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ORIGINAL PAPER

# Preparation of Human Milk Fat Substitutes from Lard by Lipase-Catalyzed Interesterification Based on Triacylglycerol Profiles

**Xiaoqiang Zou · Jianhua Huang · Qingzhe Jin · Zheng Guo · Lingzhi Cheong · Xuebing Xu · Xingguo Wang**

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**Abstract** Human milk fat substitutes (HMFSs) with high similarity in triacylglycerol profiles to HMF were prepared from lard by physical blending and enzymatic interesterfication. Firstly, on the basis of fatty acid profiles of HMF, different vegetable and single-cell oils were selected and added to lard, whose ratios were calculated by the established physical blending model. Secondly, the blending oils were interesterified by a 1,3-regiospecific lipase, Lipozyme RM IM (RML from Rhizomucor miehei immobilized on Duolite ES562; Novozymes, Bagsvaerd, Denmark), to increase the degree of similarity in triacylglycerol (TAG) profiles, and thus the distribution of palmitic acid in the sn-2 position relative to all three acylglycerol positions (% sn-2 PA), degree of similarity in sn-2 fatty acid; and TAG composition were selected as indicators. The optimized conditions were determined as blending ratios, lard:sunflower oil:canola oil:palm kernel oil:palm oil:algal oil:microbial oil = 1.00:0.10:0.50:0.13:0.12:0.02:0.02; enzyme load, 11 wt%; temperature, 60 °C; water content, 3.5 wt%; reaction time, 3 h. The obtained product was evaluated by the evaluation model based on total and sn-2 fatty acid, polyunsaturated fatty acid and TAG composition, and high scores in degrees of similarity were obtained, which indicated the great potential of the product as a fat substitute in infant formulas.

**Keywords** Human milk fat substitutes · Lard · Physical blending · Lipozyme RM IM · Enzymatic interesterification · Triacylglycerol profiles

X. Zou · J. Huang · Q. Jin · X. Wang (✉)
State Key Laboratory of Food Science and Technology, Synergetic Innovation Center of Food Safety and Nutrition, School of Food Science and Technology, Jiangnan University, 1800 Lihu Road, Wuxi 214122, Jiangsu, People's Republic of China
e-mail: wxg1002@qq.com

Z. Guo · L. Cheong · X. Xu (✉)
Department of Engineering, Aarhus University, Gustav Wieds Vej 10, 8000 Aarhus C, Denmark
e-mail: xu@mb.au.dk

## Introduction

Human milk is the best food for newborn infants with almost all essential nutrients, including proteins, fat, carbohydrates, minerals, vitamins, and other bioactive substances. The fat in human milk accounts for 3–5 %, providing more than 50 % of energy [1]. Human milk fat (HMF) is mainly composed of triacylglycerols (TAGs) (>98 %), whose chemical composition varies with such factors as lactation stages, dietary habits, seasons, genetics, and individual conditions. HMF have special intramolecular structures with most saturated fatty acid (palmitic acid) located at the sn-2 position and unsaturated fatty acid at the sn-1,3 positions of glycerol backbone [2]. This special fatty acid composition and distribution lead to formation of a great many TAG species existing in the form of USU, such as 1,3-dioleoyl-2-palmitoylglycerol (OPO) and 1-oleoyl-2-palmitoyl-3-linoleoylglycerol (OPL). These TAGs, after being ingested by infants, were digested by gastric lipase (a sn-3 preferential lipase) in the stomach and pancreatic lipase (a sn-1,3-specific lipase) in the small intestine to sn-2 monoacylglycerols (MAGs) and free fatty acids. The sn-2 MAGs can be absorbed directly by the intestine [3]. Therefore, saturated fatty acids at the sn-2 position could improve their absorption and avoid the formation of soaps with calcium, and thus many side effects. Furthermore, due to more than 70 % of sn-2 fatty acids remaining at its

original position after absorption, this special structure also affects the TAG metabolism and distribution in the infant body [4].

HMF has some medium-chain fatty acids (MCFAs), such as caproic and caprylic acids, and long-chain polyunsaturated fatty acids (LC-PUFAs), such as docosahexaenoic acid (DHA, n-3), arachidonic acid (AA, n-6), docosapentaenoic acid (DPA, n-3), and eicosapentaenoic acid (EPA, n-3) [5, 6]. MCFAs are mostly located at the sn-3 position of human milk TAGs, and the preduodenal lipases possess high activity toward MCFAs at this position [4]. The hydrolyzed MCFAs can be absorbed by the portal vein and provide energy instantly for infants, and the sn-1, 2 DAGs can facilitate fat digestion in the duodenum by increasing of the solubility of the TAGs. LC-PUFAs are very important for the development of infants, especially DHA and AA, even though they account for a very small amount in HMF (less than 1 %, individually) [7]. DHA and AA are found to be highly concentrated in human retina and brain, and are important for the function of visual and central nervous systems [8]. However, the synthesis of DHA and AA from their precursors (linolenic acid, n-3, and linoleic acid, n-6, respectively) appears to be limited for at least some human infants due to low desaturase activities [9]. Therefore, based on the special functions of MCFAs and LC-PUFAs in HMF, the supplementation of these fatty acids in human milk fat substitutes (HMFSs) should be considered for development of a better infant formula.

Lard is the fat found in nature with the most similar chemical composition and molecular structure to HMF. It has been reported that lard has more than 65 % of palmitic acid at the sn-2 position, and thus a high content of OPO [10]. However, compared with HMF, lard had lesser contents of linoleic and linolenic acids, and is free of MCFAs and LC-PUFAs. In order to improve the similarity of lard to HMF, some studies have reported different methods to prepare HMFSs. Yang et al. [10] and Wang et al. [11] reported the preparation of HMFSs by lipase-catalyzed acidolysis of lard with fatty acids from vegetable oils. da Silva et al. [12] reported the preparation of HMFSs by interesterification of lard with soybean oils. All these studies prepared HMFSs with high similarity to HMF from the perspective of fatty acid profiles. However, HMF is ingested by newborns in the forms of TAGs, and whether or not TAG species affect the digestion and metabolism of fat in infants remains unknown. Based on the principle that the chemical composition of HMF is the "golden rule" for HMFS preparation, TAG profiles should be used as the ultimate indicator for HMFS preparation and evaluation. In our laboratory, we have established two models with regard to HMFS preparation. Model I [13] is a physical blending model which can obtain blending oils with specific fatty acid composition and distribution with highest yields optimized by precise calculation, and model II [14] is a HMFS similarity evaluation model which can digitize the difference between HMFSs and HMF from the perspective of TAG profiles. Therefore, the objective of this study was to produce HMFSs with similar fatty acid profiles to HMF by the blending of lard with selected oils optimized by model I, and to increase the degree of similarity of the obtained HMFSs based on TAG profiles by enzymatic interesterification evaluated by model II. The conditions for enzymatic interesterification reactions were optimized based on the distribution of palmitic acid in the sn-2 position relative to all three acylglycerol positions (%sn-2 PA), degrees of similarity of sn-2 FA, and TAG compositions of the products.

