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Preparation of Human Milk Fat Substitutes from Palm Stearin with Arachidonic and Docosahexaenoic Acid: Combination of Enzymatic and Physical Methods

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ABSTRACT: Human milk fat substitutes (HMFSs) were prepared by a two-step process, namely, Lipozyme RM IM-catalyzed acidolysis of interesterified high-melting palm stearin with fatty acids from rapeseed oil and blending of the enzymatic product with the selected oils on the basis of the calculation model. The optimum conditions for the enzymatic reaction were a mole ratio of palm stearin/fatty acids 1:10, 60 °C, 8% enzyme load (wt % of substrates), 4 h, and 3.5% water content (wt % of enzyme); the enzymatic product contained 39.6% palmitic acid (PA), 83.7% of the fatty acids at sn-2 position were PA (sn-2 PA), and the distribution probability of PA at the sn-2 position among total PA (% sn-2 PA) was 70.5%. With the fatty acid profiles of human milk fat (HMF) as a preferable goal, a physical blending model was established for the second step to guarantee the maximum addition of selected oils. Based on the model prediction, a desirable formula constituted enzymatic product/rapeseed oil/sunflower oil/palm kernel oil/algal oil/microbial oil at a mole ratio of 1:0.28:0.40:0.36:0.015:0.017, and the final product had PA content, sn-2 PA, and %sn-2 PA at 23.5, 43.1, and 61.1%, respectively. The contents of arachidonic and docosahexaenoic acids were 0.4 and 0.3%, respectively. Relying on the total and sn-2 fatty acid compositions of HMF and "deducting score" principle, the score for the similarity between the final product and HMF was scaled as 89.2, indicating the potential as a fat substitute in infant formulas.

KEYWORDS: *human milk fat substitutes, palm stearin, acidolysis, Lipozyme RM IM, physical blending, arachidonic acid, docosahexaenoic acid*

INTRODUCTION

Human milk is a natural biological colloidal system containing 3−5% fat, providing breast-fed infants with the dietary energy required, essential nutrients, and biologically active molecules.[1,2] Human milk fat (HMF) exists in the form of globules in milk, that is, a triacylglycerol (TAG) core inside and a biological trilayer membrane outside, which keep the whole emulsion system stable.[3,4] HMF is mainly composed of TAGs (>98%); their chemical composition varies with such factors as dietary habits, lactation stage, and genetics.[5,6] Differing from other sources of oils and fats (except lard), saturated fatty acids in HMF are largely located at the sn-2 position, whereas other unsaturated fatty acids are esterified at sn-1,3 positions of the glycerol backbone. The special fatty acid distribution of HMF has considerable influence on the digestion, absorption, and subsequent metabolism of TAGs in infants. Besides 10−30% of the ingested TAGs hydrolyzed to sn-1,2 diacylglycerols (DAG) by gastric lipase (a sn-3 preferential lipase) in the stomach, most TAGs are hydrolyzed by pancreatic lipase in the small intestine.[7] The pancreatic lipase, a sn-1,3-specific lipase, hydrolyzes TAGs into sn-2 monoacylglycerols (MAG) and free fatty acids. The sn-2 MAG can be absorbed in the original form and transformed again into TAGs, which are then packed into chylomicron and transported to the blood via the lymphatic system.[8] Therefore, the major part of the fatty acids at the sn-2 position remains at the position after intestinal absorption. However, the absorption of fatty acids from sn-1,3 positions depends on such factors as the length of the carbon chain and the degree of unsaturation.[9] In addition, Aoe et al. reported that the absorption and transport rate of 1,3-dioleoyl-2-palmitoylglycerol (OPO) was higher than that of 1,2-dioleoyl-3-palmitoylglycerol (OOP), indicating that the intramolecular structure of HMF might have great influence on its metabolism in infants.[10]

HMF contains some long-chain polyunsaturated fatty acids (LC-PUFA) such as docosahexaenoic acid (DHA, n-3), arachidonic acid (AA, n-6), docosapentaenoic acid (DPA, n-3), and eicosapentaenoic acid (EPA, n-3). LC-PUFA account for a very small proportion of HMF (<1%, individually), but they are of great importance to the development of infants, especially DHA (0.32 ± 0.22%) and AA (0.47 ± 0.13%).[11] DHA and AA are highly concentrated in the phospholipid bilayer of biologically active brain and retinal neural membranes and are important in phototransduction and neuronal function.[12] Nevertheless, due to a low level of desaturase activities in infants, they rely on the dietary supply of these fatty acids.[13] Many studies have reported that supplementation of

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DHA and AA in infant formula could improve visual acuity and cognitive development.[14,15] However, potential adverse effects could occur due to excessive intake of LC-PUFA or imbalanced intake of n-6 and n-3 fatty acids.[12] Some studies concluded that infant formulas for term infants should contain at least 0.2% of total fatty acids as DHA and 0.35% as AA, with an AA to DHA ratio of approximately 1.5. Because preterm infants are born with less total body DHA and AA, the formulas for preterm infants should include at least 0.35% of DHA.[16,17]

Due to the importance of the chemical composition and intramolecular structure of HMF to infants, much attention has been given to synthesizing TAGs resembling HMF as substitutes in infant formula. Only enzymatic methods, with positional specificity, were successfully used in this field. The enzymes used were usually immobilized on the supports to get reusable biocatalysts, and proper immobilization could improve enzyme performance by improving enzyme limitations such as stability, activity, specificity, selectivity, or inhibitions.[18,19] To our best knowledge, human milk fat substitutes (HMFSs), to date, were prepared by using different starting materials, such as lard, butter, palm stearin, and tripalmitin, with sn-1,3-specific lipases such as Lipozyme RM IM, Lipozyme TL IM, and *Candida rugosa* lipase, and applying methods such as acidolysis and interesterification together with enzymatic reactors such as batch reactors and packed bed reactors.[20−25] Lipozyme RM IM (RML from *Rhizomucor miehei* immobilized on Duolite ES 562), with high activity and good stability, and acidolysis (acyl exchange of TAG with fatty acids) were the most commonly used.[26−28] The scheme for sn-1,3-specific lipase-catalyzed acidolysis reaction, taking tripalmitin and oleic acid as an example, is depicted in Figure 1. Many factors including

