The Enzymatic Preparation of Human Milk Fat Substitute Intermediate Rich in Palmitic Acid at sn-2 Position and Low-Unsaturated Fatty Acids at sn-1(3) Positions from Palm Oil Substrate

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Abstract: The lipid products that consist of structured lipids rich in palmitic acid (16:0) at the sn-2 position of triacylglycerol (TAG) and rich in low-unsaturated fatty acids (FAs) (LUFAs), such as oleic acid; 18:1 and linoleic acid; 18:2 at the sn-1(3) positions, are useful intermediates for manufacturing human milk fat substitute (HMFS), which contains functional lipid components. In this study, the HMFS intermediate (HMFS-IM) was enzymatically prepared from palm oil without using other oil sources. First, the amount of 16:0 at the sn-2 position of TAG substrate was enhanced from 18.9% to more 34.5% via a random esterification reaction using a non-stereospecific lipase, Novozym® 435, to produce a random-palm substrate. Consequently, 2-monoacylglycerol (2-MAG) rich in 16:0 at the sn-2 position over 88%, together with the FA ethyl esters substrates rich in LUFAs, such as 18:1-Et and 18:2-Et above 93.5% was prepared through ethanolysis reaction using the same lipase from the random-palm substrate and by purification with urea complexation, respectively. As the preferred modified method, a continuous use of the same lipase to these reactions were achieved while reducing the usage of enzyme to half. Finally, an HMFS-IM rich in 16:0 at the sn-2 position more than 60% and LUFAs at sn-1(3) positions was prepared using these palm oil-based products, including random-palm, palm-Et, and 2-MAG, via the interesterification reaction using a 1,3-stereospecific lipase, Lipzyme® RM-IM. Thus, HMFS-IM was successfully prepared by palm oil materials with a 65 wt% usage ratio. The concept described in this study will be useful for HMFS manufacturing from a single natural oil substrate, which is not initially rich in 16:0 at the sn-2 position.

Key words: human milk fat substitute, enzymatic preparation, palm oil, lipase

1 Introduction

Human milk fat substitute (HMFS) is an artificially produced compound that consists of reassembled triacylglycerol (TAG) composites of human milk fat (HMF), which is one of the choices for infant nutrition[1-5]. It is known that HMFS should contain palmitic acid (16:0) preferentially esterified at the TAG sn-2 position between 40% and 60%[4], around 60%[1, ~70%[4, 5], or 75%[5]. Palmitic acid is also useful for good stool consistency and absorption in breastfed infants[5, 6, 7]. Furthermore, the positioning of low-unsaturated fatty acids (FA) (LUFAs), such as oleic acid (18:1) and linoleic acid (18:2) at the TAG sn-1(3) positions at high percentages, is typical for HMF[8]. Furthermore, the specific FAs, such as medium-chain fatty acids (MCFA) and polyunsaturated fatty acids (PUFA), are also regarded as important substances for infant growth[1, 3, 4, 9].

Palm oil is an important and versatile vegetable oil that is used as an ingredient or raw material for cooking, food processing, cosmetics, oleo chemicals, biofuel, etc[10-13]. It has become the most widely used vegetable oil since 2004; its production is still expanding. Owing to its cheapness, the use of palm products, such as palm oil, palm olein, palm stearin, and some other palm-based compounds, is widespread in manufacturing HMFS. HMFS products that
are prepared using palm stearin, which is produced by partial crystallization of palm oil at controlled temperature, have been commercially available \(^{1,3-6}\).

However, palm oil products do not contain the functional FAs, such as PUFA and MCFA, at all, and therefore, access to HMFS containing these FAs is unobtainable by using palm sources only \(^{1,9}\). In addition, most of the saturated 16-carbon fatty acids, such as 16:0, are located at the sn-2 position in breast milk glycerol, whereas a large percentage of 16:0 is dominantly located at the sn-1\( (3)\) positions in the formulas of commercial palm oil, which impairs absorption of calcium and lipid and results in insoluble calcium soaps and negatively influenced early bone accretion \(^{1,2,6,9}\). Thus, palm oil should not be used as it is. Physical blending with other oil substrates and enzymatic modification or fractionation with crystallization must be performed \(^{1-4,6,9}\).

