with a desired FA, which usually has a medium-length chain (6,7). This strategy is especially useful for the synthesis of SSTG with a PUFA at the second position. Such SSTG are difficult to obtain by the common method of TAG acidolysis with 1,3-specific lipases.

Encouraging preliminary results in previous work (8) were obtained for ethanolysis of tridocosahexaenoylglycerol (TD) and trieicosapentaenoylglycerol with immobilized *Candida antarctica* lipase (Novozym 435). It was noticed that Novozym 435, which is considered a positionally nonspecific lipase and has good activity on polyunsaturated TAG, becomes 1,3-regiospecific in ethanol.

In the present work, ethanolysis with Novozym 435 was studied using trioleoylglycerol (TO) as a reaction model, and 2-MAG were obtained from various polyunsaturated TAG. 1,3-Dicapryloyl-2-oleoylglycerol (COC) and 1,3-dicapryloyl-2-docosahexaenoylglycerol (CDC) were synthesized by direct esterification of the respective purified 2-MAG with caprylic acid (CA) under vacuum.

EXPERIMENTAL PROCEDURES

Materials. Novozym 435 (immobilized *C. antarctica* lipase) and Lipozyme RM IM (immobilized *Rhizomucor miehei* lipase) were gifts from Novozymes (Chiba, Japan). CA and TO were purchased from Sigma (St. Louis, MO). Tri-α-linolenoylglycerol, triarachidonoylglycerol, trieicosapentaenoylglycerol, and TD were from Nu-Chek-Prep, Inc. (Elysian, MN). Ethylpalmitate and ethanol (0.15% water)

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were from Aldrich (Milwakee, WI). All the chemicals had >99% purity.

Ethanolysis reaction. In a typical experiment, TAG (100 mg) was mixed for 10 min with ethanol (400 mg; 8.7 mmol) at 300 rpm, 25°C for emulsification of the reaction mixture. The reaction was started by the addition of Novozym 435 (50 mg, 10% of reactants) and stopped by catalyst filtration.

2-MAG purification. Ethanol was evaporated at 25°C from the reaction mixture, which was free of Novozym 435. The concentrate was dissolved in acetonitrile/water = 95:5 (oil/solvent = 1:9) and washed three times with the same volume of hexane as the acetonitrile/water solution for ethyl ester removal. The solvent was evaporated from the acetonitrile phase, and the concentrate was dissolved in chloroform (initial oil/chloroform = 1:10), which was extracted with the same volume of water/ethanol = 9:1 for the extraction of glycerol obtained as by-product. The layers were separated, and the water layer was washed with chloroform as above. The two chloroform layers were combined, and the solvent was evaporated.

Reesterification of 2-MAG. Purified 2-MAG (0.33 mmol) was reacted with caprylic acid (0.66 mmol) and Lipozyme RM IM as catalyst (10% of the reactants) under stirring at 300 rpm, 2–5 mm Hg vacuum, and 25°C. 2-MAG were prepared and purified as described above, but starting from 0.5 g TAG, 2 g (43.5 mmol) ethanol, and 0.25 g Novozym 435.

HPLC analysis of ethanolysis reaction mixture. Samples of the reaction mixture (catalyst filtered off beforehand) dissolved in hexane/isopropanol = 9:1 were injected and separated on a silica gel column (Zorbax®Sil, 4.6 × 150 mm, particle diameter = 5 μm; Hewlett-Packard) by HPLC. A mobile phase gradient program with a binary solvent gradient made of solvent A = hexane and solvent B = hexane/ethyl acetate/isopropanol/[10% formic acid (of 90% purity) in isopropanol] = 80:10:10:1 was used (9). The column was eluted at a flow rate of 1.0 mL/min with a linear gradient of 2% solvent B to 35% solvent B in 8 min, then to 98% solvent B in 0.5 min and held at 98% solvent B for 6.5 min. The column temperature was maintained at 40°C. The lipid species were detected using an ELSD at 90°C drift tube temperature. The nitrogen flow rate of the detector was 1.1 L/min. All the components of the reaction mixture except glycerol [including the regioisomeric pairs of 1,3-diacylglycerol (1,3-DAG) and 1(3),2-DAG, and 1(3)-MAG and 2-MAG] could be clearly separated under these conditions.