## Experimental Procedures

### Materials

Pancreatin (porcine pancreas) powder was bought from Sigma (USA). Lipozyme RM IM (RML from Rhizomucor miehei immobilized on Duolite ES562) was purchased from Novozymes (Bagsvaerd, Denmark). The activity of Lipozyme RM IM was 13,746 units, where 1 unit was defined as nanomoles of oleic acid produced from the hydrolysis of triolein per minute per gram of enzyme. Sunflower oil (SFO), canola oil (CO), palm kernel oil (PKO) and palm oil (PO) were provided by Yihai Kerry Oils & Grains Industries (Shanghai, China). Microbial oil (MO) rich in AA from *Mortierella alpina* and algal oil (AO) rich in DHA from *Schizochytrium* sp. were given by Hubei Fuxing Biotechnology (Wuhai, Hubei). The fatty acid profiles of different vegetable and single-cell oils are reported in Table 1. The fatty acid profile of lard is presented in Table 1. Silicic acid 60G TLC plates (10 × 20 cm) were purchased from Shanghai Shangbang (Shanghai, China). TAG standards including OPO (1, 3-dioleoyl-2-palmitoylglycerol), OOP (1, 2-dioleoyl-3-palmitoylglycerol), PPO (1, 2-dipalmitoyl-3-oleoylglycerol), POP (1, 3-dipalmitoyl-2-oleoylglycerol), OOO (triolein), OOS (1, 2-dioleoyl-3-stearoylglycerol), SOS (1, 3-stearoyl-2-oleoylglycerol), SSO (1, 3-stearoyl-2-oleoylglycerol), PPP (tripalmitin), POS (1-palmitoyl-2-oleoyl-3-stearoylglycerol) were purchased from Larodan Fine Chemicals (Malmö, Sweden). Supelco 37 component fatty acid methyl ester mixture was purchased from Sigma-Aldrich China (Shanghai, China). Hexane, isopropanol, acetonitrile and methanol were of high-performance liquid chromatograph (HPLC) purity. Glacial acetic acid, ethyl ether, and hydrochloric acid were of analytical grade.

**Table 1** Fatty acid composition and distribution of vegetable and single-cell oils and lard

| Fatty acid (mol %) | Lard | | SFO[a] | | CO | | PKO | | PO | | AO | | MO | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Total[c] | sn-2 | $X_1$[b] | | $X_2$ | | $X_3$ | | $X_4$ | | $X_5$ | | $X_6$ | |
| | | | Total | sn-2 | Total | sn-2 | Total | sn-2 | Total | sn-2 | Total | sn-2 | Total | sn-2 |
| C8:0 | | | | | | | 3.7 | 0.9 | | | | | | |
| C10:0 | | | | | | | 3.2 | 1.9 | | | | | | |
| C12:0 | | | | | | | 49.5 | 56.1 | 0.4 | 0.3 | | | | |
| C14:0 | 1.8 | 2.3 | 0.2 | 0.1 | | | 16.2 | 16.3 | 1.3 | 0.8 | 10.5 | 19.2 | 0.4 | 0.5 |
| C16:0 | 25.6 | 70.5 | 6.5 | 1.9 | 4.8 | 0.7 | 8.2 | 4 | 42.5 | 14.8 | 31.4 | 19.5 | 10 | 12.5 |
| C16:1 | 2.3 | 4.1 | 0.4 | 0.3 | 0.3 | 0.1 | 0.2 | 0.2 | 0.2 | 0.3 | 1.8 | 2.3 | 1.3 | 1.5 |
| C17:0 | 0.3 | 0.2 | 0.2 | 0.2 | 0.2 | 0.1 | 0.3 | 0.1 | 0.2 | 0.2 | 0.2 | 0.1 | 0.3 | 0.2 |
| C17:1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.2 | 0.1 |
| C18:0 | 12.8 | 2.7 | 3.6 | 0.5 | 1.2 | 0.1 | 1.8 | 0.5 | 4.4 | 0.8 | 0.3 | 0.2 | 8.3 | 4.3 |
| C18:1 | 42.4 | 12.6 | 29.7 | 29.3 | 64.8 | 56.5 | 14.2 | 17.2 | 40.9 | 62.7 | 1.2 | 1.2 | 5 | 7.4 |
| C18:2 | 12.8 | 6.4 | 58.1 | 66.5 | 18.4 | 30.3 | 2.2 | 2.6 | 9.5 | 19.7 | 2.8 | 4 | 5.6 | 12.9 |
| C18:3 | 0.8 | 0.3 | 0.7 | 0.9 | 8.2 | 11.9 | 0.1 | | 0.3 | 0.2 | 0.3 | 0.3 | 2.2 | 4.6 |
| C20:0 | 0.3 | 0.2 | 0.1 | | 0.7 | 0.1 | 0.3 | 0.1 | 0.2 | 0.1 | 0.1 | 0.1 | 0.2 | 0.1 |
| C20:1 | 0.5 | 0.4 | 0.3 | 0.2 | 1.1 | 0.2 | | | | | 0.3 | 0.2 | 0.2 | 0.1 |
| C22:0 | 0.3 | 0.2 | 0.1 | | 0.2 | | | | | | 0.2 | 0.1 | 0.1 | 0.1 |
| C20:3 | | | | | | | | | | | 0.9 | 0.3 | 8.9 | 8.5 |
| C20:4 | | | | | | | | | | | 0.7 | 0.5 | 39.1 | 37.7 |
| C20:5 | | | | | | | | | | | 0.4 | 0.2 | 18.2 | 9.5 |
| C22:5 | | | | | | | | | | | 14.3 | 12.5 | | |
| C22:6 | | | | | | | | | | | 34.5 | 39.2 | | |
| MW[c] | 858 | | 872 | | 877 | | 696 | | 844 | | 890 | | 869 | |

*SFO* sunflower oil, *CO* canola oil, *PKO* palm kernel oil, *PO* palm oil, *MO* Microbial oil, *AO* algal oil

[a] $X_1$, $X_2$, $X_3$, $X_4$, $X_5$ and $X_6$ are the blending molar ratios of SFO, CO, PKO, PO, AO and MO to lard, respectively

[b] *Total* indicates the weight percent of a fatty acyl species among the all three acylglycerol positions, and *sn-2* indicates the weight percent of a fatty acyl species among the sn-2 fatty acyl groups

[c] *MW* is the molecular weight of triacylglycerols, which is calculated from average molecular weights of their fatty acids

## Determination of Blending Ratios

According to the fatty acid composition and distribution of HMF [15], lard was blended with SFO, CO, PKO, MO and AO at different ratios. The fatty acid profiles of the blended oils were calculated by our previously established physical blending model [13].

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

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

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

$$\%\ \mathrm{sn-2FA} = \frac{Y_{1(\mathrm{sn-2})} + \sum_{i=2}^{n} Y_{i(\mathrm{sn-2})} X_i}{3 \times \left(Y_1 + \sum_{i=2}^{n} Y_i X_i\right)} \times 100 \tag{4}$$

where $Y_1$ and $Y_i$ are the percentages of a fatty acid species among three acylglycerol positions in the lard and selected oil, $Y_{1(\mathrm{sn-2})}$ and $Y_{i(\mathrm{sn-2})}$ are the percentages of a fatty acid species among sn-2 fatty acids in the lard and selected oil, $X_i$ is the molar ratio between the selected oil and lard, in which lard is set to be 1, and n is the oil species.

The final amount of the product can be described as follows:

$$M = X_1 \left(M_1 + \sum_{i=2}^{n} X_i M_i\right) \tag{5}$$

where $M$ is the final amount of the product, $X_1$ is the moles of the lard and $M_1$ is its molecular weight, and $M_i$ is the molecular weight of the selected oil.

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.

### Enzymatic Interesterification

The blended oils (10 g) were added to a 50-mL round-bottomed flask, flushed with nitrogen and incubated in a water bath with magnetic agitation (φ 8 mm × 20 mm stirrer; Jintan Jiamei Instrument;, Jintan, China) at 500 rpm. The lipase (6–10 wt%, by the weight of substrate) with water contents from 3.5–17 wt% (by the weight of enzyme) was added to initiate the reaction and samples were withdrawn from the system periodically for chemical composition analysis. Samples were prepared in duplicate and average values of the results were used.

