Production of Specific-Structured Triacylglycerols by Lipase-Catalyzed Interesterification in a Laboratory-Scale Continuous Reactor[1]

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ABSTRACT: A laboratory-scale continuous reactor was constructed for production of specific structured triacylglycerols containing essential fatty acids and medium-chain fatty acids (MCFA) in the *sn*-2 and *sn*-1,3 positions, respectively. Different parameters in the lipase-catalyzed interesterification were elucidated. The reaction time was the most critical factor. Longer reaction time resulted in higher yield, but was accompanied by increased acyl migration. The concentration of the desired triacylglycerol (TAG) in the interesterification product increased significantly with reaction time, even though there was only a slight increase in the incorporation of MCFA. Increased reactor temperature and content of MCFA in the initial reaction substrate improved the incorporation of MCFA and the yield of the desired TAG in the products. Little increase of acyl migration was observed. Increasing the water content from 0.03 to 0.11% (w/w substrate) in the reaction substrate had almost no effect on either the incorporation or the migration of MCFA, or on the resulting composition of TAG products and their free fatty acid content. Therefore, we conclude that the water in the original reaction substrate is sufficient to maintain the enzyme activity in this continuous reactor. Since the substrates were contacted with a large amount of lipase, the reaction time was shorter compared with a batch reactor, resulting in reduced acyl migration. Consequently, the purity of the specific structured TAG produced was improved. Interesterification of various vegetable oils and caprylic acid also demonstrated that the incorporation was affected by the reaction media. Reaction conditions for lipase-catalyzed synthesis of specific structured TAG should be optimized according to the oil in use.

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KEY WORDS: Acyl migration, continuous reactor, incorporation, interesterification, lipase, medium-chain fatty acids, structured triacylglycerols.

Medium-chain triacylglycerols (MCT) have been used as rapidly digested fat in malabsorption and infant care (1–3). However, pure MCT does not provide essential fatty acids (EFA) (4,5). Therefore, alternative triacylglycerols (TAG), such as physical mixtures of MCT and conventional vegetable oil, interesterified fats with random TAG structure, and specific structured TAG with medium-chain fatty acids (MCFA) located in the *sn*-1,3 positions, have been used in absorption studies (6–9) and for clinical nutrition (10). The TAG structure affected the digestion and absorption of fat (8,9,11) and the specific structured TAG with long-chain fatty acids located at the *sn*-2 position provided a more readily absorbed source for polyunsaturated fatty acids (7–9). Therefore, the nutritional value of a TAG depends both on the fatty acid composition and the positional distribution of the acyl groups within the TAG molecule.

The interest in the production of structured lipids containing special fatty acids has been increasing continuously. Different methods for synthesis of structured TAG have been introduced among which lipase-catalyzed interesterification is superior (12–14). Even though the natural function of lipase is to catalyze the hydrolysis of TAG, interesterification can also occur because it is a reversible biological process. Under restricted water conditions, interesterification is predominant (15). Lipase-catalyzed interesterification with an *sn*-1,3 specific lipase offers high catalytic efficiency, specificity, and selectivity. It provides a useful way to improve the nutritional properties of lipids by incorporation of a required acyl group into a specific position of TAG, whereas a random chemical interesterification does not have this specificity (16). In addition, enzyme-catalyzed reactions can occur at low temperature and in nonsolvent systems (16–19).

Acyl migration is a major problem in batch reactors, which results in decreased purity of the specific structured lipids even though the lipase is *sn*-1,3 specific. The high ratio between the reaction substrate and enzyme demands long reaction time to reach equilibrium, and consequently results in acyl migration. Therefore, a continuous interesterification with immobilized lipase is of particular interest (16,18–20). Xu *et al.* (21) have recently shown the continuous enzymatic reactor to be advantageous over batch reactors in reducing the acyl-migration in pilot plant scale. Continuous processing also allows reuse of the immobilized lipase and cost reduction.

A small continuous reactor was constructed in our laboratory to produce specific structured TAG containing EFA at the *sn*-2 position and MCFA at primary positions. To elucidate the

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effects of different parameters on the incorporation and migration of MCFA in the structured TAG in the continuous reactor, different reaction times, reactor temperatures, water contents, and molar ratios in the reaction substrates were examined. The concentrations of TAG species and free fatty acids (FFA) in the interesterification products were also studied.