HPLC analysis of SSTG regioisomeric purity. The analysis was carried out on a ChromSpher 5 Lipids column (250 × 4.6 mm × 1/4″; Chrompack, Middelburg, The Netherlands).

COC and 1,2-dicapryloyl-3-oleoylglycerol were separated with a binary solvent gradient program of A = hexane and B = acetone. The column was eluted at 5% solvent B for 30 min, then to 100% solvent B in 5 min and held at 100% B for 10 min. The column was kept at ambient temperature. The mobile phase flow rate was 1 mL/min. ELSD was used at 90°C drift tube temperature. Nitrogen flow rate was maintained at 1.1 L/min.

CDC and 1,2-dicapryloyl-3-docosahexaenoylglycerol were separated with a binary solvent gradient program of A = acetone and B = acetonitrile. The elution was done with a linear gradient from 0 to 25% solvent B in 60 min. The mobile phase flow rate was 0.75 mL/min. The column was kept at ambient temperature. ELSD was used at 130°C drift tube temperature. Nitrogen flow rate was maintained at 2.4 L/min.

GC analysis of structured lipids. Samples of the reaction mixture were dissolved in diethyl ether and injected into a gas chromatograph equipped with an on-column injector and a Ultra Alloy-1 (HT) capillary column (5 m length, 0.5 mm internal diameter, 0.1 mm film thickness; Frontier Laboratories Ltd., Fukushima, Japan). The oven was heated from 40 to 360°C at 10°C/min. The on-column injector was on the oven track mode at 3°C above the oven temperature. Carrier gas was helium at 10 mL/min.

Estimation of ethanolysis reaction yield by GC analysis. A certain amount of glycerol is formed because of the complete cleavage of acyl groups of the resulting partial glycerides during the ethanolysis reaction. Owing to the difficulty in quantifying the glycerol and fatty acid ethyl esters (FAEE) by HPLC with an ELSD, the reaction yield of 2-MAG could not be evaluated by the method used to determine the glyceride composition of the reaction mixture. The yield of 2-MAG was estimated from the relative amounts of FAEE of the reaction mixture before and after complete derivatization of the component glycerides to FAEE with sodium ethylate determined by GC analysis as described below.

Five milliliters of diethyl ether was added to the final ethanolysis reaction mixture. The enzyme was removed by filtration, and the solvent was evaporated. Ten microliters of the resultant mixture containing glycerides, glycerol, and FAEE was dissolved in 1 mL diethyl ether, and 4 mg ethyl palmitate (internal standard) was added. A portion of this solution (100 μL) was mixed with 0.4 mL diethyl ether, 20 μL ethyl acetate, and 20 μL ethanolic sodium ethylate (1 M). After 5 min at room temperature, 2 μL acetic acid was added, and the mixture was dried under a nitrogen stream. The lipids were extracted with 0.5 mL diethyl ether, washed with 0.5 mL saturated NaCl, and dried with anhydrous Na_2SO_4.

The solutions of the ethanolysis reaction mixture before and after derivatization were analyzed by GC under the same conditions as for the analysis of structured lipids.