### Fatty Acid Composition Analysis

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, vol/vol/vol) as the developing solvent. The plates were then sprayed with 0.2 % 2,7-dichlorofluorescein in methanol and visualized under UV light. The TAG band was scraped off and methylated with 3 mL of 4 % $H_2SO_4$ in methanol at 90 °C for 20 min under nitrogen. The fatty acid methyl esters were extracted twice with 2 mL hexane, dried with anhydrous sodium sulfate; and concentrated using nitrogen.

The fatty acid composition analysis was carried out on a GC-14B gas chromatograph, equipped with a flame-ionization detector (Shimadzu, Tokyo, Japan) and a fused-silica capillary column (PEG-20 M, 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 10 °C/min; and then held at 180 °C for 4 min and to 215 °C at the rate of 4 °C/min. The injection port and detector temperatures were both set at 250 °C. The separated fatty acid methyl esters were identified by comparison retention time with the standards, and the relative contents expressed as mol % were then calculated.

### Sn-2 Fatty Acid Composition Analysis

TAGs were isolated by TLC as described above and the TAG band was scraped off and extracted twice with 2 mL of ethyl ether. After removal of the solution by nitrogen, TAGs were hydrolyzed according to the method described by Luddy et al. [16]: 1 mL 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 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 MAGs was scraped off, methylated, and analyzed as mentioned above.

### TAG Composition Analysis

The separation and identification of TAG species were carried out according to our previously reported studies [13, 14]. The separation was performed on a Lichrospher C18 column (5 μm, 4.6 × 250 mm; Hanbon Science & Technology, Jiangsu, China), and 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 70 to 60 % in the first 30 min, then to 55 % in 40 min, staying at 55 % for 20 min, and then to 70 % in 5 min. The identification was carried out on a 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; an MS multiplier voltage, 700 V; and the measurement range, m/z 250–1,200. The sample concentration was 20 mg/mL and the injection volume was 10 μL.

### Evaluation of HMFSs Based on TAG profiles

The degrees of similarity of HMFSs were evaluated by our established model from the perspective of TAG profiles by employment of four indicators, including total (C10:0, C12:0, C14:0, C16:0, C16:1, C18:0, C18:1 and C18:2) and sn-2 fatty acid (C12:0, C14:0, C16:0, C16:1, C18:0, C18:1 and C18:2) composition, and PUFA (C18:3, C20:2, C20:3, C20:4, C20:5, C22:2, C22:4, C22:5, C22:5 and C22:6) and TAG (OLaLa, MMLa, LLaO, MOLa, PMLa, LaOO, POLa, MPL, SMM, POL, PPL, PPM, OOO, POO, PPO and POS) composition (obtained from RP-HPLC) [14]. The results from four aspects of HMFSs should precisely reflect their degrees of similarity in chemical composition to HMF. The model was given as follows:

$$G_{\mathrm{FA/sn\text{ - }2FA/PUFA/TAG}} = 100 - \sum_{i=1}^{n} E_{i(\mathrm{FA/sn\text{ - }2FA/PUFA/TAG})} \tag{6}$$

$$E_{i(\mathrm{FA/sn\text{ - }2FA/PUFA/TAG})} = 100 \times \left( C_{i(\mathrm{FA/sn\text{ - }2FA/PUFA/TAG})} \frac{D_{i(\mathrm{FA/sn\text{ - }2FA/PUFA/TAG})}}{\sum_{i=1}^{n} D_{i(\mathrm{FA/sn\text{ - }2FA/PUFA/TAG})}} \right) \tag{7}$$

$$C_{i(\text{FA/sn - 2FA/PUFA/TAG})} = \frac{\left|B_{i(\text{FA/sn - 2FA/PUFA/TAG})} - A_{\text{i(FA/sn - 2FA/PUFA/TAG)}}\right|}{A_{i(\text{FA/sn - 2FA/PUFA/TAG})}} \quad (8)$$

where $G_{\text{FA/sn-2FA/PUFA/TAG}}$ is the degree of similarity of HMFSs to HMF in the aspect of total fatty acid composition, the percentages of fatty acids in the sn-2 position relative to the three acylglycerol positions, PUFA or TAG composition for the $n$ different fatty acyl species, $E_{i(\text{FA/sn-2FA/PUFA/TAG})}$ is the deducted degree of similarity of the content of fatty acyl species $i$, the percentage of fatty acyl species $i$ in the sn-2 position relative to its three acylglycerol positions, content of polyunsaturated fatty acyl species $i$ or triacylglycerol $i$ of HMFSs that is outside the range of that of HMF; $D_{i(\text{FA/sn - 2FA/PUFA/TAG})} / \sum_{i=1}^{n} D_{i(\text{FA/sn - 2FA/PUFA/TAG})}$ is the weight of the fatty acyl species $i$, sn-2 fatty acyl species $i$, polyunsaturated fatty acyl species i or triacylglycerol $i$ of HMF relative to its total amount; $C_{i(\text{FA/}sn\text{-2FA/PUFA/TAG})}$ is the floating coefficient, which is dependent on the content of fatty acyl species $i$, the percentage of fatty acyl species $i$ in the sn-2 position relative to its three acylglycerol positions, content of polyunsaturated fatty acyl species $i$ or triacylglycerol $i$ of HMFSs. $B_{i(\text{FA/}sn\text{-2FA/PUFA/TAG})}$ is the content of fatty acyl species $i$, the percentage of fatty acyl species $i$ in the sn-2 position relative to its three acylglycerol positions, content of polyunsaturated fatty acyl species $i$ or triacylglycerol $i$ of HMFSs, and $A_{i(\text{FA/}sn\text{-2FA/PUFA/TAG})}$ is the upper or lower limit of corresponding indicator $i$ in HMF. When $B$ is higher than the upper limit of the corresponding indicator in HMF, $A$ is selected as the upper limit, and vice versa. If $B$ is within the range, $C$ is set to zero.

## Results and Discussion

### Determination of Blending Ratios

Human milk fat has special fatty acid composition and distribution, and unique TAG profiles. All these characteristics are closely related to the healthy development of infants. Therefore, the evaluation of the quality of HMFSs should be based not only on their fatty acid profiles but also on their TAG profiles. The fatty acid and TAG profiles of HMF from different stages of lactation have been reported in our previous studies [14, 15].