MATERIALS AND METHODS

Materials. Safflower oil, sunflower oil, linseed oil, borage oil, and high-oleic sunflower oil were purchased from Róco (Copenhagen, Denmark), FDB (Lyngby, Denmark), Nomeco A/S (Copenhagen, Denmark), Roche (Basel, Switzerland), and Århus Olie A/S (Århus, Denmark), respectively. The fatty acid compositions of the oils are given in Table 1. Capric acid and caprylic acid were purchased from Sigma Chemical (St. Louis, MO), and lauric acid was donated by Henkel KGaA (Düsseldorf, Germany). MCT, containing 60 mol% caprylic acid and 40 mol% capric acid, was obtained from Grünau GmbH (Illertissen, Germany). Lipozyme IM, in which *Rhizomucor miehei* is immobilized on a macroporous anion exchange resin, was donated by Novo Nordisk A/S (Bagsværd, Denmark). All the solvents used for gas–liquid chromatography (GLC) and high-performance liquid chromatography (HPLC) were of HPLC grade (Struers Kebo, Albertslund, Denmark).

Interesterification. The bioreactor was a glass column (l = 38 cm, o.d. = 5 cm, i.d. = 2.6 cm) packed with 58 g of Lipozyme IM (l = 28 cm) and covered with aluminum foil to prevent photo-induced oxidation. The reaction substrates kept in a circulation water bath (HETO, Birkerød, Denmark) were pumped through the enzyme reactor by a peristaltic pump (Type 4912A; LKB product AB, Stockholm, Sweden). The substrate mixture was fed upward into the column and the column temperature was maintained constant by the circulation water bath (Fig. 1). The interesterification products were collected under nitrogen and the reaction medium was solvent-free.

Experiment 1. The dead volume of the enzyme column was measured. The enzyme column was first filled with a mixture of borage oil and caprylic acid at a molar ratio of 1 to 8, and then the MCT was used as the substitute. The eluate from the enzyme column was collected from the starting point of feeding MCT, and the fatty acid profile of each fraction

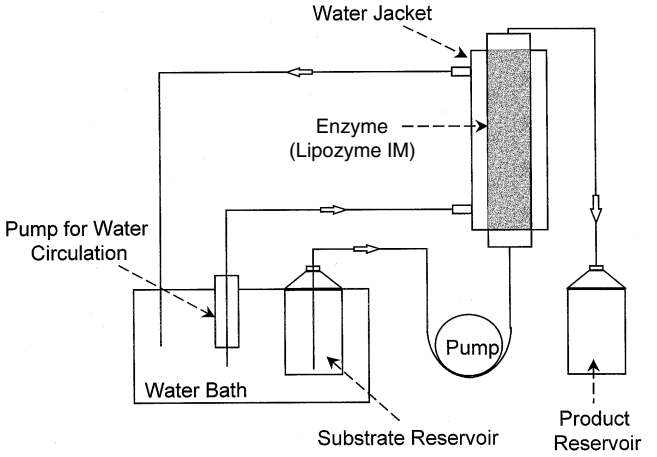

FIG. 1. The diagram of the continuous reactor used for lipase-catalyzed interesterification.

was studied. Both MCT and the enzyme column were kept at 60°C and the flow was kept at 1.5 mL/min. The dead volume was calculated by Equation 1:

$$V_0 = t_0 \cdot u \qquad [1]$$

where V_0 is the apparent value of the dead volume of the enzyme column, t_0 is the appearing time of capric acid in the collected fractions, and u is the flow rate of the substrate.

Experiment 2. The reaction time of the substrates in the reactor was calculated on the basis of porosity of the Lipozyme IM (l = 16 cm, porosity = 0.44), the feeding flow rate of the substrate, and the volume of the enzyme column. The flow rate of the reaction substrate in the enzyme column was varied from 2.8 to 0.09 mL/min, which corresponded to a reaction time of 13 to 425 min. The substrates used for interesterification consisted of sunflower oil and capric acid at a molar ratio of 1 to 6 and contained 0.03% of water. Both the substrate and the enzyme column were kept at 60°C.

Experiment 3. The effects of reaction temperature were examined between 30 and 70°C. The substrate used for interesterification consisted of sunflower oil and caprylic acid at a molar ratio of 1 to 4, and was kept at a flow rate of 1 mL/min. The substrate contained 0.03% water.

Experiment 4. The effect of water content between 0.03 and 0.11% was examined. The original reaction substrate contained

TABLE 1
The Main Fatty Acid Composition (mol%) of the Oils[a]

	Sunflower oil		Safflower oil		Borage oil		Linseed oil		Sunflower oil (high-oleic)	
Fatty acid	TAG	*sn*-2	TAG	*sn*-2	TAG	*sn*-2	TAG	*sn*-2	TAG	*sn*-2
16:0	6.6		7.1		10.7		5.6		4.5	
18:0	5.1		2.4		3.4		3.0		4.7	
18:1n-9	21.9	20.9	11.2	10.9	14.7	14.2	19.8	23.1	77.0	89.9
18:2n-6	61.0	71.3	75.5	85.6	36.5	40.3	15.3	21.1	6.4	6.8
18:3n-6					23.3	41.7				
18:3n-3							53.2	52.4		

[a]TAG, triacylglycerol.