RESULTS AND DISCUSSION

Optimization of the reaction conditions for 2-MAG synthesis. Figure 1 shows the typical time course change in the glyceride composition during the ethanolysis of TO with Novozym 435 at 4:1 ethanol/TO weight ratio. As the reaction proceeded, the intermediate 1(3),2-DAG accumulated for a while, and then it was further deacylated to 2-MAG to give more than 98% 2-MAG content among glycerides in 4 h. FFA, 1,3-DAG, and 1-MAG contents were under the detection limit of the analytical method used. Although it is known that under some conditions Novozym 435 shows low regiospecificity for the glycerol backbone, the 2-MAG generated were not further deacylated to glycerol. In a similar

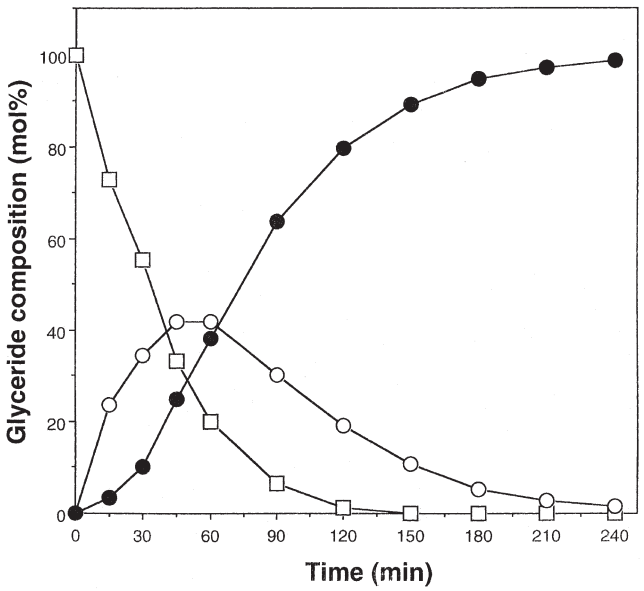

FIG. 1. Time course change of glyceride composition during ethanolysis of trioleoylglycerol (TO). The reaction was performed at an ethanol/TO molar ratio = 77:1 (weight ratio = 4:1) and 25°C. (○) 1,2-DAG; (●) 2-MAG; and (□) TAG.

application, Novozym 435 was used as catalyst for ethanolysis of TAG at a low molar ratio of reactants (three succesive additions of one mol equiv. of ethanol against 1 mol equiv. of TAG), which resulted in complete conversion of TAG, giving 3 mol equiv. of FAEE and 1 mol equiv. of glycerol (10). In comparing the two systems, the only significant difference in the reaction conditions that can account for these different results is the amount of ethanol employed in our system. Hence, the influence of ethanol/TAG ratio on the reaction yield of 2-MAG was investigated in order to elucidate the effect of ethanol concentration on catalyst regiospecificity (Table 1). The reaction yield of 2-monooleoylglycerol (2-MO) represents the molar percentage of the initial TAG transformed into 2-MAG at the end of the reaction. As expected, the reactions performed with lower ratios of ethanol/TAG gave lower 2-MAG reaction yields and lower regioisomeric purity of 2-MO, indicating that a larger part of the glycerides was completely deacylated to glycerol. The reactions at higher ethanol excess were faster, but the final yield was roughly constant at an ethanol/TAG molar ratio = 77:1 (weight ratio = 4:1) and above. Under the reaction conditions used, acyl migration should be limited as a result of the low water content of the reaction mixture, the absence of FFA, the low reaction temperature (temperature effect is described later), and short reaction times. Therefore, it is not likely that the formation of glycerol was caused by the spontaneous acyl migration of the acyl groups from the second position of 2-MAG or 1(3), 2-DAG to the outer positions, followed by enzymatic deacylation from these positions. Thus, it is very probable that the formation of glycerol was caused by enzymatic deacylation directly from the mid-position of the glycerides as well as the outer positions, implying that a larger amount of ethanol makes the enzyme more 1,3-regiospecific. A possible explanation for this "ethanol effect" is that ethanol, a polar solvent, may fix the tertiary structure of the enzyme, hindering the substrate from accessing the catalytic pocket with the acyl group at the second position.