High similarity in fatty acid composition can be easily achieved by physical blending of selected oils and fats. However, in terms of fatty acid distribution, it is quite difficult to attain high similarity because of the unique PA distribution of HMF. Lard had high PA content at sn-2 position and, by blending with selected vegetable oils, lard can meet the requirements in both fatty acid composition and distribution and attain high similarity to HMF. Based on the difference of fatty acid profiles between lard and HMF, sunflower oil (SFO), canola oil (CO), palm kernel oil (PKO), microbial oil (MO) and algal oil (AO) were selected and their ratio to lard was determined by the physical blending model [13]. The equations derived from the model according to fatty acid profiles of human milk fat were given as follows:

$$\text{C12}:0: 49.0X_3 \geq 3.1(1 + X_1 + X_2 + X_3 + X_4) \quad (9)$$

$$49.0X_3 \leq 10.1(1 + X_1 + X_2 + X_3 + X_4) \quad (10)$$

$$\text{C14}:0: 16.2X_3 + 10.5X_5 \geq 5.7(1 + X_1 + X_2 + X_3 + X_4) \quad (11)$$

$$16.2X_3 + 10.5X_5 \leq 14.8(1 + X_1 + X_2 + X_3 + X_4) \quad (12)$$

$$\text{C16}:0: 25.2 + 6.2X_1 + 4.2X_2 + 8.2X_3 + 42.3X_4 \geq 19.6(1 + X_1 + X_2 + X_3 + X_4) \quad (13)$$

$$25.2 + 6.2X_1 + 4.2X_2 + 8.2X_3 + 42.3X_4 \leq 29.0(1 + X_1 + X_2 + X_3 + X_4) \quad (14)$$

$$\text{C18}:2: 10.8 + 56.4X_1 + 18.0X_2 + 2.1X_3 + 9.7X_4 \geq 7.1(1 + X_1 + X_2 + X_3 + X_4) \quad (15)$$

$$10.8 + 56.4X_1 + 18.0X_2 + 2.1X_3 + 9.7X_4 \leq 20.2(1 + X_1 + X_2 + X_3 + X_4) \quad (16)$$

$$\text{sn}-2\ \text{C16}:0: 70.3 + 4.0X_3 + 14.6X_4 \geq S(25.2 + 6.2X_1 + 4.2X_2 + 8.2X_3 + 42.3X_4) \quad (17)$$

$$\text{C20}:4: 39.0X_6 = 0.5(1 + X_1 + X_2 + X_3 + X_4 + X_5 + X_6) \quad (18)$$

$$\text{C22}:6: 34.0X_5 = 0.3(1 + X_1 + X_2 + X_3 + X_4 + X_5 + X_6) \quad (19)$$

$$\text{Quality}: M = 854 + 870X_1 + 870X_2 + 696X_3 + 844X_4 + 890X_5 + 869X_6 \quad (20)$$

where $X_1$, $X_2$, $X_3$, $X_4$, $X_5$ and $X_6$ were molar ratios of SFO, CO, PKO, PO, AO and MO to lard, respectively, $S$ was %sn-2 PA, which were determined to be 60, 65, 70 and 75 %, and $M$ was the final maximum quality of the blending oils, which was optimized by the Matlab R2010a (MathWorks). In Eqs. 9 and 10, 49.0 was the content of C12:0 in PKO, and 3.1 and 10.1 were the lower and upper limits of C12:0 in HMF, respectively. In Eqs. 11 and 12, 16.2 and 10.5 were the contents of C14:0 in PKO and AO, respectively, and 5.7 and 14.8 were the lower and upper limits of C14:0 in HMF, respectively. In Eqs. 13 and 14, 25.2, 6.2, 4.2, 8.2 and 42.3 were the contents of C16:0 in lard, SFO, CO, PKO and PO, respectively, and 19.6 and 29.0 were the lower and upper limits of C16:0 in HMF, respectively. In Eqs. 15 and 16, 10.8, 56.4, 18.0, 2.1 and 9.7 were the contents of C18:2 in lard, SFO, CO, PKO and PO, respectively,

**Table 2** The ratios for blending oils with %sn-2 PA of 60, 65, 70 and 75 % calculated by the physical blending model (Eqs. 1–5)

| % sn-2 PA[a] (%) | Lard | SFO[b] $X_1^b$ | CO $X_2$ | PKO $X_3$ | PO $X_4$ | AO $X_5$ | MO $X_6$ |
|---|---|---|---|---|---|---|---|
| 60 | 1.00 | 0.19 | 0.71 | 0.16 | 0.25 | 0.02 | 0.03 |
| 65 | 1.00 | 0.14 | 0.59 | 0.14 | 0.18 | 0.02 | 0.03 |
| 70 | 1.00 | 0.10 | 0.50 | 0.13 | 0.12 | 0.02 | 0.02 |
| 75 | 1.00 | 0.06 | 0.42 | 0.12 | 0.08 | 0.02 | 0.02 |

[a] % sn-2 PA indicates the distribution of palmitic acid in the sn-2 position relative to the all three acylglycerol positions

[b] Abbreviations of oils used for blending are given in Table 1

**Table 3** Theoretical and actual fatty acid profiles of the blending oils with %sn-2 PA of 60, 65, 70 and 75 % calculated by the physical blending model (Eqs. 1–5)

| Fatty acid (mol %) | Theoretical values | | | | | | | | Actual values | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 60 %[a] | | 65 % | | 70 % | | 75 % | | 60 % | | 65 % | | 70 % | | 75 % | |
| | Total[b] | sn-2 | Total | sn-2 | Total | sn-2 | Total | sn-2 | Total | sn-2 | Total | sn-2 | Total | sn-2 | Total | sn-2 |
| C8:0 | 0.3 | 0.1 | 0.3 | 0.1 | 0.3 | 0.1 | 0.3 | 0.1 | 0.2 | 0.1 | 0.3 | 0.1 | 0.3 | 0.1 | 0.3 | 0.1 |
| C10:0 | 0.2 | 0.1 | 0.2 | 0.1 | 0.2 | 0.1 | 0.2 | 0.1 | 0.2 | 0.1 | 0.2 | 0.1 | 0.2 | 0.1 | 0.2 | 0.1 |
| C12:0 | 3.4 | 3.8 | 3.4 | 3.8 | 3.4 | 3.8 | 3.4 | 3.8 | 3.2 | 2.9 | 3.2 | 3.5 | 3.1 | 3.3 | 3.6 | 3.1 |
| C14:0 | 2.2 | 2.4 | 2.2 | 2.5 | 2.2 | 2.5 | 2.2 | 2.5 | 2.7 | 2.3 | 2.3 | 1.9 | 2.5 | 2 | 1.9 | 2.4 |
| C16:0 | 20 | 35.6 | 20 | 38.6 | 20 | 41.5 | 20 | 44.6 | 20.4 | 36.7 | 20.6 | 38.5 | 20.7 | 42.5 | 20.4 | 45.2 |
| C16:1 | 1.3 | 1.9 | 1.1 | 2.1 | 1.2 | 2.3 | 1.3 | 2.5 | 1.2 | 1.3 | 0.9 | 1.7 | 1.5 | 1.3 | 1.6 | 2.1 |
| C17:0 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.1 | 0.2 | 0.1 | 0.2 | 0.2 | 0.3 | 0.1 |
| C17:1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| C18:0 | 7.8 | 1.8 | 7.2 | 1.9 | 7.7 | 2 | 8.2 | 2.1 | 6.8 | 2.8 | 7.7 | 2.8 | 7.5 | 2.8 | 7.1 | 3 |
| C18:1 | 41.7 | 30.3 | 41.6 | 28.5 | 41.6 | 26.8 | 41.7 | 25.1 | 41.8 | 29.7 | 40.5 | 28.6 | 40.3 | 26.1 | 41.2 | 25.7 |
| C18:2 | 18.3 | 18.4 | 19.5 | 17 | 18.8 | 15.5 | 18.4 | 14.3 | 18.7 | 18.6 | 19.5 | 17.2 | 19.2 | 17.5 | 19.5 | 14.8 |
| C18:3 | 2.4 | 3.6 | 2.1 | 3.4 | 2.2 | 3.4 | 1.9 | 2.9 | 2.4 | 3.8 | 2.4 | 3.6 | 2.3 | 2.5 | 1.7 | 1.6 |
| C20:0 | 0.3 | 0.2 | 0.3 | 0.2 | 0.3 | 0.2 | 0.3 | 0.2 | 0.3 | 0.2 | 0.3 | 0.3 | 0.3 | 0.2 | 0.3 | 0.3 |
| C20:1 | 0.4 | 0.3 | 0.4 | 0.3 | 0.4 | 0.3 | 0.4 | 0.3 | 0.4 | 0.2 | 0.4 | 0.2 | 0.4 | 0.2 | 0.4 | 0.2 |
| C22:0 | 0.2 | 0.1 | 0.2 | 0.1 | 0.2 | 0.1 | 0.2 | 0.1 | 0.2 | 0.1 | 0.2 | 0.1 | 0.2 | 0.1 | 0.2 | 0.1 |
| C20:3 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 |
| C20:4 | 0.5 | 0.4 | 0.5 | 0.4 | 0.5 | 0.4 | 0.5 | 0.4 | 0.5 | 0.3 | 0.5 | 0.4 | 0.5 | 0.3 | 0.5 | 0.4 |
| C20:5 | 0.2 | 0.1 | 0.2 | 0.1 | 0.2 | 0.1 | 0.2 | 0.1 | 0.2 | 0.1 | 0.2 | 0.1 | 0.2 | 0.1 | 0.2 | 0.1 |
| C22:5 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| C22:6 | 0.3 | 0.4 | 0.3 | 0.4 | 0.3 | 0.4 | 0.3 | 0.4 | 0.3 | 0.4 | 0.3 | 0.4 | 0.3 | 0.4 | 0.3 | 0.4 |