In the case of physical blending, the relatively small amounts of TAG components containing specific FAs, such as PUFA, can be added into the HMFS intermediates (HMFS-IMs) rich in 16:0 at the sn-2 position and LUFAs at the sn-1\( (3)\) position. The physical blending technique reduces the possibility of reorganization in the HMFS, which often occurs during the modification of FA distribution in TAG molecules using lipase via intra- and interesterification reactions. In our previous study, 1,3-18:1-2-PUFA-TAG was physically blended with HMFS-IM to prepare PUFA-containing HMFS with a high amount of TAG components containing specific FAs, such as PUFA, can be added into the HMFS intermediates using an sn-1,3-specific lipase and subsequent esterification of 2-MAG using FA sources. That is, 2-MAG rich in 16:0 and FA ethyl(Et)ester (FA-Et) rich in LUFAs-Et (palm-Et) were prepared through enzymatic ethanalysis reaction using Novozym \(^{435}\), and the subsequent extraction using an appropriate solvent and purification with urea (Schemes 1b, c and d). Then, the enzymatic reaction was carried out using these palm-based products consisting of random-palm, 2-MAG, and palm-Et by Lipozyme \(^{®}\) RM-IM-mediated esterification reaction to produce the HMFS-IM (Scheme 1e). At the same time, the model reactions were also performed to investigate the esterification or interesterification reactions between random-palm, 2-MAG, and commercial FA or commercial FA-Et substrates (Schemes 1m1 and m2).

## 2 Experimental Procedures

### 2.1 Materials

#### 2.1.1 Substrates

Commercially available palm oil (Miyoshi Oil & Fat Co. Ltd., Tokyo, Japan) was used as TAG substrate for the preparation of HMFS-IM. The lipid and FA compositions of the palm oil is shown in Table 1. Commercially available FA and FA-Et were used for the model reactions. Oleic acid (O) and linoleic acid (L) were purchased from Tokyo Chemical Industry Co. Ltd. O-Et and L-Et were purchased from Kanto Chemical Co. Inc. The FA compositions of these FA and FA-Et substrates were as the following: O [16:0, 4.1%; 18:1, 89.7%; 18:2, 6.1%; and others, 0.1%], L [18:2, 100.0%], O-Et [16:0, 11.6%; 18:1, 1.4%; 18:2, 82.2%; and 18:3, 4.8%], and L-Et [16:0, 0.0%; 18:0, 0.0%; 18:1, 0.0%; and 18:2, 100.0%].

#### 2.1.2 Enzymes

Novozym \(^{®}\) 435 (Candida antarctica, 10,000 PLU/g, regiospecificity: random, MIK Pharm Co., Ltd., Tokyo, Japan) and Lipozyme \(^{®}\) RM-IM (Rhizomucor miehei, 150 IUN/g, regiospecificity: sn-1 and -3 positions, Novozymes Japan Ltd., Chiba, Japan) were used for enzymatic reactions.
2.1.3 Reagents
All other special-grade reagents were commercially available.

2.2 Enzymatic Synthesis
2.2.1 Randomization of palm oil and preparations of 2-MAG and palm-Et substrates
2.2.1.1 Preparation of random-palm via interesterification of palm oil
2.2.1.1.1 Reaction in hexane
Palm oil (1.0 g) was dissolved in hexane (10 mL) in a round flask. After Novozym® 435 (0.3 g; 30 wt%) was added, the mixture was stirred at 60°C with magnetic agitation at 550 rpm for 3 h (Scheme 1a). After removing the enzyme by filtration, the solvent in the filtrate was evaporated under reduced pressure to produce the randomized oil (random-palm) (yield: 95%). The FA composition at the sn-2 position is shown in Table 2.

Scheme 1 The preparations of HMFS-IMs from palm oil. (a) The randomization reaction of palm oil, (b) ethanolation reaction of random-palm, (c) continuous reaction of randomization and ethanolation reaction, (d) purification with urea complexation, and (e) interesterification reaction between random-palm, 2-MAG, and palm-Et substrates. (m1) and (m2) are the model reactions. (m1) Esterification reaction between random-palm, 2-MAG, and commercial FA substrates. (m2) Interesterification reaction between random-palm, 2-MAG, and commercial FA-Et substrates.
2.2.1.2 Preparations of 2-MAG and palm-Et substrate via ethanolysis reaction