0.03% water, which was measured by the Karl Fischer method. The lipase contained 2.3% water. Reaction substrates were prepared with water contents varying between 0.03 and 0.11% by adding different amounts of water. The substrate used for interesterification consisted of sunflower oil and caprylic acid at a molar ratio of 1 to 4. The temperature was 60°C and the flow rate of the reaction substrate was 1.1 mL/min.

Experiment 5. The molar ratios between caprylic acid and sunflower oil in the reaction substrates varied between 1:1 and 8:1. The water content was 0.03%, reaction temperature was 60°C, and the flow rate was 1.1 mL/min.

Experiment 6. Various specific structured TAG were synthesized by changing the reaction substrate. The substrates used for interesterification consisted of 1:6 (molar ratio) of oil (safflower oil, linseed oil, or borage oil) and caprylic acid. Water content was 0.03%, temperature was 60°C, and flow rate was 0.2 mL/min.

Analytical procedures. Ten mg of the interesterification products was dissolved in 150 μL of chloroform and applied on a silica thin-layer chromatography (TLC) plate (Merck art. 5721, Darmstadt, Germany). The TLC plate was developed in a closed chamber with heptane/isopropanol/acetic acid (95:5:1, vol/vol/vol) for 45 min. Following spraying with 2,7-dichlorofluorescein, the bands were visualized in ultraviolet light. The TAG were scraped off, extracted with diethylether, and methylated with 2 M KOH in methanol (22). Regiospecific analysis of TAG was conducted by Grignard degradation of approximately 30 mg of the interesterification products with allyl magnesium bromide (23,24).

The TAG components in the interesterification products were analyzed with a JASCO high-performance liquid chromatograph (JASCO Corporation, Tokyo, Japan) equipped with a Supelcosil LC-C_{18} column (l = 25 cm, i.d. = 4.6 mm, particle size = 5 μm; Supelco, Inc., Bellefonte, PA), two PU-980 pumps, an HG-980-30 solvent mixing module, an AS-950 autosampler, and a UV-970 UV/VIS detector. A SEDEX 55 evaporative light-scattering detector (ELSD; SEDERE, Alfortville, France) was also coupled to JASCO and was set at 30°C. Separation of TAG was performed with a binary solvent system of acetonitrile and isopropanol/hexane (2:1). Flow rate was 1 mL/min at ambient temperature. The interesterification products were dissolved in chloroform (100 mg/mL) and 10 μL were injected. The TAG components in the interesterification products were identified by collection of the HPLC fractions, followed by methylation and GLC analysis.

The concentration of free fatty acids in the products was determined by AOCS Official Method (25).

The fatty acid profiles were determined by GLC using an HP 6890 Series gas chromatograph (Hewlett-Packard, Waldbronn, Germany) equipped with a flame-ionization detector and a fused-silica capillary column (SP-2380, 60 m × 0.25 mm i.d.; Supelco). Oven temperature was programmed from 70 to 160°C at a rate of 15°C/min, increased to 180°C at a rate of 1°C/min, further to 185°C at a rate of 0.5°C/min, and finally to 200°C at a rate of 20°C/min and held for 10 min. The injector and detector temperatures were 250 and 260°C, respectively. The fatty acid methyl esters were identified by comparing their retention times with authentic standards from Sigma Chemical.

RESULTS AND DISCUSSION

Dead volume. The concentration of capric acid in the eluate from the enzyme column increased sharply after 80 min (at a flow rate 1.5 mL/min), corresponding to a dead volume of 120 mL (Fig. 2). However, the mixture of borage oil and caprylic acid was still the dominant contributor in this fraction. The concentration of linoleic acid decreased to a minimum after 135 min, while the concentration of capric acid reached the maximum. Therefore, the first 200 mL of eluate from the enzyme column should be discarded in order to minimize the interference from the initial reaction substrate. This also implied that this reactor is more suitable for continuous production of the same structured lipids. For a smaller scale production, a lipase column of reduced size is preferable in order to decrease the dead volume.