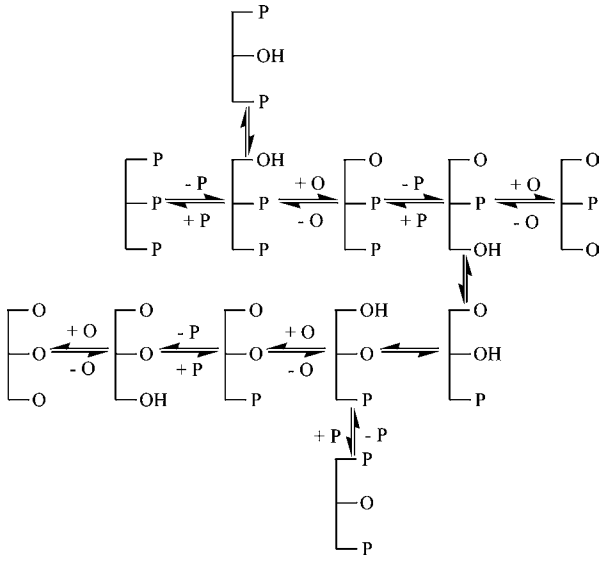

Figure 1. Scheme for sn-1,3-specific lipase-catalyzed acidolysis of tripalmitin with oleic acid. P, palmitic acid; O, oleic acid.

enzyme properties and reaction conditions can result in byproduct formation. Therefore, finding a type of enzyme with good activity, specificity, stability, and selectivity and optimizing the reaction conditions are of great importance for the quality (purity) of the products.

Different types of palm stearin (characterized by melting point or iodine value), fractionated from palm oil, can be used as the starting materials for the HMFS preparation due to their high palmitic acid (PA) content as well as easy availability and cheap prices. Nowadays, palm stearin is mainly used as a material for specialty fat production. Preparation of HMFSs by exploration of palm stearin can not only decrease the production cost but also increase the added value of palm oil. In terms of the reported processes relying on palm stearin and tripalmitin, due to the large amount of PA existing, much higher substrate molar ratios were used, which resulted in higher cost for the materials and downstream procedures and thus made the entire processes uneconomical. Meanwhile, some studies used a physical blend of palm stearin with vegetable oils to prepare HMFSs. The products had fatty acid compositions similar to that of HMF, but the fatty acid distribution was quite different; that is, the saturated fatty acids were equally distributed at three positions. Due to the physiological functions of LC-PUFA for infants, incorporation of these fatty acids into HMFSs has been intensively investigated. However, most of these studies used the acidolysis as the reaction method and the concentrated LC-PUFA as acyl donors, which led to great loss of LC-PUFA and thus increased the production cost. With respect to LC-PUFA, considering their high value and low content in HMF, physical addition by the precise calculation could be a preferred supplementation method.

On the basis of the fatty acid profiles of HMF, the synthesized HMFSs should at least meet the following requirements: (i) the content of total PA ranges from 20 to 30%; (ii) % sn-2 PA should be >60%; (iii) the contents of other major fatty acids (>1%) and some PUFA (>0.1%) are within the ranges of the corresponding fatty acids in HMF. In this study, two steps, including the first step, enzymatic acidolysis of interesterified palm stearin with fatty acids from rapeseed oil to increase the percentage of PA esterified at sn-2 position among total PA (% sn-2 PA), and the second step, blending of the enzymatic product with selected oils to adjust the contents of other major fatty acids to the range of corresponding fatty acids in HMF based on the calculation model, are for the first time reported to prepare HMFSs. The production cost could be extremely decreased by the increase of production yield, which makes the process economically viable. Therefore, the objective of this study is to optimize the conditions for the acidolysis step and establish the model to calculate the amount of selected oils for the second step. The TAG composition of product at different steps was analyzed, and the similarity of the final product to HMF was also evaluated by the related model.

MATERIALS AND METHODS

Materials. Lipozyme RM IM was purchased from Novozymes A/S (Bagsvaerd, Denmark). Pancreatin (porcine pancreas) powder was bought from Sigma Corp. (USA). Three types of palm stearin (PA = 62.6, 70.3, and 83.4%, respectively), rapeseed oil (RSO), sunflower oil (SFO), and palm kernel oil (PKO) were provided by Shanghai Kerry Oils & Grains Industries Co., Ltd. (Shanghai, China). Microbial oil (MO) rich in AA and algal oil (AO) rich in DHA was provided by Fuxing Biotechnology Co., Ltd. (Wuhai, Hubei). Fatty acid profiles of RSO, SFO, PKO, MO, AO, and different types of palm stearin are shown in Table 1. Silicic acid 60G TLC plates (10 × 20 cm) were purchased from Shanghai Shangbang Co., Ltd. (Shanghai, China). Methanol, glacial acetic acid, acetonitrile, and isopropanol were of high-performance liquid chromatography (HPLC) purity. Hexane, hydrochloric acid, and ethyl ether were of analytical grade.

Fractionation of High-Melting Palm Stearin. High-melting palm stearin (PA > 90%) was fractionated by acetone with a concentration of 5 mL/g palm stearin (PA = 70.3%) at 40 °C for 3 h.

Table 1. Fatty Acid Profiles of Sunflower Oil (SFO), Rapeseed Oil (RSO), Palm Kernel Oil (PKO), Algal Oil (AO), Microbial Oil (MO), and Different Types of Palm Stearin (PS)

	SFO		RSO		PKO		AO		MO					
fatty acid	total	sn-2	total	sn-2	total	sn-2	total	sn-2	total	sn-2	PS 1 total	PS 2 total	PS 3 total	PS 4 total
C8:0					4.4	0.3								
C10:0					4.8	1.0								
C12:0	1.3		0.9		55.7	42.3								
C14:0	1.7		1.0		15.1	15.6	8.7	13.1	0.4	0.5	1.3	1.4	1.0	1.4
C16:0	6.8	1.0	6.7	1.1	6.4	8.7	24.7	14.6	10.9	4.1	62.6	70.3	83.4	91.3
C18:0	5.2	0.4	2.7	0.4	1.1	1.4	0.7	1.1	6.1	2.0	5.4	5.6	3.9	3.1
C18:1n-9	25.4	25.8	58.1	54.6	10.2	25.2	1.4	2.4	8.6	13.6	25.4	18.8	10.1	3.6
C18:2n-6	59.0	72.3	22.5	33.9	2.1	5.4	0.4	2.2	4.0	12.8	5.3	3.9	1.6	0.7
C18:3n-3	0.6	0.4	7.8	9.5			0.3	0.3	2.8	5.9				
C20:0							0.4	0.5	3.6	0.8				
C20:3n-6							0.9	0.3	4.5	9.6				
C20:4n-6							0.7	0.5	49.0	45.9				
C22:0							0.0	0.0	4.4	1.2				
C22:5n-3							15.4	13.7						
C22:6n-3							42.0	47.8						