2.2.1.2.1 Ethanolysis reaction

2-MAG was prepared by Novozym® 435-mediated ethanolysis in a manner similar to the previous report but with slight modifications. The reactants containing palm substrates (palm oil or random-palm; about 1 g), Novozym® 435 (30 or 40 wt% for TAG substrates), and ethanol (400 wt% for TAG substrates) were stirred at 60°C with magnetic agitation at 550 rpm for 3.0 to 7.0 h (Scheme 1b). Furthermore, 3.0 h was selected as the appropriate reaction time (Fig. 1). At the end of the reaction, Novozym® 435 was removed by filtration, and the solvent was evaporated under reduced pressure. In the reaction mixture, 10 mL of hexane was added, and it was stored under an ambient atmosphere for 10 min. Then, the 2-MAG fraction rich in saturated FA (SAFA) was obtained as precipitate after the filtration. The product yield was 64% when 80 wt% of enzyme was used (Table 3). The FA composition is shown in Table 2. Moreover, the solution was evaporated under reduced pressure to produce the mixture rich in FA-Et components, which was further purified as described in 2.2.1.2.2.

2.2.1.2.2 Continuous reaction of solvent-free randomization and subsequent ethanolysis reaction

Solvent-free randomization and subsequent ethanolysis reactions were continuously conducted (Scheme 1c). [Randomization] Palm oil (1.0 g) and Novozym® 435 (0.3 or...
0.4 g; 30 or 40 wt %) were added into the round flask. The mixture was stirred at 60°C with magnetic agitation at 550 rpm for 3 h. [Ethanolysis] Ethanol (4 g; 400 wt %) was added to the reaction mixture and it was stirred at 60°C with magnetic agitation at 550 rpm for 3.0 h. At the end of the reaction, Novozym® 435 was removed by filtration, and the solvent was evaporated under reduced pressure. Then, 10 mL of hexane was added to the mixture and stored under an ambient atmosphere for 10 min. Finally, 2-MAG was obtained as precipitate after the filtration. The total yield was 73% when 40 wt % of enzyme was used.

2.2.1.2.3 Purification of palm-Et using urea complexation (Scheme 1d)

The FA-Et-rich reactant (1 g) that was obtained in 2.2.1.2.1 was dissolved in ethanol (15 mL), and urea (1–3 g; 100–300 wt %) was added. After dissolving in a heating bath at 90°C, the solution was allowed to cool to room temperature. After storing overnight, the precipitate was removed. The filtrate was evaporated to dryness, warm water was added to dissolve urea, and the oily phase was separated by hexane. The extracted hexane phase was stirred at 250 rpm for 1 h, and the precipitated urea crystal was removed by filtration. After evaporating under reduced pressure, palm-Et rich in LUFA-Et was obtained. As discussed in the following text, with Fig. 2, the mixture with a 5 by 2 ratio of 18:1 and 18:2 was obtained with the yield of 88% when 150 wt % of urea was used for the extraction. The FA composition of the HMFS-IM is shown in Figs. 3a and b. When the reaction time was set to 1 h, the HMFS-IM obtained had an 82% yield for FA reaction system in the presence of a 4 Å molecular sieve.

### Table 3

|                      | Two step scheme | Continuous scheme |
|----------------------|-----------------|-------------------|
| Total reaction time  | 6.5 h           | 6.0 h             |
| Enzyme usage         | 80 wt%          | 40 wt%            |
| Yield of random-palm | 95%             | 100%              |
| Yield of 2-16:0-MAG  | 64%             | 73%               |
| Purity of 2-16:0-MAG | 89%             | 89%               |

Fig. 2 The FA compositions of the extracted mixtures and the recovery yields as the function of the amount of urea used for the extraction. The FA composition before the extraction is shown as a comparison at the left column (before).

1m1 and m2). The crude mixture obtained after filtration was concentrated to remove hexane. Then, the crude mixture was purified by 7% water-containing Florisil® column chromatography (diethyl ether/hexane = 85/15, v/v, 200 mL) to give a TAG-containing fraction. Here, the condition of the LUFA mixture was determined by calculating the amount of palm-Et that was needed to make the HMFS composition close to breast milk composition. The FA composition of the HMFS-IM is shown in Figs. 3a and b. When the reaction time was set to 1 h, the HMFS-IM obtained had an 82% yield for FA reaction system in the presence of a 4 Å molecular sieve.