Reaction time. Different reaction times of the reaction substrate in the lipase column were investigated (Fig. 3A). The incorporation of capric acid in the TAG products was defined as the molar percentage of capric acid in the TAG, and the migration was defined as the molar percentage of capric acid at the *sn*-2 position of the TAG. The incorporation of capric acid increased rapidly in the first 2 h, and 42% of capric acid was incorporated into the TAG. In the next 5 h, there was a slow but steady increase of incorporation, and 48% of capric acid was incorporated into the TAG after a 7 h of reaction (Fig. 3A). The acyl migration increased smoothly from 0.4 to 4.2% over the whole reaction period (Fig. 3A). Even though longer reaction time corresponds to higher incorporation of MCFA in the TAG, a parallel increase of the migration of MCFA also occurred. Therefore, for interesterification a compromise con-

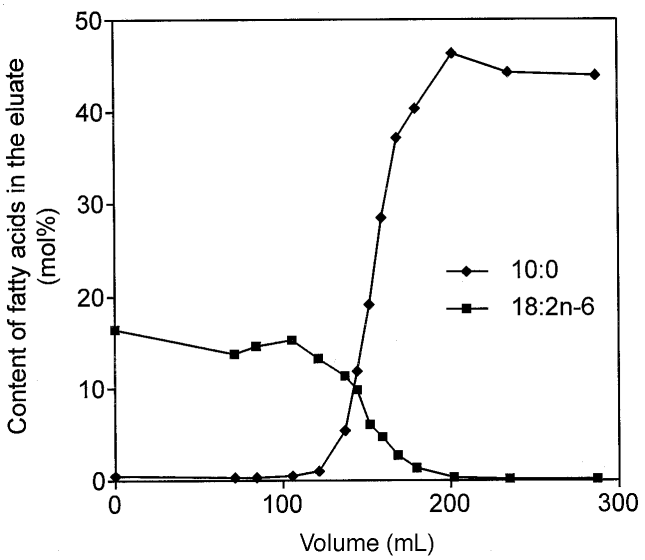

FIG. 2. Measurement of the apparent dead volume of the enzyme column. The concentrations of capric acid and linoleic acid in the collected fractions were measured by using gas–liquid chromatography.

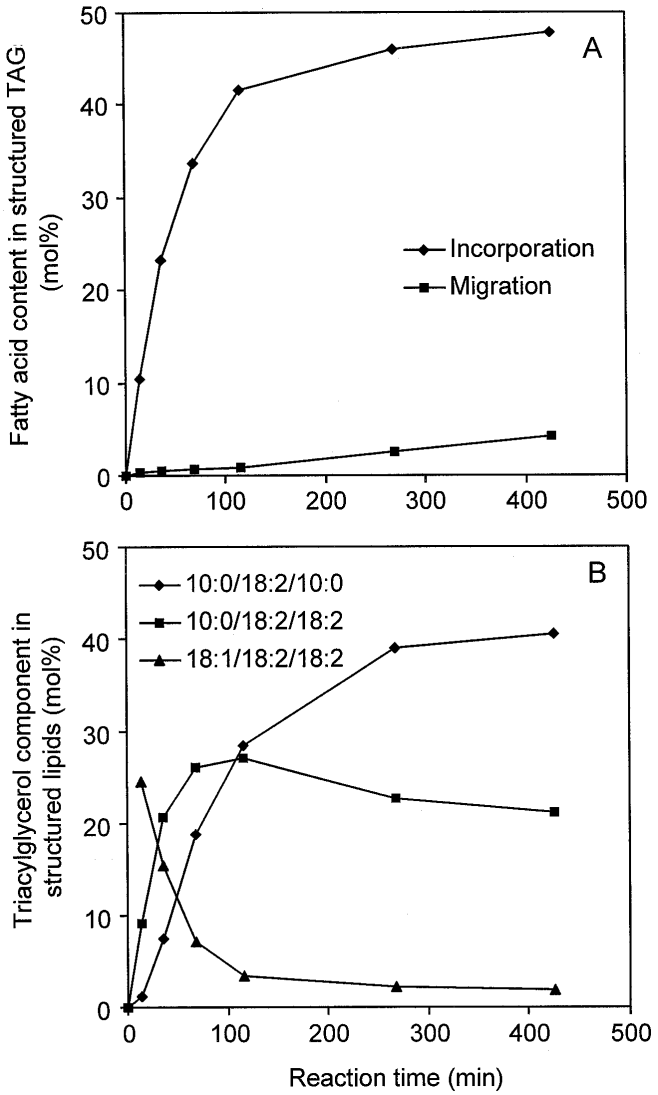

FIG. 3. Effect of reaction time of the reaction substrate in the lipase column on (A) the incorporation and migration of capric acid in the structured lipids and (B) the concentrations of specific structured triacylglycerol in the interesterification products.