[a] Refers to the distribution of palmitic acid in the sn-2 position relative to the all three acylglycerol positions, with the ratios of oils yielding the percentages given in Table 2

[b] Abbreviations are given in Table 1

and 7.1 and 20.2 were the lower and upper limits of C18:2 in HMF. In Eq. 17, 25.2, 6.2, 4.2, 8.2 and 42.3 were the contents of C16:0 in lard, SFO, CO, PKO and PO, respectively; 70.3, 4.0 and 14.6 were the percentages of palmitic acid in sn-2 position of lard, PKO and PO, respectively. In Eq. 18, 39.1 and 0.5 was the contents of C20:4 in MO and HMF, respectively; and in Eq. 19, 34.0 and 0.3 were the contents of C22:6 in AO and HMF, respectively.

The ratios for the blending oils with %sn-2 PA of 60, 65, 70 and 75 % are shown in Table 2.The fatty acid composition and distribution of the blending oils calculated by the model and their actual values determined by the GC are presented in Table 3.

## Effect of Blending Ratios

The products with similar fatty acid composition and distribution to human milk fat were obtained by physical blending lard with other vegetable oils, whereas the TAG composition of the blending oils was a mixture of TAGs of

selected oils, which was different from that of HMF. Therefore, to obtain HMFSs mimicking HMF in TAG profiles, lipozyme RM IM, a 1,3-specific lipase, was chosen as the catalyst to interesterify the blending oils and the degree of similarity in TAG composition was chosen as the reaction indicator. However, because of the existence of TAG isomers and the occurrence of acyl migration [10], two assistant indicators reflecting the variation of acyl migration and TAG isomers were chosen as %sn-2 PA and degree of similarity in the sn-2 fatty acid composition. According to the attributes of HMF, HMFSs should have %sn-2 PA more than 60 % and a high degree of similarity in sn-2 fatty acid and TAG composition. The %sn-2 PA and degree of similarity in sn-2 fatty acid and TAG composition at different blending ratios as a function of reaction time are shown in Fig. 1.

Four different oils blended with lard with different %sn-2 PA obtained from model calculation had high contents of palmitic acid in sn-2 position (sn-2 PA). As reaction time increased, the content of sn-2 PA decreased, which led to the decrease of %sn-2 PA. The blending oil with %sn-2 PA of 65 % decreased to 60 % after 2.5 h reaction; however, the blending oil with %sn-2 PA of 70 and 75 % kept the contents more than 60 % after 6 h reaction. The sn-2 fatty acid composition was compared with that of HMF and their degrees of similarity were calculated by the evaluation model (Eqs. 6–8). The sn-2 fatty acid composition was affected by acyl migration. The degrees of similarity in sn-2 fatty acid composition of the four types of the blending oils firstly increased and then decreased. However, their degrees of similarity in TAG composition firstly increased and then flattened. As seen in Fig. 1, the blending oils with %sn-2 PA of 70 and 75 % has similar degrees of similarity in sn-2 fatty acid and TAG composition, which were obviously higher than those of %sn-2 PA of 60 and 65 %. Meanwhile, after the interesterification reaction, their %sn-2 PA were both higher than 60 %, which were within the range of that of HMF. However, the blending oil with %sn-2 PA of 70 % had higher yield than that of %sn-2 PA of 75 % when the same amount of lard was used. Therefore, the blending oil with %sn-2 PA of 70 %, that is, the blending ratio of lard:SFO:CO:PKO:PO:AO:MO = 1.00:0.10:0.50:0.13:0.12:0.02:0.02, was chosen for the subsequent experiments.

## Effect of Enzyme Load

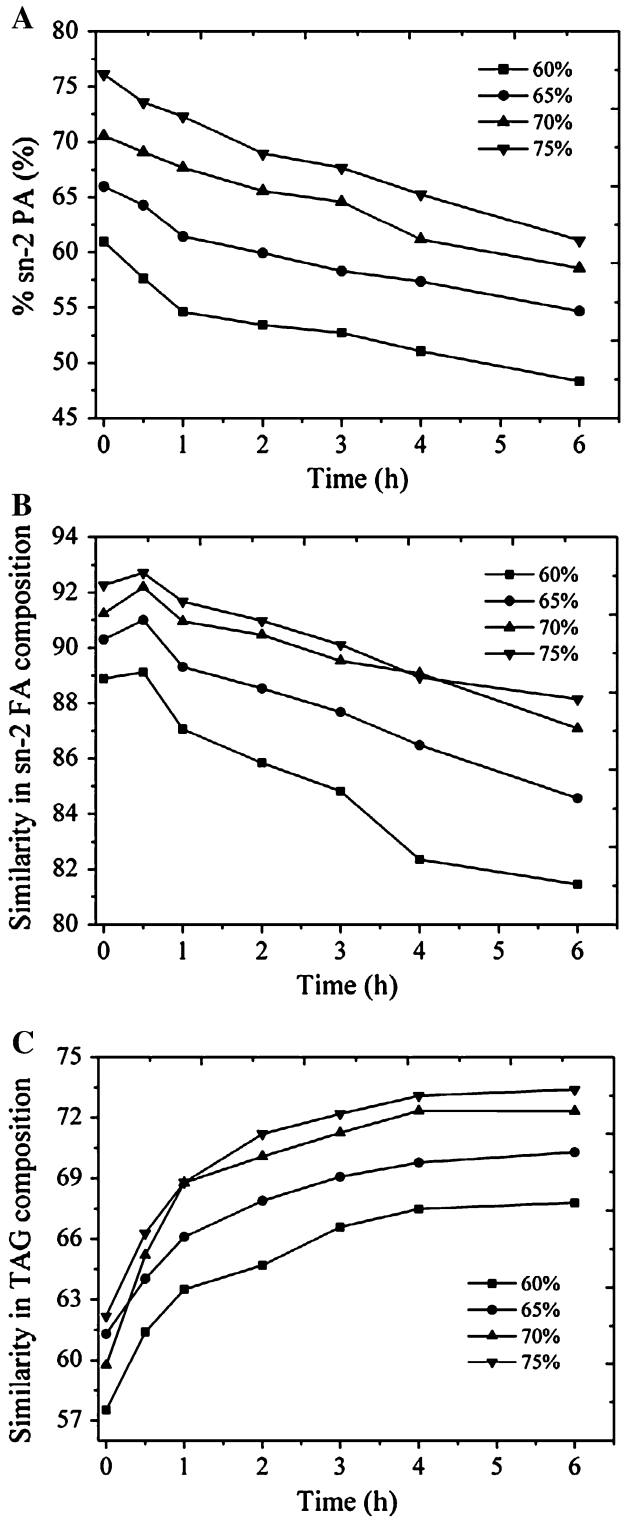
Increasing the enzyme load in the system would accelerate the reaction rate, and thus shorten the time to the reaction equilibrium [10]. However, the major indicator in the reaction is the degree of similarity in TAG composition which might achieve its maximum point before equilibrium. There is an equilibrium in the enzymatic reactions, namely, hydrolysis and re-esterification [17]. Large amounts of enzyme existing in the system might lead to more DAGs and the immobilization carriers might result in more acyl migration. Therefore, considering the enzyme cost and other side reactions, it is reasonable to optimize the enzyme load in the reaction system to get products with high quality. According to Fig. 2, %sn-2 PA decreased with the increase of amounts of enzyme load and, in terms of degrees of similarity of sn-2 fatty acid composition, they had maximum peaks when the enzyme load was in the range of 5–11 wt%. However, when the enzyme load was 14 wt%, the degrees of similarity decreased with the increase of reaction time. The degrees of similarity of TAG composition at enzyme loads of 11 and 14 wt % both had maximum points at reaction time of 4 and 3 h, respectively, whereas their values at enzyme loads of 5 and 8 wt% both had an increasing flat trend and lower than these of 11 and 14 wt%, which were mostly due to the difference of reaction rates. Therefore, the enzyme load of 11 wt% was selected for the subsequent reactions, and the optimal reaction time for reactions with this enzyme load was 3 h.