2.2.2.2 Preparation of HMFS-IM using palm-Et

Lipozyme® RM-IM (30 wt % of total substrate mass) and FA (O; 5.8 mmol, L; 3.4 mmol) or FA-Et (O; 5.8 mmol, L; 3.4 mmol) were added to the mixture of random-palm substrate (1.0 mmol; 1.0 g), 2-MAG (3.3 mmol; 140 wt %), and it was dissolved in 10 mL of hexane completely with and without the addition of 4 Å molecular sieves (0.3 g). The enzymatic interesterification reactions were carried out at 37°C with agitation at 550 rpm from 1.0 to 6.0 h (Schemes 1m1 and 1m2).

### Table 3

Comparison of the two-step reaction and continuous reaction consisting of randomization and ethanolysis reaction. The yield was calculated for 2-16:0-MAG specie and the purity was estimated for 2-16:0-MAG specie in the 2-MAG product in mol %.
The crude mixture obtained after filtration was concentrated to remove hexane. Then, the crude mixture was purified by water-containing Florisil® column chromatography (diethyl ether/hexane 85/15, v/v, 200 mL) to give a TAG-containing fraction. Here, the condition of the LUFA mixture was determined by calculating the amount of 18:1 in palm-Et that was needed to make the HMFS composition close to breast milk. The lipid and FA compositions of the HMFS are shown in Fig. 3c. Using 1 h as the reaction time, HMFS-IM was obtained with 82% yield.

2.3 Measurements

2.3.1 Estimation of lipid composition

The lipid compositions of the commercial materials and products were analyzed using an iatroscan MK-6s thin-layer chromatography flame ionization detector (TLC-FID) (LSI Medience Corporation, Tokyo, Japan). The TLC-FID analysis was conducted using an iatroscan MK-6s with a silica gel rod S-V (LSI Medience Corporation, Tokyo, Japan) and a mixture of benzene/chloroform/acetic acid (35/15/1, v/v/v) as the development solvent.

2.3.2 Estimation of FA composition

The FAs of acyl glycerol substrates were methyl-esterified, as described by Jham et al., and subjected to gas chromatography (GC) analysis to determine the FA compositions. GC analysis was carried out using GC-18A (Shimadzu, Kyoto, Japan) equipped with a fused silica capillary column, HR-SS-10 (0.25 mm x 25 m: Shinwa Chemical Industries, Ltd., Kyoto, Japan) and an FID detector. The column temperature was programmed to change as follows: the temperature was increased at a rate of 2°C/min from 170°C to 186°C, and then subsequently increased at a rate of 7°C/min from 186°C to 200°C. The temperatures of the injector and the detector were set to 250°C for the analysis.

2.3.3 Estimation of FA composition at the sn-2 position of TAG

At first, the corresponding 2-MAG was prepared from the targeted TAG product from the Novozym® 435-mediated ethanolysis reaction. Briefly, the TAG product (around 1.0 g) and Novozym® 435 (40 wt%) were mixed in ethanol (4.0 g) and the reaction was carried out at 60°C with agitation at 550 rpm for 3 h. After the reaction, the filtration and evaporation of solvent under reduced pressure were performed to produce a crude reaction mixture. Then, the crude mixture was purified by silica gel chromatography using a mixture of benzene/chloroform/acetic acid (35/15/1, v/v/v) as the development solvent to give a corresponding 2-MAG fraction. Subsequently, the composition of 2-MAG was measured as described in Section 2.3.2.

3 Results and Discussion

3.1 Preparation of random-palm substrate from palm oil using Novozym® 435

The randomization of palm oil was conducted to enhance the ratio of 16:0 at the sn-2 position in the reaction mixture with and without solvent, respectively (Scheme 1a). As shown in Table 2, the contents of 16:0 at the sn-2 position increased from 18.9% to 36.7% and 34.5%. However, the content of 18:1 at the sn-2 position was decreased by both reactions. Thus, the random-palm substrate with relatively enriched 16:0 at the sn-2 position was obtained; This pro-
cEDURE was applicable for other palm substrate such as palm olein (data not shown). Ethanolysis reaction was then performed (Scheme 1b) to the random-palm substrate.

3.2 Preparation of 2-MAG from random-palm substrate using Novozym® 435

At first, the ethanolysis reaction (Scheme 1b) was conducted according to the report by Irimescu et al.21. In their report, 40 wt% of enzyme and 2,000 wt% of ethanol solvent against an oil substrate were used for a 4 h reaction. When the reaction condition was applied for the random-palm, the researchers observed that more than 30% of DAG species remained after 4 h. By using this condition as the standard in this study, modification of the reaction condition was applied in terms of enzyme concentration.