The sediment containing high-melting palm stearin was collected after filtration by Buchner funnel and removal of acetone by vacuum-rotary evaporator at 80 °C and 1.33 × 10^3 Pa for 1 h. The yield for high-melting palm stearin by fractionation was 54.3%.

Chemical Interesterification. Palm stearin, after melting at 105 °C under vacuum (1.33 × 10^3 Pa), was mixed with 0.5% sodium methoxide powder and interesterified for 60 min at 80 °C with magnetic stirring. It was then cooled to 70 °C, and 5% aqueous citric acid (20%) was added to inactivate the catalyst.[29] After 15 min of stirring, the mixture was washed with warm water five times. The interesterified fat was then bleached with 1% of bleaching earth for 30 min at 90 °C under vacuum, followed by filtration through a Whatman no. 4 filter paper. Finally, the interesterified palm stearin was washed with 95% ethanol (w/v = 1:2) three times at 60 °C to remove the produced lower glycerides.

Preparation of Free Fatty Acids. Free fatty acids were prepared from rapeseed oil as described by Senenayeke and Shahidi.[30] Twenty-five grams of oil was saponified by mixing with KOH (5.75 g), water (11 mL), and 95% (v/v) aqueous ethanol (66 mL) for 1 h at 60 °C by refluxing. Distilled water (50 mL) and hexane (2 × 100 mL) were then added to the mixture. The organic layer with unsaponifiable matters was discarded, and the aqueous layer containing the saponifiable matters was acidified with 3 M HCl to a pH of 1.0. The fatty acids were released after acidification and then extracted with hexane (50 mL). The hexane layer was dried over anhydrous sodium sulfate, and the fatty acids were then recovered by removal of the solvent with a rotary evaporator at 40 °C.

Enzymatic Acidolysis. Palm stearin (2 g) was mixed with fatty acids at different substrate molar ratios ranging from 4 to 12 in 25 mL round-bottom flasks, and then the lipase (5−14 wt %, by the weight of total substrates) with water content from 3.5 to 17 wt % (by the weight of enzyme) was added to start the reaction. Samples were flushed with nitrogen and incubated in a water bath with magnetic agitation at 250 rpm.

Triglyceride Isolation. A 50 μL aliquot of the reaction product was taken from the reaction system and isolated by thin layer chromatography (TLC) plates with hexane/diethyl ether/acetic acid (80:20:1, v/v/v) as the developing solvent.[31] The plates were then sprayed with 0.2% 2,7-dichlorofluorescein in methanol and visualized under UV light. The band corresponding to TAG was scraped off and methylated with 3 mL of 4% H_2SO_4 in methanol at 90 °C for 20 min under nitrogen. The FA methyl esters were extracted twice with 2 mL of hexane, dried with anhydrous sodium sulfate, and concentrated with nitrogen.

Fatty Acid Composition Analysis. The fatty acid composition of the products was analyzed with a GC-14B gas chromatograph, equipped with a flame ionization detector (Shimadzu, Tokyo, Japan) and a fused-silica capillary column (PEG-20M, 30 m × 0.32 mm × 0.5 μm). The column was initially held at 100 °C for 4 min, followed by temperature programming to 180 °C at the rate of 15 °C/min, then held at 180 °C for 4 min, and raised to 215 °C at the rate of 4 °C/min. The injection port and detector temperatures were both set at 250 °C. The fatty acid methyl esters were identified by comparison of retention time with the standards, and the relative contents expressed as mole percent were then calculated.

Pancreatic Lipase Hydrolysis. TAGs were isolated by TLC as described above, and the band corresponding to TAGs was scraped off and extracted twice with 2 mL of ethyl ether. TAGs were obtained after removal of solvent by nitrogen concentration. Hydrolysis of TAGs was carried out according to the method described by Luddy et al.[32] One milliliter of 1 M Tris-HCl buffer (pH 8.0), 0.25 mL of 0.05% bile salts, 0.1 mL of 2.2% $CaCl_2$, and 10 mg of pancreatic lipase were added to the TAGs. The mixture was incubated in a water bath at 40 °C for 3 min with vigorous shaking, and then 1 mL of 6 M HCl and 2 mL of diethyl ether were added and centrifuged. Diethyl ether was dried by anhydrous sodium sulfate and evaporated by nitrogen to 200 μL. The hydrolytic products were separated on silica gel G TLC plates, and the developing solvent system was hexane/diethyl ether/acetic acid (50:50:1, v/v/v). The band corresponding to sn-2 MAG identified by the 2-oleoylglycerol was scraped off, methylated, and analyzed as mentioned above.

Deacidification by Molecular Distillation. Molecular distillation (KDL1, UIC GmbH, Alzenau, Germany) was used to remove the fatty acids in the products with the following conditions: evaporator temperature, 185 °C; condenser temperature, 40 °C; heat exchanger temperature, 60 °C; rotation speed of the wiped film, 120 rpm; feeding speed, 2 mL/min; absolute pressure, 2 Pa; preheating temperature, 50 °C; condensing temperature, 50 °C. The heating of the evaporator was provided by the jacket circulated with heated oil from an oil bath.