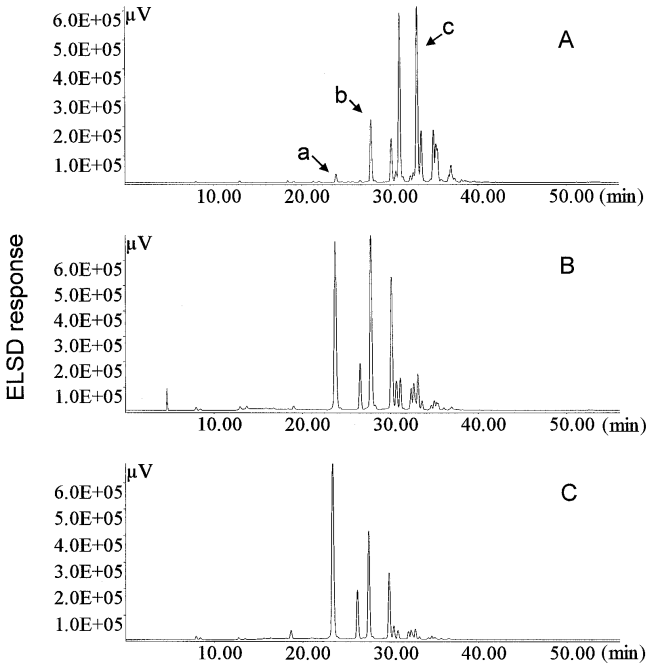

FIG. 4. Separation of interesterification products using reversed-phase high-performance liquid chromatography with evaporative light-scattering detector (ELSD) detection. Comparison of the products of sunflower oil and capric acid under different reaction times: (A) 13 min, (B) 116 min, and (C) 425 min. Peaks a, b, and c represent dicaproyl-linoleoyl-glycerol, dilinoleoyl-caproyl-glycerol, and dilinoleoyl-oleoyl-glycerol, respectively.

sidering both the degree of incorporation and migration of capric acid in the TAG must be made.

Reaction time used in earlier studies with continuous reactors was shorter, and a linear flow rate between $5 - 10 \times 10^{-5}$ m/s was used (18,19). The extent of transesterification of rapeseed oil and lauric acid reached a constant level only after 30 min with 5% migration and 20% incorporation of lauric acid at 70°C (19). In our studies a much lower linear flow rate was used, which resulted in improved incorporation (48%) and similar acyl migration (4%) at 60°C. The differences between these studies may be influenced by several factors, e.g., the construction of the reactor system, the molar ratio between oil and MCFA, water content in the reaction substrate, and incorporation difference between capric and lauric acids.

HPLC analysis of the interesterification products (Fig. 4) demonstrated a similar tendency for the yield of the desired TAG, 1,3-dicaproyl-2-linoleoyl glycerol, during the first 2 h (Fig. 3B). However, in the following 2.5 h, an 11% increment of the desired TAG in the interesterification products was obtained, even though the increment of the incorporation of capric acid was only 4%, and the yield of the by-product 1(3)-caproyl-2,3(1,2)-dilinoleoyl glycerol also decreased. A further 2.5 h reaction time only improved the yield of the desired TAG by 1% and a nearly constant level of products was achieved, but the acyl migration increased from 2.5 to 4.2%. From these results, the optimal reaction time was determined to be 4.5 h.

Compared with a batch reactor, the continuous reactor has less acyl migration even when using longer reaction times. At an incorporation of 48% capric acid, the migration in the batch reactor was 10% (26), but only 4% in the continuous reactor; and the reaction times were 50 and 7 h, respectively. In the continuous reactor, less acyl migration results from shorter reaction time owing to the efficient contact between substrates and lipase. This is a significant advantage since the isomers of the structured TAG with MCFA at different positions (*sn*-1 and *sn*-3, or *sn*-2) have different lymphatic absorption efficiency (7).

Reactor temperature. A reactor temperature of 30°C was chosen as the starting point for the interesterification between caprylic acid and sunflower oil. The incorporation of caprylic acid increased from 13 to 40% when the reactor temperature was changed from 30 to 60°C, while the migration of caprylic acid into the *sn*-2 position was only 0.3 to 0.6% (Fig. 5A). A further increase of temperature to 70°C only increased the in-