The reaction using 100 wt% instead of 40 wt% of enzyme increased the reaction rate, and the desirable compositions containing 28.1% MAG, 0.9% DAG, and 67.6% FA-Et species were yielded from the four-hour reaction. However, the usage of enzyme was too high and wasteful. Instead, increasing the enzyme concentration while decreasing of the amount of ethanol was found to make the reaction rate increase. The use of 40 wt% of enzyme and 400 wt% of ethanol successfully produced the desirable compositions (filled symbol, Fig. 1). It is of note that the further decrease in the amount of enzyme loading failed to produce better results. As shown in Fig. 1, when 30 wt% of enzyme was used for the reaction, the large amount of DAG species (about 19%) remained at 3 h, and about 7% of DAG species still remained at 7 h; whereas fewer than 4% of DAG species remained in the 40 wt% condition at 3 h. Note that while the weight ratio of 100 wt% of enzyme and 2,000 wt% of ethanol was 1 by 20 (w/w), those of 30 or 40 wt% of enzyme and 400 wt% of ethanol were 1 by 13 or 1 by 10 (w/w), respectively, meaning that the initial weight ratio of the enzyme and ethanol was not crucial for the reaction behavior. Consequently, it may be a valid argument that the enzyme must have undergone denaturation by the absorption of ethanol onto enzymes24. Therefore, a large amount of enzyme may be needed to finish the reaction.

Considering that the amount of enzyme should be low, the use of 40 wt% enzyme in the 400 wt% ethanol solvent for 3 h reaction time was received as the appropriate condition. Through the reaction and subsequent extraction, 2-MAG fraction containing 88% of 2-16:0-MAG was obtained, which will be useful substrate for the preparation of HMFS-IM.

3.3 Continuous reaction consisting of Novozym® 435-mediated randomization and ethanolysis reactions

In the previous two sections, the randomization and ethanolysis reactions were individually operated. However, the process inevitably needed a step that can remove existing enzymes and add new enzymes at the second reaction. To improve these terms, the continuous operation of randomization and ethanolysis reactions were investigated (Scheme 1c).

In Table 3, the reaction yield and the purity of 2-16:0-MAG between the two-step reaction method and continuous reaction method under the fixation of 40 wt% of enzyme loading were compared. For the continuous method, the composition of 2-16:0-MAG in the 2-MAG fraction was about 89%, and it was found to be similar to that of the two-step reaction. The reaction yield of 2-16:0-MAG slightly increased from 64% to 73%, in addition to the reduction of enzyme usage to half (from 80 wt% to 40 wt%). Thus, the continuous setup was received as the preferable protocol.

Meanwhile, when the continuous scheme under the condition with 30 wt% of enzyme loading was conducted, the remaining amount of DAG was large (16.6% ± 0.1%) and was undeniably similar to the ethanolysis reaction in the two-step reaction scheme. Therefore, 40 wt% of enzyme, affording small amount of DAG (2.5 ± 0.2%), was determined as appropriate for the continuous reaction scheme.

3.4 Extraction of palm-Et via the purification using urea complexation

Urea is known to form complexes with FA substrates in a warm cosolvent, such as polar alcohol25,26. In this study, LUFa-rich FA-Et fraction was obtained via purification using urea complexation (Scheme 1d). As shown in Fig. 2, the amount of the added urea greatly influenced the FA composition and final yield of the palm-Et substrate. First, the remaining amounts of stearin acid (18:0) and 16:0 were effectively reduced when the 100 wt% of urea was used, but the product yield decreased when the amount of urea was increased from 150 wt% to 250 wt% without the distinct change in lipid composition of FA-Et fraction. Then, the 300 wt% of urea even reduced the composition of 18:1 in the extracted FA-Et, which was accompanied by a decrease in FA-Et fraction. These results indicate that complexation with urea selectively occurred for SAFA-Et25,26, but it can also occur with unsaturated FA when an excess amount of urea was used. Urea, in excessive amounts, kinetically forms complexes with FA-Et substrates. Therefore, it was determined that 150 wt% of urea will be accepted as the appropriate amount that can produce high yields of FA-Et (88%) and LUFa-Et substrates (93.5%). It should be noted that such high compositions of LUFa-Et substrates could not be obtained by a simple solvent-extraction method without using urea.