Reaction temperature is also a very important parameter for this system, as higher temperatures increase the rate of acyl migration. Thus, the final reaction yields of 2-MAG decrease (Table 2). High reaction yields were obtained at temperatures of 25°C or less. At temperatures higher than 35°C, the 2-MAG yield decreased considerably owing to the complete deacylation to glycerol. Although the reaction yield decreased with increased reaction temperatures, neither 1,3-DAG nor 1(3)-MAG (which indicates the occurrence of acyl migration) was detected in the reaction mixtures. Most probably, kinetic properties prevented their accumulation. The reaction rates of 1,3-DAG and 1(3)-MAG deacylation are probably much higher than acyl migration rates, so they are deacylated immediately after formation.

2-MAG synthesis from polyunsaturated TAG. Some other homogeneous TAG of different PUFA (linoleic, α-linolenic,

TABLE 1
Effect of Reactant Ratio on Glyceride Composition and Reaction Yield of 2-MO[a]

Ethanol/TO molar ratio (weight ratio)	Time (min)	Glyceride composition[b] (mol%)				2-MO reaction yield[c] (mol%)
		TO	1(3),2-DO	1(3)-MO	2-MO	
192.5:1 (10:1)	120	0	2.2	0	97.8	83.6
96.3:1 (5:1)	180	0	0.9	0	99.1	84.2
77:1 (4:1)	240	0	1.4	0	98.6	87.9
57.8:1 (3:1)	240	0	1.2	0	98.8	82.5
38.5:1 (2:1)	300	0	1.7	2.2	96.1	65.1
19.3:1 (1:1)	360	0.7	7.2	4.5	87.6	57.7

[a]The reactions were performed at 25°C.
[b]Glyceride composition (without glycerol) of the reaction mixture.
[c]2-Monooleoylglycerol (2-MO) reaction yield represents the molar percentage of the initial TAG transformed into 2-MAG at the end of the reaction. Abbreviations: TO, trioleoylglycerol; 1(3),2-DO, 1(3),2-dioleoylglycerol.

TABLE 2
Effect of Temperature on Glyceride Composition and 2-MO Reaction Yield[a]

Temperature (°C)	Time (min)	Glyceride composition[b] (mol%)		2-MO reaction yield[c] (mol%)
		1(3),2-DO	2-MO	
15	300	1.6	98.4	95.1
20	240	1.3	98.7	97.4
25	240	1.4	98.6	87.9
30	180	2.3	97.7	80.6
35	180	1.5	98.5	78.8
40	180	1.9	98.1	52.4
60	240	6.3	93.7	35.1

[a]The reactions were performed at ethanol/triolein molar ratio = 77:1 (weight ratio = 4:1).
[b]Glyceride composition (without glycerol) of the reaction mixture. 1(3)-MAG were not detected in any of the reaction mixtures.
[c]2-MO reaction yield represents the molar percentage of the initial TAG transformed into 2-MAG at the end of the reaction. For abbreviations see Table 1.

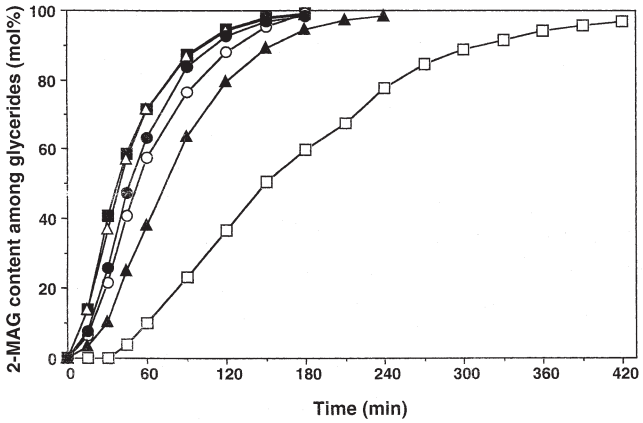

FIG. 2. Comparison of 2-MAG formation during ethanolysis reaction with various substrates. The reactions were performed at ethanol/TAG weight ratio = 4:1 and 25°C. (○) Tri-α-linolenoylglycerol; (●) trilinoleoylglycerol; (□) tridocosahexaenoylglycerol; (■) trieicosapentaenoylglycerol; (△) triarachidonoylglycerol; (▲) TO. For abbreviation see Figure 1.