Separation and Identification of TAG Species. TAG species were analyzed by a reverse-phase high-performance liquid chromatograph (RP-HPLC), equipped with an evaporative light-scattering detector (ELSD). The ELSD was set at 55 °C at a nitrogen nebulizer gas flow rate of 1.8 mL/min and a gain of 1. The separation was carried out on a Lichrospher C18 column (5 μm, 4.6 × 250 mm; Hanbon Science & Technology Co., Ltd., Jiangsu, China), eluted with a binary gradient of acetonitrile (A) and isopropanol (B) at a flow rate of 0.8 mL/min with a linear gradient of solvent A from 65 to 60% in the first 10 min, then to 55% in 40 min, staying at 55% for 30 min, and then to 65% in 5 min. The sample concentration was 20 mg/mL, and the injection volume was 10 μL.

The TAG identification was carried out by HPLC−atmospheric pressure chemical ionization mass spectrometry (HPLC-APCI-MS). The MS conditions were as follows: APCI source block and probe temperatures, 100 and 400 °C, respectively; MS multiplier voltage, 700 V; measurement range, m/z 250−1200.

Evaluation of Human Milk Fat Substitutes. Different HMFSs have different fatty acid compositions and positional distributions. Therefore, it is necessary to evaluate the quality of HMFSs by using some evaluation criteria. A model established by Wang et al. was used in this study to evaluate the degree of similarity of the produced HMFSs to HMF. On the basis of the two equally important elements that determine the chemical structure of HMF, that is, total and sn-2 fatty acid composition, a "deducting score" principle was used in the model to evaluate the degree of similarity.[33] The model was then given as

$$G = G_1 + G_2 \quad (1)$$

$$G_1 = 50 - 50\sum_{i=1}^{n}\left(\frac{|B_i - A_i|}{A_i}\frac{D_i}{\sum_{i=1}^{n} D_i}\right) \quad (2)$$

$$G_2 = 50 - 50\sum_{i=1}^{n}\left(\frac{|B_{i(\text{sn-2})} - A_{i(\text{sn-2})}|}{A_{i(\text{sn-2})}}\frac{D_{i(\text{sn-2})}}{\sum_{i=i}^{n} D_{i(\text{sn-2})}}\right) \quad (3)$$

where G is the score that the evaluated HMFSs get, and the maximum score for G is 100; G_1 and G_2 are the scores that the total and sn-2 fatty acids get; B_i and $B_{i(\text{sn-2})}$ are the contents of the total and sn-2 fatty acids; A_i and $A_{i(\text{sn-2})}$ are the lower or the upper limit of 95% reference range of total and sn-2 fatty acids, the values of lower or upper limits being obtained from HMF. When B is higher than the upper limit of the content of corresponding fatty acid, A is selected as the upper limit, and vice versa. If B is within the range, $|B_i - A_i|/A_i$ and $|B_{i(\text{sn-2})} - A_{i(\text{sn-2})}|/A_{i(\text{sn-2})}$ are kept at 0. D_i and $D_{i(\text{sn-2})}$ are mean values of the contents of total and sn-2 fatty acids in HMF, respectively.

RESULTS AND DISCUSSION

Enzymatic Acidolysis (First Step). *Selection of Suitable Palm Stearin and Substrate Ratio.* Palm stearin after interesterification, characterized as high PA content at sn-2 position, is a good starting material for HMFS preparation. The fatty acid composition at sn-1,3 positions can be altered by transesterification of palm stearin with free fatty acids. However, it is difficult to modify palm stearin to meet the requirements of HMFSs in terms of fatty acid profiles only by transesterification. Therefore, to modify palm stearin for the preparation of HMFSs, a two-step process, that is, a first step to increase the % sn-2 PA of palm stearin to >60% by replacement of sn-1,3 PA with suitable fatty acids, and a second step to mix the enzymatic products with the suitably selected low PA content oils to further decrease the total PA and adjust other fatty acids could be an alternative strategy. The % sn-2 PA of the enzymatic products from the first step had great influence on the amount of blended oils at the second step and the quality of the final product. Therefore, the % sn-2 PA in the acidolysis reaction was selected as the major index and the contents of PA and sn-2 PA, which were respectively negatively and positively correlated to the % sn-2 PA, were chosen as the assistant indices.

Four types of palm stearin with different contents of PA were selected as the starting materials and reacted with free fatty acids from rapeseed oil at different substrate ratios to choose a suitable palm stearin and substrate ratio for HMFS preparation; the results are shown in Table 2. As seen in Table 2, at the same conditions, % sn-2 PA in the products increased with the increase of the content of PA in palm stearin and substrate molar ratios. When the substrate molar ratio was fixed at 1:10,

Table 2. Contents of Total PA, sn-2 PA, and % sn-2 PA of Different Types of Palm Stearin (PS) Reacted with Free Fatty Acids from Rapeseed Oil at Different Substrate Molar Ratios[a]

		substrate ratio (PS/FAs, mol/mol)				
		1:4	1:6	1:8	1:10	1:12
PS 1	total PA (%)	38.6	35.1	33.2	29.7	28.3
	sn-2 PA (%)	54.7	54.1	53.5	52.7	53.3
	% sn-2 PA	47.2	51.4	53.7	59.1	62.8
PS 2	total PA (%)	42.4	38.5	36.4	34.1	32.2
	sn-2 PA (%)	63.6	62.9	63.1	62.7	61.5
	% sn-2 PA	50.0	54.5	57.8	61.3	63.7
PS 3	total PA (%)	45.1	42.3	39.2	36.3	34.7
	sn-2 PA (%)	76.5	75.3	74.7	73.5	72.4
	% sn-2 PA	56.5	59.3	63.5	67.5	69.5
PS 4	total PA (%)	47.3	44.4	42.1	39.3	37.8
	sn-2 PA (%)	85.2	84.5	84.1	83.5	81.3
	% sn-2 PA	60.0	63.4	66.6	70.8	71.7

[a]Reaction conditions: temperature, 60 °C; enzyme load, 11 wt %; water content, 3.5 wt %; reaction time, 4 h.