3.5 Preparation of HMFS-IM substrate

In this section, the HMFS-IM was prepared with random-palm, 2-16:0-rich-MAG, and FA substrates, such as palm-Et prepared from palm oil and commercially available FA and FA-Et substrates (Scheme 1d). In the previous study, FA
was used for the preparation of HMFS substrate, in which water yielded in the esterification reaction (Scheme 1m1). Furthermore, the use of FA-Et substrate should induce EtOH in the interesterification reaction (Scheme 1m2). Since the reaction behaviors greatly differs between them, the researchers tried to investigate the reaction behaviors by comparing the model reactions using commercial FA and FA-Et substrates, respectively.

At first, the esterification reaction was conducted using an FA substrate in the presence and absence of 4 Å molecular sieves, respectively (Fig. 3a). In both reaction conditions, as shown in Fig. 3a, the composition of 16:0 at the sn-2 position gradually decreased as the function of reaction time, indicating the occurrence of randomization. Therefore, to maintain the richness in 16:0 at the sn-2 position, a short reaction should be ensured to avoid randomization. When the influence of molecular sieves was considered, less difference at 1 h reaction time was observed in the FA compositions between the two reactions. However, the amount of 16:0 at the sn-2 position become larger than the other one when the reaction was performed in the absence of a molecular sieve. At the end, the reaction seemed to reach an equilibrium where FA compositions at the sn-2 position became similar to the corresponding total composition at 6 h reaction time. The result indicates that the use of molecular sieves accelerates the reorganization of FA locations. Moreover, the reaction yield of TAG species for the 1 h reaction was about 82% in the reaction with molecular sieves, whereas it was 73% in the reaction without molecular sieves. Because there was a large amount of free water in the reaction mixture in the reaction system without a molecular sieve, there was also a large amount of free FA substrate produced in the reaction system via hydrolysis, resulting in the decrease of the reaction yield. Following the appropriate reaction condition, the reaction with molecular sieve for 1 h was obtained, which produced a high yield of TAG fractions rich in 16:0 at the sn-2 position and LUFA at the sn-1(3) positions.

Meanwhile, when the reaction using FA-Et substrate was conducted without a molecular sieve, a higher yield was achieved compared with the reaction system using FA and a molecular sieve. It follows that the production of EtOH as byproduct did not reduce the reaction yield. The reorganization rate seemed to be midway between the reaction systems using FA substrate with and without molecular sieves when the amount of 16:0 at the sn-2 position was compared. Thus, as the appropriate reaction condition for the FA-Et reaction system, the reaction without molecular sieves for 1 h was obtained, which produced a high yield of TAG fractions rich in 16:0 at the sn-2 position and rich in LUFA at the sn-1(3) positions (Fig. 3b).

Finally, the HMFS-IM from palm oil products was prepared according to the established protocol for the model reaction using a commercial FA-Et substrate. Here, palm-Et was used as the FA substrate. After 1 h reaction in the absence of a molecular sieve, similar to the reaction of FA-Et reaction, the researchers obtained the TAG fraction rich in SAFA at the sn-2 position at about 62.5% and rich in LUFA at the sn-1(3) position at about 76.3%, with a product yield of 82% (Fig. 3c). The product possessing these FA compositions was received as good HMFS-IM sources because they resemble the FA compositions of HMF at each location.

4 Conclusion

HMFS-IM consisting of TAG fractions rich in 16:0 at the sn-2 position and rich in LUFA at the sn-1(3) positions was prepared by palm oil materials without other oil sources via a three-step enzymatic reaction, namely randomization, ethanolysis reaction, and interesterification, using Novozym® 435 and Lipozyme® RM-IM as enzymes (Fig. 4). As a result, palm oil produced 33.6 wt% of HMFS-IM against the initial weight of palm oil with 65 wt% of usage ratio (i.e., HMFS-IM was prepared using 8 g of random-palm, 11 g of 2-16:0-rich-MAG, and 22 g of LUFA-rich-Et). Compared with the two-step reaction that includes randomization and ethanolysis, continuous operation of these reactions without changing the enzyme could effectively reduce the usage of enzymes. The protocol presented in this study will aid HMFS manufacturing from a single oil

![Fig. 4](image_url) The scheme of mass transfer for the preparation of HMFS-IM from the palm oil substrate.
substrate that is not initially rich in 16:0 at the sn-2 position and does not use fractionation of TAG species.

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