arachidonic, eicosapentaenoic, and docosahexaenoic acids) were subjected to ethanolysis (Fig. 2). All of the experiments were performed at an ethanol/TAG weight ratio = 4:1 and 25°C. In most cases, the reactions finished in 3 to 4 h with final 2-MAG content >97% and reaction yields >70% (Table 3). 1-MAG were not detected in any of the reaction mixtures. For TD, the reaction was slower, but >93% 2-MAG reaction yield was obtained in 7 h. The reaction in ethanol is much faster than ethanolysis in an organic media (5,6). Similar reaction yields were obtained in this work for 2-MO and 2-monolinoleoylglycerol in 4 and 3 h, respectively, compared to 24 h by ethanolysis in methyl *tert*-butyl ether with immobilized *Rhizopus delemar* lipase (6).

Synthesis of SSTG from 2-MAG. One important application of 2-MAG would be their use as synthetic blocks for enzymatic SSTG synthesis. DHA is a nutritionally valuable PUFA on which fungal lipases (well-known as strictly 1,3-regiospecific enzymes) have very low activity. For this reason, SSTG with DHA at the mid-position are very difficult to synthesize by transesterification using 1,3-regiospecific enzymes. The synthesis of SSTG was conducted here using purified 2-MO and 2-monodocosahexaenoylglycerol (2-MD) as starting materials. The 2-MAG were esterified with a stoichiometric amount of CA by Lipozyme IM under vacuum. Figures 3A and 3B show the time courses of the syntheses of COC and CDC, respectively. In both cases, the substrate (2-MAG) was rapidly esterified to 1(3),2-DAG, and then 1(3),2-DAG were slowly esterified to TAG. The glyceride composition for COC final reaction mixture was 94.8% COC, 1.7% 1-capryloyl-2,3-dioleoylglycerol, and 3.5% 1(3)-capryloyl-2-oleoylglycerol at 4 h, whereas that of the CDC reaction mixture was 96.3% CDC and 3.7% 1(3)-capryloyl-2-docosahexaenoylglycerol at 8 h. Regioisomeric purity of the final products was confirmed to be 100% by silver ion HPLC. Schmid *et al.* (11) used the same solvent-free system for esterification of 2-monopalmitoylglycerol with oleic acid at 50°C. The higher temperature resulted in lower regioisomeric purity of the product (82% palmitic acid in the second position of TAG), presumably owing to faster acyl migration (11).

In our preliminary work (8), the reesterification of 2-MD was performed with ethyl caprylate (EtC) under vacuum without the purification of 2-MD. Novozym 435 was filtered,

TABLE 3
Effect of Acyl Species on Glyceride Composition and Reaction Yield of 2-MAG[a]

TG species	Time (min)	Glyceride composition[b] (mol%) 1(3),2-DAG	2-MAG	2-MAG[c] reaction yield (mol%)
Trioleoylglycerol	240	1.4	98.6	87.9
Trilinoleoylglycerol	180	1.6	98.4	90.4
Tri-α-linolenoylglycerol	180	0.8	99.2	71.9
Triarachidonoylglycerol	180	1.2	98.8	82.4
Trieicosapentaenoylglycerol	150	2.0	98.0	75.4
Tridocosahexaenoylglycerol	420	2.9	97.1	93.7

[a]The reactions were performed at ethanol/TAG weight ratio = 4:1 and 25°C.
[b]Glyceride composition (without glycerol) of the final reaction mixture. 1(3)-MAG were not detected in any of the reaction mixtures.
[c]2-MAG reaction yield represents the molar percentage of the initial TAG transformed into 2-MAG at the end of the reaction.