the % sn-2 PA in the enzymatic products from four types of palm stearin with contents of PA of 62.6, 70.3, 83.4, and 91.3% were 59.1, 61.3, 67.5, and 70.8%, with the corresponding contents of PA of 29.7, 34.1, 36.3, and 39.3% and sn-2 PA of 52.7, 62.7, 73.5, and 83.5%, respectively. It could be easily observed that the % sn-2 PA in the enzymatic product from PS 4 was 11.7% higher than that in PS 1. When the effect of substrate ratio was compared, the % sn-2 PA in the enzymatic products from PS 4 at the substrate ratios of 1:4, 1:6, 1:8, 1:10, and 1:12 were 60.0, 63.4, 66.6, 70.8, and 71.7% with the corresponding contents of PA of 47.3, 44.4, 42.1, 39.3, and 37.8% and sn-2 PA of 85.2, 84.5, 84.1, 83.5, and 81.3%, respectively. However, the rate of increase of % sn-2 PA became slower as the substrate molar ratio increased to a higher level. The % sn-2 PA at the substrate molar ratio of 1:12 was only 0.9% higher than that at 1:10. Therefore, considering the quality of the final products and the cost for the subsequent deacidification procedure, the palm stearin with 91.3% of PA content and a substrate molar ratio of 1:10 were chosen for the latter experiments. At these conditions, the % sn-2 PA for the enzymatic product was 70.8%, with the corresponding contents of PA of 39.3% and sn-2 PA of 83.5%, after 4 h of reaction.

Effect of Temperature. The effect of temperature on the contents of PA, sn-2 PA, and % sn-2 PA as a function of time is shown in Figure 2, panels A, B, and C, respectively. The increase of the reaction temperature favors the decrease rate of the content of PA and also decreases the reaction time to equilibrium. However, higher temperature leads to more byproducts due to the higher rate of occurrence of acyl migration, which decreases the content of sn-2 PA. In the lipase-catalyzed acidolysis reaction, an elevated temperature can decrease the viscosity of the reagents and increase mass transfer and, thus, increase the effective collisions among different molecules, which could considerably increase the reaction rate. Meanwhile, it is also a two-step reaction including the initial hydrolysis and subsequent re-esterification.[34] 1,2-DAG, produced by hydrolysis, is the precursor of acyl migration. The

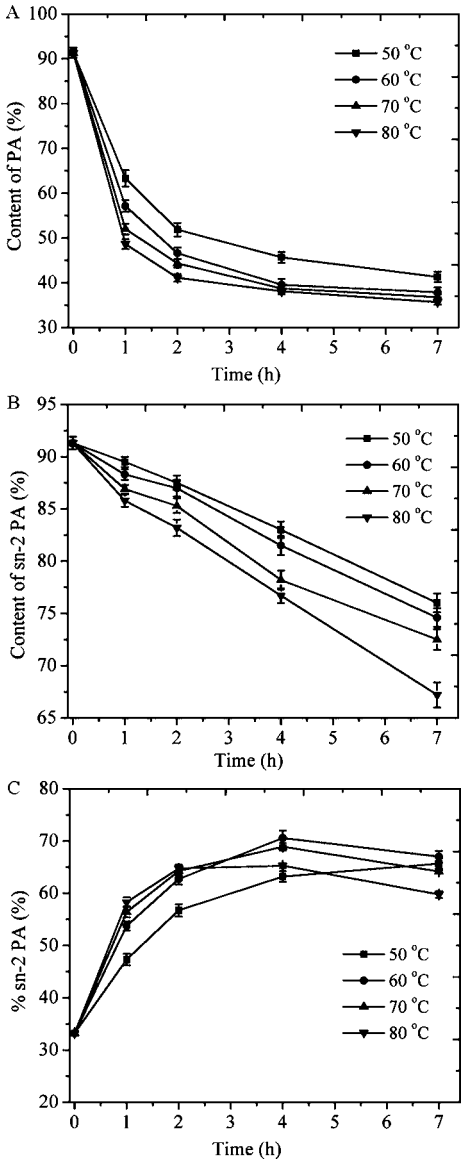

Figure 2. Effect of temperature on the contents of PA (A), sn-2 PA (B), and % sn-2 PA (C) as a function of reaction time. Reaction conditions: enzyme load, 11 wt % (relative to the amount of total substrate); substrate molar ratio, 1:10; water content, 3.5 wt % (relative to the amount of enzyme).

major index investigated in the experiments is negatively proportionate to the content of PA and positively proportionate to the content of sn-2 PA. Therefore, to increase the yield and quality of the final products, decreasing the content of PA and increasing the content of sn-2 PA should be taken into consideration.

In this part of the experiment, some solutions could be considered to get higher % sn-2 PA, such as increasing the reaction temperature with shorter reaction time or decreasing the reaction temperature with longer reaction time. However, a compromise should be achieved to get the maximum value of % sn-2 PA. As shown in Figure 2C, when the temperature was fixed at 60 °C, the % sn-2 PA of the product was 70.2% after equilibrium was achieved, with corresponding 39.5% of total PA content and 83.2% of sn-2 PA content. Compared with other temperatures, the products at 50 °C had a higher content of sn-2 PA with the value of 85.7%, whereas the content of total PA was 45.2%, which resulted in the lower % sn-2 PA with a value of 63.2%. Even though the reaction time was extended to 7 h and the content of PA got to a relatively low level, due to the large amount of acyl migration occurring during such long reactions, the % sn-2 PA was still low, with a value of 65.7%. In terms of reactions at 70 and 80 °C, the contents of PA in the products were low, whereas, because of serious acyl migration at high temperatures, the % sn-2 PA values were 68.9 and 65.3%, respectively, after 4 h of reaction. The high acyl migration rate at high temperature might also be due to the real changes in enzyme properties, which might be affected by the change of variables and the complexity of the reaction involved. Therefore, the possibility of lipase specificity changes at high temperature or after several recycling times might be another reason for the decrease of the content of sn-2 PA. Decreasing the reaction time to 2 h led to the contents of sn-2 PA remaining at higher levels, whereas, due to the relatively high contents of PA, the % sn-2 PA was not as high as expected. Furthermore, high temperature will lead to a high deactivation rate of enzyme, resulting in lower recycling times and thus increasing the cost of production. Therefore, on the basis of the above-mentioned reasons, 60 °C was selected as the reaction temperature for the subsequent reactions.