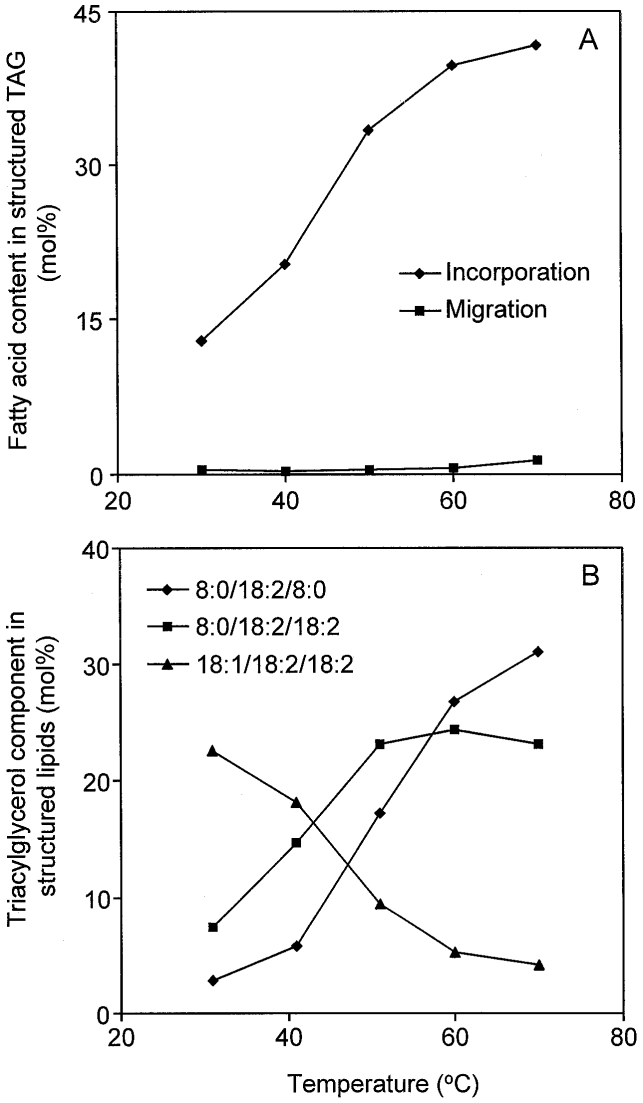

FIG. 5. Effect of reactor temperatures on the interesterification between sunflower oil and caprylic acid. (A) The variation of incorporation and migration of caprylic acid in the structured lipids. (B) The variation of the concentrations of specific structured triacylglycerol in the interesterification products.

corporation of caprylic acid by 2%, while the acyl migration also increased from 0.6 to 1.4%.

Increased acyl migration in a continuous reactor with the same lipase has previously been observed (19). Acyl migrations (2 and 3%) have been observed for the reaction time of 20 and 40 min, respectively, when the reactor temperature was 50°C. Similarly, 3 and 5% acyl migrations have been observed when the reactor temperature was 70°C. Our results further demonstrated the effect of reactor temperature on the acyl migration.

The yield of the desired TAG (1,3-dicapryloyl-2-linoleoyl glycerol) increased constantly with increased reactor temperature (Fig. 5B). This suggested that higher reactor temperature should be used to increase the content of the desired TAG in the interesterification products. High temperature also accelerates inactivation of the enzyme (15) and increases the acyl migration. Therefore, 60°C was the optimal condition for maximal yield of the specific structured TAG when using Lipozyme IM in this reactor. The increased incorporation at high temperature may result from a higher activity of enzyme and improved contact between reaction substrate and enzyme as the viscosity of the substrate decreases.

Water content. A trace amount of water is necessary to maintain the hydration layer around the lipase molecule and maintain the enzyme activity, but it also promotes hydrolysis when the amount of water reaches a critical level (15). The effect of water content on the interesterification in this continuous reactor was therefore studied.

Water content in the reaction substrates had little effect on both incorporation and migration of caprylic acid (Fig. 6A). The incorporation decreased 2% when the water content in the reaction substrate was varied from 0.03 to 0.11% (w/w).

