GRASAS Y ACEITES 74 (1)
January-March 2023, e495
ISSN-L: 0017-3495
https://doi.org/10.3989/gya.0444211

Preparation of human milk fat substitute and improvement of its oxidative stability

H.A. Liu[a,b,*], J.Y. Huang[b*], T.M. Olajide[c], T. Liu[c], Z.M. Liu[a], X.Y. Liao[b,✉] and X.C. Weng[b,c,✉]

[a]State Key Laboratory of Dairy Biotechnology, Shanghai Engineering Research Center of Dairy Biotechnology, Dairy Research Institute, Bright Dairy & Food Co., Ltd., Shanghai, 200436, People's Republic of China

[b]School of Life Sciences, Shanghai University, 351, Nanchen Road, Shanghai, 200444, People's Republic of China

[c]School of Environmental and Chemical Engineering, Shanghai University, 351, Nanchen Road, Shanghai, 200444, People's Republic of China

*These authors contributed equally to this work.
✉Corresponding authors: wxch@staff.shu.edu.cn; xyliao@shu.edu.cn.

Submitted: 12 April 2021; Accepted: 26 May 2022; Published online: 24 March 2023

SUMMARY: 1,3-Dioleoyl-2-palmitoylglycerol (OPO) was synthesized by enzymatic interesterification using palm stearin rich in tripalmitin (PPP) and ethyl oleate. Enzymatic interesterification parameters such as temperature, water content, enzyme load, and substrate molar ratio were optimized. High contents of C52 (primarily OPO and its isomeric compounds) production (46.7%) and *sn*-2 palmitic acid (PA) content of 75.3% were detected. In addition, OPO-human milk fat substitute (HMFS) was blended with coconut, soybean, algal and microbial oils at a weight ratio of 0.70:0.18:0.11:0.004:0.007 to simulate fatty acids in human milk fat (HMF) according to the mathematical model. The main and important fatty acids in the Final-HMFS were within the ranges of those present in HMF. The Final-HMFS could promote the absorption of fats and minerals and the development of retina tissues in infants. The mixture of L-ascorbyl palmitate (L-AP) and vitamin E (VE) resulted in a synergistic antioxidant effect both in OPO-HMFS and OPO-HMFS emulsions. This finding has great significance in improving the quality and extending shelf-life of HMFS.

KEYWORDS: *1,3-dioleoyl-2-palmitoylglycerol; Enzymatic interesterification; Human milk fat substitutes; Oxidative stability; Physical blending.*

RESUMEN: ***Preparación de sustitutos de grasa de leche humana y mejora de su estabilidad oxidativa.*** Se sintetizó el 1,3-dioleoil-2-palmitoilglicerol (OPO), utilizando estearina de palma rica en tripalmitina (PPP) y oleato de etilo, mediante interesterificación enzimática. Se optimizaron los parámetros de la interesterificación enzimática, como la temperatura, el contenido de agua, la carga de enzimas y la relación molar del sustrato. Se lograron altos rendimientos de C52 (principalmente OPO y sus isómeros, 46,7%) y un contenido de ácido palmítico (PA) en *sn*-2 del 75,3%. Además, el sustituto graso de leche humana OPO (HMFS), se mezcló con aceites de coco, soja, algas y microbianos, en una proporción en peso de 0,70:0,18:0,11:0,004:0,007 para simular los ácidos grasos de la leche humana (HMF) de acuerdo con un modelo matemático. Los ácidos grasos principales e importantes en HMFS-Final estaban casi dentro de los rangos de los presentes en HMF. El HMFS-Final podría promover la absorción de grasas y minerales y el desarrollo de los tejidos de la retina en los bebés. La mezcla de palmitato de L-ascorbilo (L-AP) y vitamina E (VE) resultó tener un efecto antioxidante sinérgico, tanto en la emulsión OPO-HMFS como en la OPO-HMFS. Este hallazgo tiene una gran importancia para mejorar la calidad y prolongar la vida útil de HMFS.

PALABRAS CLAVE: *1,3-dioleoil-2-palmitoilglicerol; Estabilidad oxidativa; Interesterificación enzimática; Mezcla física; Sucedáneos de grasa de leche humana.*

Citation/Cómo citar este artículo: Liu HA, Huang JY, Olajide TM, Liu T, Liao XY, Weng XC. 2023. Preparation of human milk fat substitute and improvement of its oxidative stability. *Grasas Aceites* **74** (1), e495. https://doi.org/10.3989/gya.0444211

1. INTRODUCTION

Human milk is recognized as the gold standard of infant nutrition because it provides not only the energy and optimum balanced nutrition for growing infants, but also immunological protection to newborns (Wang *et al*., 2019). A population-based birth cohort study of neonates from Brazil showed that the durations of breastfeeding positively correlated with the intelligence quotient (IQ), educational attainment, and income of adults (Victoria *et al*., 2015). Thus, breastfeeding has not only clear short-term benefits, but also influences long-term consequences on human capital. However, the rate of breastfeeding continues to decline because of medical or personal reasons. In these situations, some infants are partly or even fully fed infant formulas. Due to the demand for high-quality infant formulas in recent years, extensive simulation studies on the composition and distribution of HMF fatty acids have been undertaken.