## Effect of Temperature

The temperature imposes effects on the reaction rate and acyl migration in the enzymatic reaction system [18]. The increase of temperature accelerates the movement rate of the reaction molecules and thus enhances the frequency of their effective collision. However, a higher temperature could lead to a higher acyl migration rate of DAGs and thus decrease %sn-2 PA, and also cause irreversible inactivation of the enzyme [19]. According to Fig. 3, %sn-2 PA related to acyl migration decreased with the increase of reaction temperature. The degrees of similarity of fatty acid composition had an increasing–decreasing trend at the reaction temperatures of 40, 50 and 60 °C as a function of reaction time. However, for reactions at 70 °C, their maximum point was not found, which might occur before 0.5 h due to a higher reaction rate. The degrees of similarity of TAG composition of reactions at 40 °C could not achieve a great extent until the reaction time exceeded 4 h. However, for reactions at 50, 60 and 70 °C, they could have high degrees of similarity of TAG composition within 3 h. The degrees of similarity of reactions at 60 and 70 °C were similar, and both higher than these of reactions at 50 °C. Therefore, 60 °C was selected as the reaction temperature for subsequent reactions, and the optimal reaction time for reactions at this temperature was 3 h.

## Effect of Water Content

Water content in the reaction system affects the enzyme activity and the amounts of partial glycerides (DAGs and




◀**Fig. 1** Effect of blending ratios on % sn-2 PA (**a**) and degrees of similarity in sn-2 FA (**b**) and TAG (**c**) composition as a function of reaction time. Reaction conditions: temperature, 60 °C; enzyme load, 8 wt %; water content, 3.5 wt %. The values *60*, *65*, *70* and *75* % refer to % sn-2 PA which were obtained by blending lard with sunflower oil, canola oil, palm kernel oil, palm oil, algal oil and microbial oil, whose ratios were determined by the physical blending model (Eqs. 1–5). % sn-2 PA refers to the distribution of palmitic acid in the sn-2 position relative to the all three acylglycerol positions, and *sn-2 FA* to fatty acid in the sn-2 position

MAGs) [20], and thus influences the reaction rate and acyl migration. Higher water content would lead to higher amounts of partial glycerides and free fatty acids, and, when water content reaches a certain extent, the major reaction changes from esterification to hydrolysis. Therefore, in order to obtain products with high yield and quality, the water content in the system should be optimized. As seen in Fig. 4, the %sn-2 PA and degrees of similarity in fatty acid composition decreased with the increase of water content. However, for degrees of similarity of TAG composition, their variations were complicated, which was mostly caused by the variations of contents of partial glycerides at different water content levels. As the water contents increased, reactions at different water contents, except 3.5 wt%, had an increasing–decreasing trend, and, meanwhile, higher water contents led to lower degrees of similarity of TAG composition. For reactions at a water content of 3.5 wt%, the obtained product had the highest %sn-2 PA, degrees of similarity in sn-2 fatty acid, and TAG composition.

According to the reactions at conditions of water content of 3.5 wt%, temperature 60 °C, enzyme load 11 wt%, %sn-2 PA and degrees of similarity of fatty acid composition decreased with the increase of reaction time. After 3 h reaction, the %sn-2 PA was 63.0 %, higher than the required value (60 %), the degree of similarity of fatty acid composition was 89.8 %, and, most importantly, the degree of similarity of TAG composition achieved a high value, 71.9 %. Therefore, after overall consideration, 3 h was selected as the reaction time.

After optimization, the conditions selected for enzymatic preparation of HMFSs from lard based on their TAG composition were as follows: blending ratios, lard:SFO:CO:PKO:PO:AO:MO = 1.00:0.10:0.50:0.13:0.12:0.02:0.02; enzyme load, 11 wt%; temperature, 60 °C; water content, 3.5 wt%; reaction time, 3 h.

## Characterization and Similarity Evaluation of the Final Product

Under the optimized conditions, the fatty acid composition and distribution of the product are presented in Table 4, and TAG composition is shown in Table 5. Compared with the products before enzymatic interesterification, the content of sn-2 PA decreased from 42.5 to 38.2 %, and the content of sn-2 oleic acid increased from 26.1 to 28.6 %. However, their fatty acid composition was not obviously varied. In terms of TAG composition, the blending product contained more TAGs