Effect of Enzyme Load. The effect of enzyme load on the contents of PA, sn-2 PA, and % sn-2 PA as a function of time is shown in Figure 3, panels A, B, and C, respectively. As the enzyme load increased, the content of PA in the products decreased. When the enzyme load was located in the range from 8 to 14 wt %, the contents of PA were similar to one another after the reaction equilibrium was reached. The enzyme load has a minor influence on the acyl migration, which was largely dependent on the amount of DAG produced in the hydrolysis stage. At the initial stage of the acidolysis reaction, lower acyl migration rates could be observed by investigation of the content of sn-2 PA in the enzymatic products due to the lower content of DAG compared with reactions after the achievement of the equilibrium. As seen in Figure 3B, the content of sn-2 PA decreased sharply as the reaction time increased. Therefore, to increase % sn-2 PA in the products, it was necessary to decrease the reaction time by increasing the enzyme load. Compared with the content of PA and % sn-2 PA at different enzyme loads as a function of reaction time, the time to equilibrium was similar and the maximum % sn-2 PA was achieved with values of 71.3, 70.4, and 69.9%, with the corresponding contents of PA of 39.6, 39.3, and 38.7% and sn-2 PA of 84.7, 83.0, and 81.1%, respectively, when the enzyme load ranged from 8 to 14% at a reaction temperature of 60 °C. Afterward, because the content of PA reamined constant while acyl migration proceeded, the % sn-2 PA decreased rapidly. In terms of the reactions with the enzyme load of 5 wt % at 60 °C, due to the relatively lower reaction rate compared with the reactions with higher enzyme load, the % sn-2 PA was always lower. After overall consideration, the enzyme load was selected as 8 wt % for further study.

Effect of Water Content. In the enzymatic acidolysis reactions, water acts as the reactant in the hydrolysis stage and a product in the esterification stage.[34] The effect of water content on the reaction system is mainly reflected in the amount of DAG. The increase of water content will favor the content of DAG and, as a consequence, result in an increase of reaction rate as well as acyl migration. In this reaction system, the effect of water content on the contents of PA, sn-2 PA, and % sn-2 PA is shown in Figure 4, panels A, B, and C,

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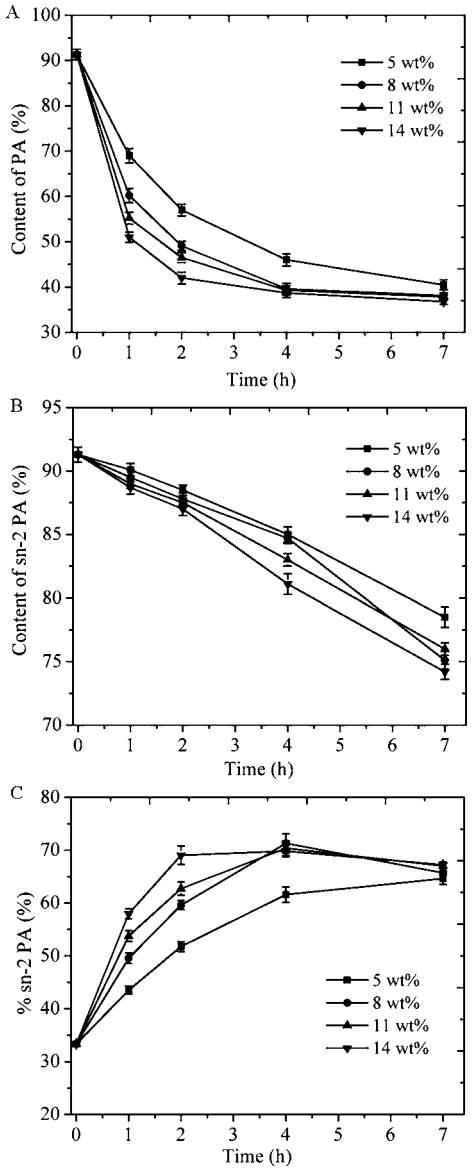

Figure 3. Effect of enzyme load on the contents of PA (A), sn-2 PA (B), and % sn-2 PA (C) as a function of reaction time. Reaction conditions: temperature, 60 °C; substrate molar ratio, 1:10; water content, 3.5 wt %.

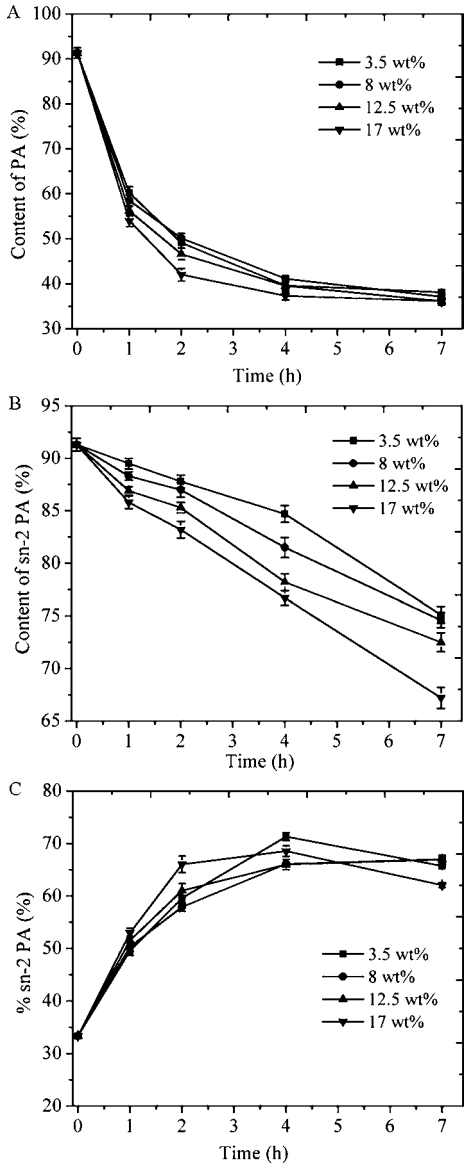

Figure 4. Effect of water content on the content of PA (A), sn-2 PA (B), and % sn-2 PA (C) as a function of reaction time. Reaction conditions: temperature, 60 °C; substrate molar ratio, 1:10; enzyme load, 8 wt %.

respectively. The water content had a minor influence on the content of PA, which was largely because the water content in the enzyme was adequate to produce enough intermediate reactant for esterification, even at the initial content of 3.5 wt %. However, the slight increase in the initial reaction rates could be observed with the water content increasing from 3.5 to 17 wt %. As seen in Figure 4B, acyl migration rate, in general, increased with the increase of the water content. The % sn-2 PA at different water contents as a function of time could be divided into two stages, just like what happened in reactions at different temperatures and substrate ratios. Before the achievement of the equilibrium, the % sn-2 PA increased with the increase of reaction time. However, after equilibrium was achieved, due to the minor decrease in PA content and the obvious decrease in sn-2 PA, the % sn-2 PA decreased with the increase of time. The % sn-2 PA decreased with the increase of water content when they got to maximum points. Meanwhile, the increase of water content increased the content of DAG, which led to the loss of TAG. Therefore, on the basis of the above results, the initial water content of the enzyme (3.5 wt %) was selected for further study.