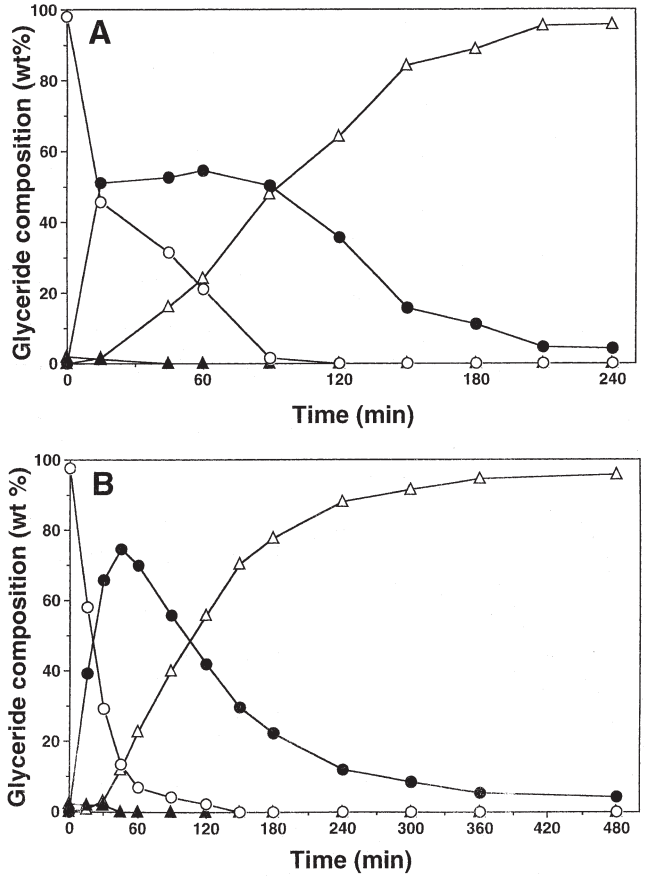

FIG. 3. Time course of 1,3-dicapryloyl-2-oleoylglycerol (A) and 1,3-dicapryloyl-2-docosahexaenoylglycerol (B) synthesis at a stoichiometric ratio of reactants. The reaction was performed at 25°C and 2–5 mm Hg vacuum. (△) TAG; (▲) 1(3)-MAG; (○) 2-MAG; (●) 1(3),2-DAG.

and then the excess of ethanol was evaporated from the final reaction mixture of ethanolysis prior to addition of excess of EtC and Lipozyme IM for reesterification. High excess of EtC (EtC/TD molar ratio = 20:1) was necessary in order to diminish the reesterification of 2-MD with DHA residues present in the reaction mixture as ethyldocosahexaenoate (resulting from the ethanolysis reaction). The reesterification reaction was very fast (it finished in only 1.5 h), and the final content of CDC among glycerides was approximately 85%. This procedure avoids the intermediary purification of 2-MAG but has some drawbacks, such as working with big reaction volumes (low volumetric productivity), the need for high energy-consuming molecular distillation for removal of ethyl esters from the final reaction mixture, and the use of more expensive EtC (10 times more expensive than CA).

The purification of 2-MAG is usually performed by crystallization from an organic solvent, but it is not suitable for polyunsaturated 2-MAG because of their very low freezing temperature. Another alternative is silica gel column purification, but the quantity of solvent involved is very high and partial migration to 1-MAG is inevitable even when boric acid-treated silica gel is used. The selective extraction used in this work for purification needed a lower quantity of solvents, was faster, and the recovery yield of 2-MAG was high. Purified 2-MD (97.8%; 171.4 mg) and 2-MO (98%; 157.3 mg) were obtained with recovery yields of 93 and 89%, respectively. The reaction yields after the purification were 87.1% for 2-MD and 76.2% for 2-MO.

The use of purified 2-MAG as starting materials for SSTG synthesis minimizes the volume of the esterification reaction mixture by requiring only a stoichiometric amount of FA. The reaction system is very simple and requires minimal downstream separation and purification operations.

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