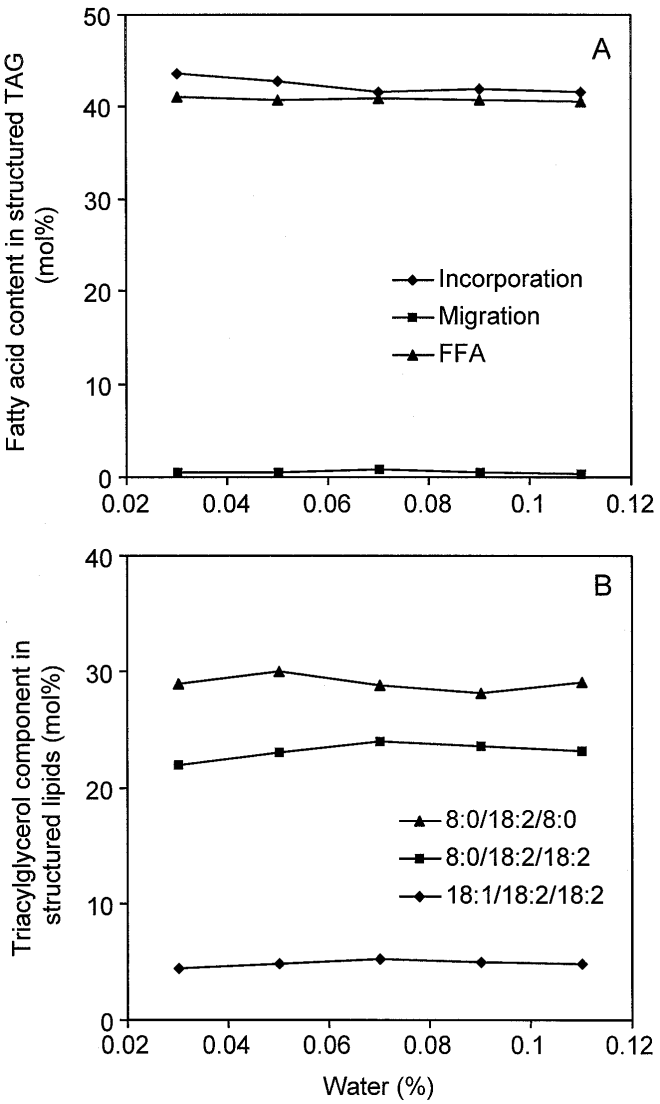

FIG. 6. Effect of water content in the reaction substrate on (A) the incorporation and migration of caprylic acid in the structured lipids and the concentration of free fatty acids in the product, and (B) the concentrations of specific structured triacylglycerol (TAG) in the interesterification products.

Acyl migration was not affected by the water content and was less than 1%. Similar results were obtained when capric acid was used instead of caprylic acid (data not shown). Huang and Akoh (27) also reported that the amount of added water had little effect in the transesterification of triolein and caprylic acid ethyl ester using a similar lipase when the water content varied from 0 to 0.06%. In the transesterification of rapeseed oil and lauric acid, a fast increase of incorporation of lauric acid was found when the water content in the reaction substrate increased from 0 to 0.2%. Thereafter the extent of transesterification reached a constant level, and about 2% of FFA was formed by hydrolysis (19). The differences between these studies may result from the variance of reaction time, molar ratio, and reaction substrate. A reaction time of 60 min was used in our study and the interesterification did not reach equilibrium, whereas a residence time of 35 min was used in the other continuous process and transesterification reached equilibrium (19). Different solubilities of water in caprylic acid and lauric acid may also contribute to the observed difference. The reaction substrate used in our study had a molar ratio of 1:4 between oil and acid, while the substrate used in the other continuous reactor had a mass ratio of 3:1, corresponding to a molar ratio of 1:1.5.

The water content in the reaction substrate did not affect the concentrations of different TAG (Fig. 6B). Both the concentrations of the structured TAG (1,3-dicapryloyl-2-linoleoyl glycerol) and the original TAG from sunflower oil were kept almost constant. This result suggests that water content is not a critical factor in the production of structured TAG in this continuous reactor, and the water in the original reaction substrate is sufficient to maintain the enzyme's activity.

The degree of hydrolysis has been reported to increase continuously with the addition of water in the reaction substrates (19). The concentration of FFA in the interesterification products was also studied. No additional FFA were produced when the water content increased (Fig. 6A). This suggests that the water content also does not affect the amount of diacylglycerols produced in the interesterification. More FFA should otherwise be produced in the interesterification products. This may be due to the high molar ratio between oil and FFA in the reaction substrate, which suppresses the hydrolysis.

Extra water in the reaction substrate had different effects for different reaction substrates (13). Extra water (1%) increased the incorporation of eicosapentaenoic acid into tricaprylin and trilaurin, but it did not affect the incorporation for tricaprin. Water content also had diverse effects for different lipases (27,28). Extra water (0.01%; w/w reaction substrate) could make SP435 lose catalytic capacity completely, but it did not affect the capacity of IM60 at all (27). Therefore, the water content of the reaction substrate has to be carefully adjusted for the individual reaction systems to achieve both an acceptable reaction rate and product yield.

Molar ratio between oil and fatty acids. The effect of molar ratio on incorporation and acyl migration was studied when the interesterification reaction had not reached equilibrium. The incorporation of caprylic acid increased steadily with the increased molar ratio of caprylic acid to sunflower oil (from 1:1 to 8:1), while the migration of caprylic acid into the *sn*-2 position was less than 0.5% (Fig. 7A). Therefore a high molar ratio can improve the reaction rate, for example, when a high molar ratio reaction substrate is used, a shorter reaction time is needed, resulting in reduced acyl-migration.