After optimization, the conditions selected for the first stage were as follows: substrate molar ratio, 1:10; temperature, 60 °C; enzyme load, 8 wt %; water content, 3.5 wt %; reaction time, 4 h. Under these conditions, the fatty acid composition and positional distribution of the enzymatic product are shown in Table 3. The TAG chromatographs of the palm stearin before and after acidolysis reaction under the optimum conditions are shown in Figure 5A,B. The content of PPP decreased from 90.5% in the starting material to 3.8% in the enzymatic product, and the contents of PPO and POO increased from 4.3 and 0.3 to 25.9 and 24.6%, respectively.

Blend of the Enzymatic Product with Selected Oils (Second Step). In contrast with fatty acid composition and positional distribution of HMF (PA, 20−30%; oleic acid, 25−35%; linoleic acid, 10−20%; stearic acid, 5−9%; myristic acid,

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Table 3. Fatty Acid Composition and Positional Distribution of the Enzymatic Product under Optimum Conditions

fatty acid	total	sn-2	% sn-2[a]	sn-1,3[b]
C12:0	0.3	0.1	11.1	0.4
C14:0	0.4	0.1	8.3	0.6
C16:0	39.6	83.7	70.5	17.6
C18:0	3.5	2.4	22.9	4.1
C18:1n-9	36.6	10.2	9.3	49.8
C18:2n-6	14.6	2.7	6.2	20.6
C18:3n-6	4.8	0.6	4.2	6.9

[a]The percentage of fatty acids located at the sn-2 position was calculated as sn-2 × 100%/(3 × total). [b]FA composition at sn-1,3 positions was calculated as (3 × total − sn-2)/2.

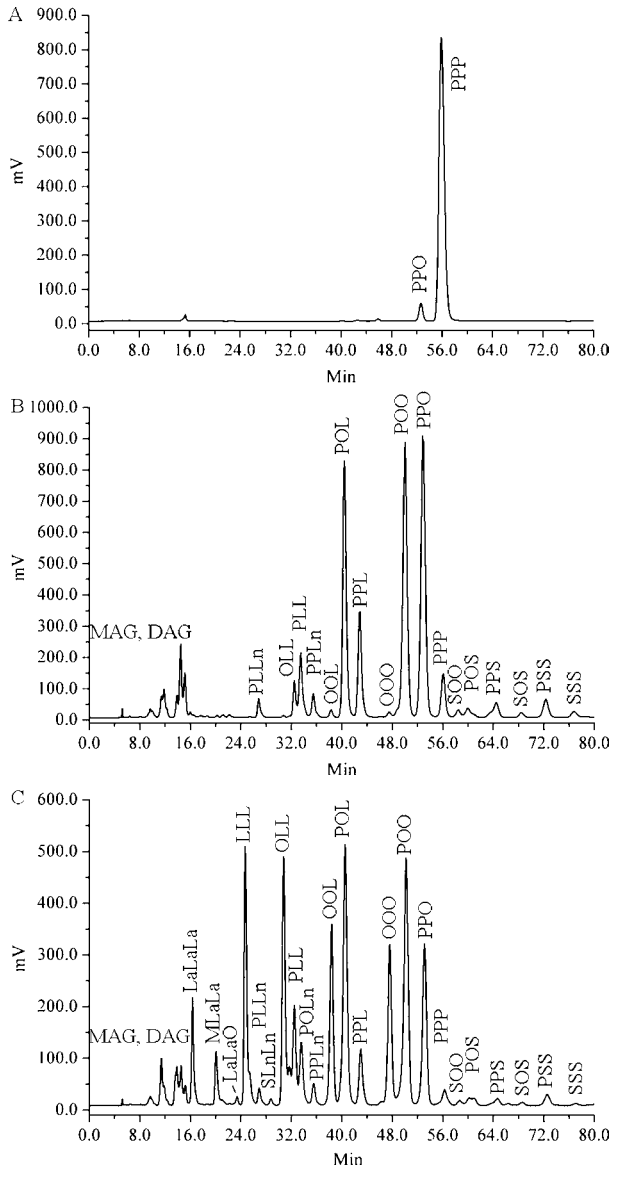

Figure 5. HPLC chromatograms of TAG species of the starting material (A), enzymatic product (B), and final product (C). Abbreviations: La, lauric acid; M, myristic acid; P, palmitic acid; S, stearic acid; O, oleic acid; L, linoleic acid; Ln, linolenic acid.

4−9%; lauric acid, 3−7%; % sn-2 PA > 60%; linoleic acid/linolenic acid, 5−15; DHA, 0.2−0.4%; and AA, 0.35−0.6%), the content of PA in the enzymatic product from the first stage was much higher, requiring the further decrease by blending with other oils with low PA content, and, at the same time, adjusting the contents of other fatty acids to the proper ranges. The fatty acid composition and distribution of the blended oils can be theoretically calculated as

$$\text{FA}\,\% = \frac{Y_1 + \sum_{i=2}^{n} Y_i X_i}{1 + \sum_{i=2}^{n} X_i} \tag{4}$$

$$\text{sn-2 FA}\,\% = \frac{Y_{1(\text{sn-2})} + \sum_{i=2}^{n} Y_{i(\text{sn-2})} X_i}{1 + \sum_{i=2}^{n} X_i} \tag{5}$$

$$\text{sn-1,3 FA}\,\% = \frac{3 \times (Y_1 + \sum_{i=2}^{n} Y_i X_i) - (Y_{1(\text{sn-2})} + \sum_{i=2}^{n} Y_{i(\text{sn-2})} X_i)}{2 \times (1 + \sum_{i=2}^{n} X_i)} \tag{6}$$

$$\%\ \text{sn-2 FA} = \frac{Y_{1(\text{sn-2})} + \sum_{i=2}^{n} Y_{i(\text{sn-2})} X_i}{3(Y_1 + \sum_{i=2}^{n} Y_i X_i)} \times 100 \tag{7}$$

where Y_1 and Y_i are the contents of total fatty acids in the enzymatic product and selected oils, $Y_{1(\text{sn-2})}$ and $Y_{i(\text{sn-2})}$ are the contents of sn-2 fatty acids in the enzymatic product and selected oils, and X_i is the molar ratio between the selected oil and the enzymatic product.