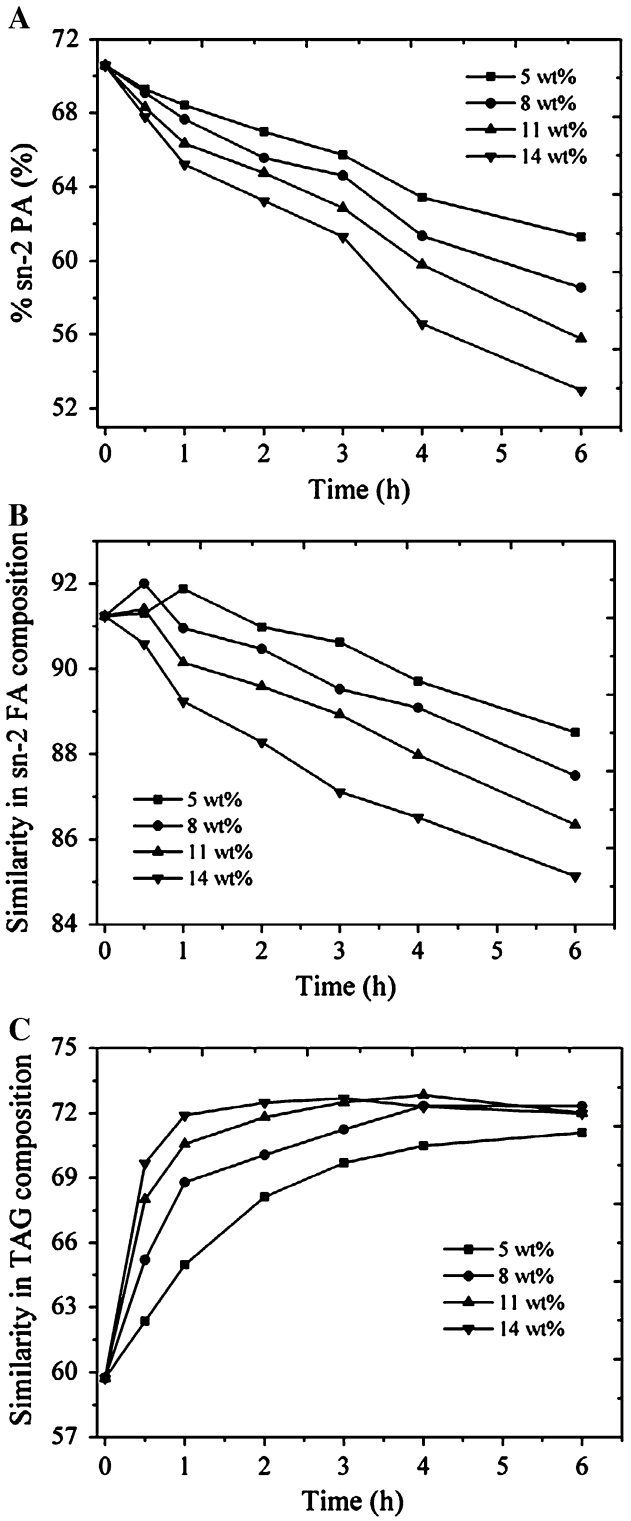



**Fig. 2** Effect of enzyme load on % sn-2 PA (**a**) and degrees of similarity in sn-2 FA (**b**) and TAG (**c**) composition as a function of reaction time. Reaction conditions: blending ratio, lard:SFO:CO:PKO:PO:AO: MO = 1.00:0.10:0.50:0.13:0.12:0.02:0.02; temperature, 60 °C; water content, 3.5 wt%. The original % sn-2 PA was 70 % under the blending ratio. For abbreviations, see Fig. 1

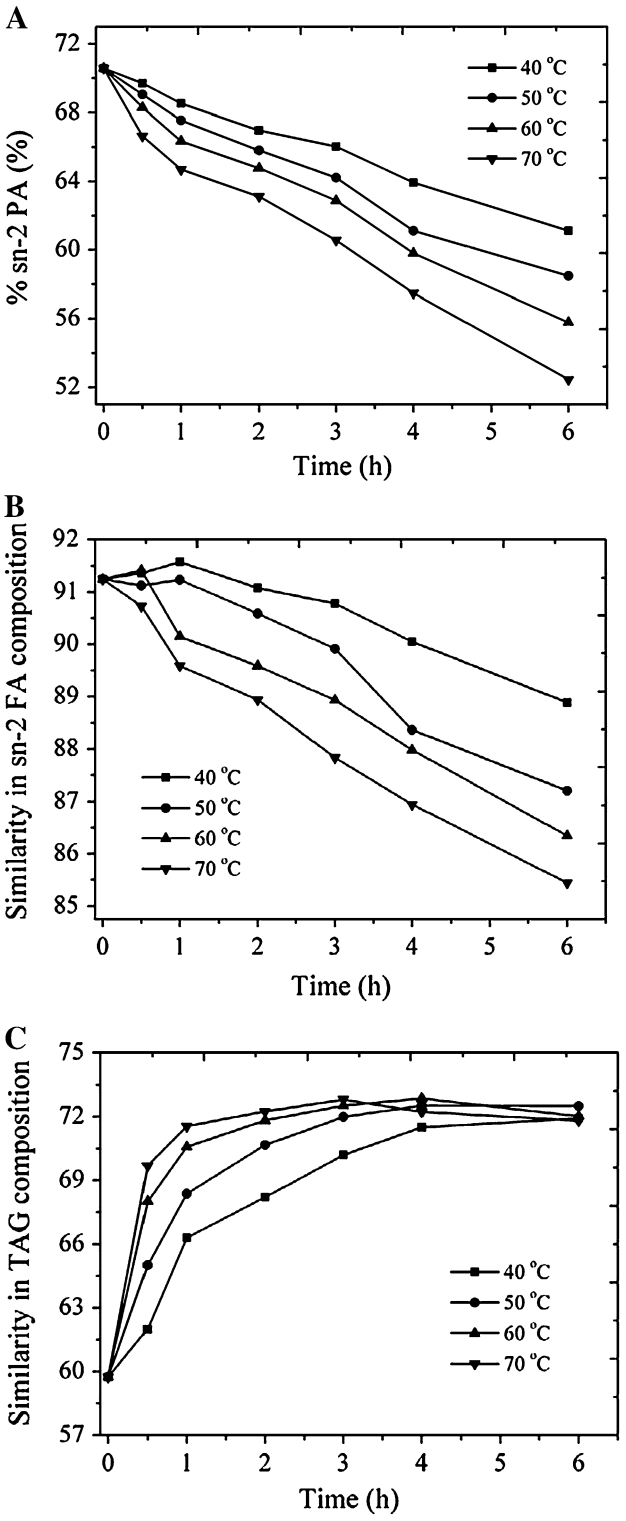



**Fig. 3** Effect of temperature on % sn-2 PA (**a**) and degrees of similarity in sn-2 FA (**b**) and TAG (**c**) composition as a function of reaction time. Oil mixture ratio and water content are those given in Fig. 2, except enzyme load (11 wt%). For abbreviations, see Fig. 1