The analyses of TAG components in the interesterification products also showed that higher molar ratio in the reaction substrate can improve the yield of the desired structured TAG (1,3-dicapryloyl-2-linoleoyl glycerol) in the interesterification products. The yield of the structured TAG was still in a steady increase stage at a molar ratio of 8 to 1 (Fig. 7B). Huang and Akoh (27) reported that there was no increase in the yield of 1,3-dicapryloyl-2-oleoyl glycerol in the transesterification between triolein and caprylic acid ethyl ester when the molar ratio increased from 8 to 10. This difference can be contributed

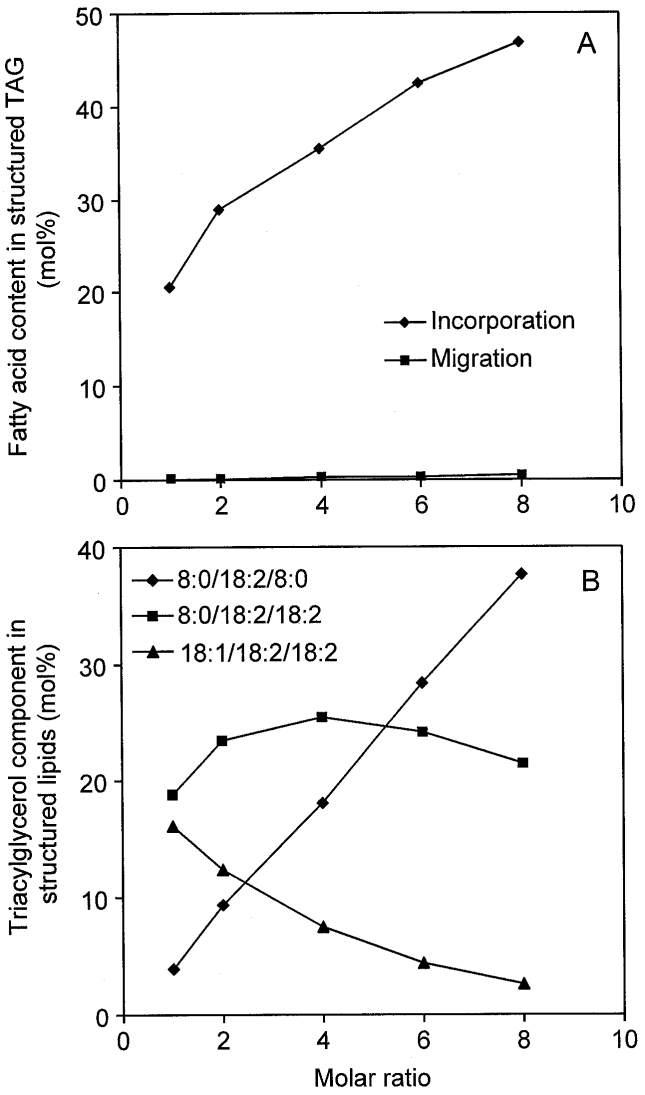

FIG. 7. Effect of molar ratio of caprylic acid to sunflower oil in the reaction substrate on (A) the incorporation and migration of caprylic acid in the structured lipids, and (B) the concentrations of specific structured TAG in the interesterification products. See Figure 6 for abbreviation.

TABLE 2
Incorporation and Migration of Caprylic Acid in the Structured Triacylglycerols Produced in the Continuous Reactor[a]

Reaction substrate	Incorporation (mol%)	Migration (mol%)	Flow (mL/min)
Safflower oil	47	0.3	0.2
Borage oil	35	0.3	0.2
Linseed oil	39	0.5	0.2

[a]The molar ratio between oil and caprylic acid was 1:6, and the reactor temperature was 60°C.

by the different reaction time, since our interesterification has not yet reached equilibrium. With increasing molar ratio, however, other problems will arise, such as high viscosity and difficulties in separating the TAG from FFA. Therefore, a suitable molar ratio in the reaction substrate should be chosen according to the specific requirements for the products.

Interesterification between caprylic acid and different oils. Different vegetable oils were used in the synthesis of structured TAG with caprylic acid (Table 2). The incorporation of caprylic acid in the structured TAG varied with the oils in the reaction substrates even under similar reaction conditions. These results agree well with the report on the synthesis of structured lipids with eicosapentaenoic acid and different MCT (13). This further suggests that the interesterification conditions have to be optimized according to the process being considered.

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