On the basis of the fatty acid profiles of the HMF, RSO, SFO, PKO, AO, and MO, which are rich in oleic acid (58.1%), linoleic acid (59.0%), lauric acid (55.7%), docosahexaenoic acid (42.0%), and arachidonic acid (49.0%), respectively, were selected to blend with the enzymatic product. The final amount of the product can be described as

$$M = X_1(M_1 + \sum_{i=2}^{n} X_i M_i) \tag{8}$$

where M is the final amount of the product, X_1 is the moles of enzymatic product, M_1 is its molecular weight, and M_i is the molecular weight of the selected oils.

To guarantee the quality of the final product and the maximum yield, Matlab R2010a (MathWorks, Natick, MA, USA) was used to optimize the entire blending process, and the final optimized ratio obtained was 1:0.28:0.40:0.36:0.015:0.017 (the enzymatic product/SFO/RSO/PKO/AO/MO). Under this blending ratio, the theoretically calculated and actual fatty acid compositions and positional distributions of the final product are presented in Table 4. In the final product, the contents of PA, sn-2 PA, and %sn-2 PA were 23.5, 43.1, and 61.1%, and the contents of LC-PUFA including AA, DPA, and DHA were 0.4, 0.1, and 0.3%, respectively. A small discrepancy was observed between the theoretical and actual values, largely due to the existence of some lower glycerides in the system. Compared with other reported studies, the yield of the final product was doubled yet with less substrate molar ratio, indicating that the process has the promise for further industrial trials and the oxidative stability of the product was enhanced due to the retention of a higher content of tocopherols by physical blending than the reported products for which the final process was molecular distillation, causing significant loss of these antioxidative substances.

The TAG chromatogram of the final product is shown in Figure 5C. The contents of TAG with unsaturated fatty acids in the final product were dramatically increased due to the

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Table 4. Theoretical and Actual Values of the Final Product Obtained under Optimum Blending Conditions

	theoretical values				actual values			
fatty acid	total	sn-2	% sn-2	sn-1,3	total	sn-2	% sn-2	sn-1,3
C8:0	0.7	0.1	2.4	1.1	0.7	0.1	4.8	1.0
C10:0	0.8	0.2	7.2	1.1	0.8	0.2	8.3	1.1
C12:0	9.8	7.1	24.2	11.2	9.6	6.9	24.0	11.0
C14:0	3.2	2.7	28.1	3.5	3.5	3.0	28.6	3.8
C16:0	23.1	42.6	61.6	13.3	23.5	43.1	61.1	13.7
C18:0	3.2	1.6	16.7	4.0	3.4	1.4	13.7	4.4
C18:1n-9	34.0	23.5	23.0	39.3	34.6	22.8	22.0	40.5
C18:2n-6	19.7	18.7	31.6	20.3	19.1	19.0	33.2	19.2
C18:3n-6	4.0	2.2	18.6	4.9	3.7	2.3	20.7	4.4
C20:4n-6	0.4	0.4	31.1	0.4	0.4	0.4	30.0	0.4
C22:5n-3	0.1	0.1	29.7	0.1	0.1	0.1	26.7	0.1
C22:6n-3	0.3	0.3	37.9	0.3	0.3	0.3	35.6	0.3

addition of the selected oils, and the content of PPP was further decreased from 3.8% in the enzymatic product to 1.0% in the final product. Pons et al. reported that the major TAG species in HMF were OOL (5.9%), POL (15.4%), OOO (6.3%), POO (28.9%), and PPO (4.3%).[35] In the final product, the contents of OOL, POL, OOO, POO, and PPO were 8.2, 14.0, 8.1, 14.9, and 9.8%, respectively. However, the similarity of the product could not be evaluated on the basis of the TAG composition due to isomers existing in some TAG species. More precise similarity evaluation should be based on fatty acid profiles.

HMFS Evaluation. The degree of similarity of major fatty acid profiles of the final product to those of HMF was evaluated by the related model. The scores for the total and sn-2 fatty acids were 44.8 and 44.4, respectively, and the total score was 89.2. AA and DHA were the most important PUFA for the development of infants and their contents in the product were also within the ranges of these in human milk fat, which were reported in our previous study.[36] All of these results indicated that the product had a high degree of similarity to HMF and could be used as a fat substitute in infant formula.

In conclusion, the preparation of HMFSs was achieved by lipase-catalyzed acidolysis of interesterified high-melting palm stearin with fatty acids from rapeseed oil and blending of the enzymatic product with RSO, SFO, PKO, AO, and MO. The optimum conditions for enzymatic reactions were as follows: substrate molar ratio, 1:10; temperature, 60 °C; enzyme load, 8 wt %; reaction time, 4 h; water content, 3.5 wt %. Under these conditions, the contents of PA, sn-2 PA, and % sn-2 PA were 39.6, 83.7, and 70.5%, respectively. On the basis of the fatty acid profiles of HMF, the maximum amount of the selected oils was determined by the established model as 1:0.28:0.40:0.36:0.015:0.017 (the enzymatic product/RSO/SFO/PKO/AO/MO). Under this blending ratio, the contents of PA, sn-2 PA, and % sn-2 PA were 23.5, 43.1, and 61.1% and the contents of AA and DHA were 0.4 and 0.3%, respectively; the score for degree of similarity of the product evaluated by the related model was 89.2, indicating that the product was suitable for use as a fat substitute in infant formula.

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Notes

The authors declare no competing financial interest.

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