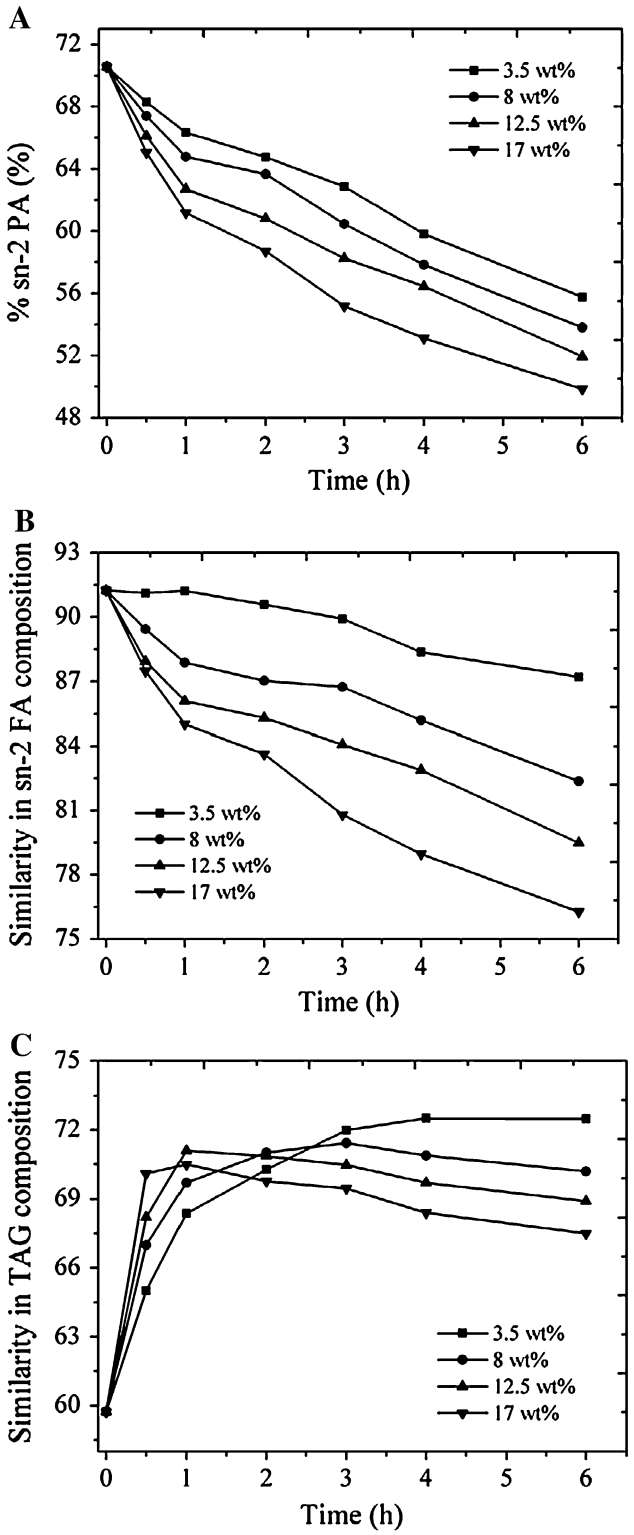



**Fig. 4** Effect of water content on %sn-2 PA (**a**) and degrees of similarity in sn-2 FA (**b**) and TAG (**c**) composition as a function of reaction time. Oil mixture ratio and temperature are those given in Fig. 2, except enzyme load (11 wt%). For abbreviations, see Fig. 1

**Table 4** The fatty acid composition and distribution of the final product under the optimized conditions

| Fatty acid (mol %) | Total[a] | sn-2 | % sn-2 | sn-1,3 |
|---|---|---|---|---|
| C8:0 | 0.2 | 0.1 | 16.7 | 0.3 |
| C10:0 | 0.2 | 0.1 | 16.7 | 0.3 |
| C12:0 | 3.4 | 3.1 | 30.4 | 3.6 |
| C14:0 | 2.1 | 2.6 | 41.3 | 1.9 |
| C16:0 | 20.1 | 38.2 | 63.3 | 11.1 |
| C16:1 | 1.8 | 1.1 | 20.4 | 2.2 |
| C17:0 | 0.2 | 0.1 | 16.7 | 0.3 |
| C17:1 | 0.1 | 0.1 | 33.3 | 0.1 |
| C18:0 | 6.7 | 2.4 | 11.9 | 8.9 |
| C18:1 | 42.4 | 28.6 | 22.5 | 49.3 |
| C18:2 | 18.2 | 19.9 | 36.4 | 17.4 |
| C18:3 | 2.5 | 2.3 | 30.7 | 2.6 |
| C20:0 | 0.3 | 0.1 | 11.1 | 0.4 |
| C20:1 | 0.4 | 0.3 | 25.0 | 0.5 |
| C22:0 | 0.2 | 0.1 | 16.7 | 0.3 |
| C20:3 | 0.1 | 0.1 | 33.3 | 0.1 |
| C20:4 | 0.5 | 0.2 | 13.3 | 0.7 |
| C20:5 | 0.2 | 0.1 | 16.7 | 0.3 |
| C22:5 | 0.1 | 0.1 | 33.3 | 0.1 |
| C22:6 | 0.3 | 0.4 | 44.4 | 0.3 |

The optimized conditions were blending ratios, lard:sunflower oil:canola oil:palm kernel oil:palm oil:algal oil:microbial oil = 1.00 :0.10:0.50:0.13:0.12:0.02:0.02; enzyme load, 11 wt%; temperature, 60 °C; water content, 3.5 wt%; reaction time, 3 h

[a] Abbreviations are given in Table 1

with unsaturated fatty acids such as LLL, OLLn, OLL, OOL and OOO, compared with these of lard, which were derived from the added vegetable oils. After enzymatic interesterification, the TAG composition of the product was greatly varied. The contents of TAGs such as OLL, OOO, PPP and POS decreased from 5.1, 14.8, 1.4 and 10.5 to 3.4, 10.2, 0.4 and 4.7 %, respectively, while the contents of TAGs such as PLL, POLn, POL and SOO increased from 2.7, 0.1, 11.9 and 1.6 to 5.2, 2.3, 17.6 and 2.8 %, respectively.

The degrees of similarity of the products before and after enzymatic intereterfication to HMF were evaluated by the established model by employment of four indexes, namely, fatty acid composition and distribution and PUFA and TAG compositions, which are shown in Table 6. The degrees of similarity in total and sn-2 fatty acid and PUFA composition were slightly decreased after enzymatic interesterificaiton, whereas the degree of similarity in TAG composition was greatly increased from 59.7 to 71.9, which increased the overall similarity of the product to HMF.

**Table 5** TAG composition of lard, blending oils and final product under the optimum conditions

| TAG[a] | Lard | Blending oils | Final product |
|---|---|---|---|
| LLL | | 1.5 | 0.8 |
| OLLn | | 1.5 | 1.7 |
| PLLn | | | 0.6 |
| OLL | 1.1 | 5.1 | 3.4 |
| OOLn | | 2.8 | 1.0 |
| PLL | 2.0 | 2.7 | 5.2 |
| POLn | | 0.1 | 2.3 |
| OOL | 3.3 | 12.0 | 12.5 |
| POL | 16.2 | 11.9 | 17.6 |
| PPL | 1.2 | 1.1 | 3.2 |
| OOO | 3.7 | 14.8 | 10.2 |
| POO | 36.7 | 24.6 | 26.0 |
| PPO | 11.4 | 8.1 | 7.2 |
| PPP | 0.2 | 1.4 | 0.4 |
| SOO | 2.7 | 1.6 | 2.8 |
| POS | 20.8 | 10.5 | 4.7 |
| PSS | 0.6 | 0.2 | 0.3 |

*P* palmitic acid, *S* strearic acid, *O* oleic acid, *L* linoleic acid, *Ln* linolenic acid

**Table 6** Similarity evaluation (Eqs. 6–8) of the products before and after enzymatic interesterification

| Similarity | Blending oils before interesterification | Blending oils after interesterification[a] |
|---|---|---|
| $G_{FA}^{b}$ | 93.8 | 92.5 |
| $G_{sn\text{-}2FA}$ | 91.2 | 90.3 |
| $G_{PUFA}$ | 62.1 | 61.5 |
| $G_{TAG}$ | 59.7 | 71.9 |

[a] Interesterificaiton reactions were carried out under optimized conditions as described in Table 4

[b] $G_{FA}$, $G_{sn\text{-}2FA}$, $G_{PUFA}$ and $G_{TAG}$ indicate the degree of similarity of HMFSs to HMF in the aspect of total fatty acid composition, % sn-2 PA, PUFA and TAG composition, respectively

## Conclusions

The preparation of HMFSs based on TAG profiles was achieved by a two-step method, namely, physical blending of different oils with lard to adjust the fatty acid profiles, and enzymatic interesterificaiton to increase their degree of similarity in TAG composition. The optimized conditions obtained for both steps were blending ratios, lard:sunflower oil:canola oil:palm kernel oil:palm oil:algal oil:microbial oil = 1.00:0.10:0.50:0.13:0.12:0.02:0.02; enzyme load, 11 wt%; temperature, 60 °C; water content, 3.5 wt%; reaction time, 3 h. The obtained product had high degrees of similarity in total and sn-2 fatty acid, PUFA and TAG composition with the values of 92.5, 90.3, 61.5 and 71.9, respectively. This process for HMFS preparation has advantages of low cost and high yield, and the product has high similarity to HMF in TAG profiles, indicating the potential for industrialization.

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