Human milk contains about 2 to 6% fat, of which more than 98% is in the form of triacylglycerols (TAGs), providing approximately 50–60% of the dietary energy required for infants (Wang *et al*., 2019). Commercial infant formulas are usually manufactured from cow milk and vegetable oils which may have similar fatty acid (FAs) compositions to human milk, but quite different position distribution in TAGs. In human milk, saturated fatty acids (SFAs), predominantly PA (20-30% of total FA), are largely located at the sn-2 position (70% of all PA); whereas the sn-1 and sn-3 positions are occupied by unsaturated fatty acids (USFAs), such as oleic acid (OA) (Ghide and Yan, 2021). Thus, the characteristic component of human milk TAGs is OPO. However, cow milk and vegetable oils generally contain SFAs esterified to the sn-1 and sn-3 positions and USFAs at sn-2 position. These differences not only affect the nutrition and digestion of fats, but also cause stool hardness and constipation due to the preferential hydrolysis of PA in the sn-1,3 positions by pancreatic lipase to form calcium soap which is water-insoluble and can hardly be absorbed in the intestine of babies (Lee and Chang, 2021). In contrast, when PA is esterified to the sn-2 position, the 2-monoacylglycerol formed is readily absorbed (Ghide and Yan, 2021), therefore, the kinetics of hydrolysis of different TAGs may induce a different digestive process.

The synthesis of OPO mainly takes place in two steps: the first step is the synthesis of TAGs enriched in PA at position 2 by chemical or enzymatic methods. Zou *et al*. (2012b) produced TAGs enriched in PA at position 2 by chemical interesterification of palm stearin and sodium methoxide as catalyst. Jimenez *et al*. (2010) synthesized TAGs enriched in PA at position 2 by enzymatic interesterification of palm stearin and Novozym 435 as catalyst. In the second step, OPO was obtained by the synthesis of TAGs enriched in PA at position 2 with acyl donors using sn-1,3 regiospecific lipase. Esteban *et al*. (2011) obtained TAGs with 66% PA at sn-2 position and 67.5% OA at sn-1,3 positions, by acidolysis of PA-enriched TAGs and several OA-rich free fatty acid fractions in solvent-free media at 50 °C. Besides, OPO was also obtained by enzymatic interesterification. For instance, Lee *et al*. (2010) synthesized a high OPO content (31.43%) from a PPP-rich fraction and ethyl oleate by a lipase-catalyzed interesterification.

HMF contains long-chain polyunsaturated fatty acids (LC-PUFAs) such as docosahexaenoic acid (DHA, 0.2~0.4 mol%, Table 3), arachidonic acid (ARA, 0.3~0.6 mol%, Table 3), which are crucial for infant growth and development (Qin *et al*., 2014, Haddad *et al*., 2012). Specifically, DHA and ARA are present in large amounts in the membranes of the brain and retina (Hoffman *et al*., 2009). Appropriate amounts of these fatty acids play a complex role in the development and function of the neuronal and retinal tissues after birth (Hoffman *et al*., 2009). They can be synthesized from linolenic and linoleic acid precursors by adults but infants lack this capacity and for this reason an adequate supply of LC-PUFAs are recommended in infant formulas (Ab *et al*., 2017).

When preparing HMFS products, the oxidative stability of HMFS must be acceptable during processing and storage. Some unsaturated fatty acids in HMFS, especially DHA and ARA, are easily oxidized to form lipid hydroperoxides and other small oxidation compounds that produce off-flavors via further decomposition. Researchers have reported that the ingestion of a diet high in polyunsaturated fatty acids which are insufficiently protected by antioxidants may increase the risk of atherosclerosis (Sakai *et al*., 1995). Oxidation reduces the nutritional value and safety of HMFS and in order to prevent the oxidation of oil, different methods have been developed to protect against the lipid oxidation effect, although adding antioxidants remains the most effective one.

The aim of this work was to produce HMFS containing OPO and LC-PUFAs, whose structures and contents are highly similar to HMF's. The first step involved enzymatic interesterification of the PPP-rich fraction with ethyl oleate to prepare OPO-HMFS and the second step involved the blending of the OPO-HMFS with other oils in order to adjust the contents of LC-PUFAs to simulate the fatty acid profiles in HMF based on some mathematical model. In order to improve the yield of OPO and reduce production costs, the interesterification reaction conditions were optimized and a linear restraint function was established to calculate the physical mixing ratio of oils in the second step. Improvement in the oxidative stability of HMFS-Emulsion by adding antioxidants was also achieved.

2. MATERIALS AND METHODS

2.1. Chemicals and enzymes

Lipozyme RM IM (*Rhizomucormiehei* immobilized in an ion exchange resin) and Lipozyme TL IM (*Thermomyces lanuginosus* immobilized on a silica support) were purchased from Novozymes A/S (Bagsvaerd, Denmark). Pancreatic lipase powder was purchased from Sigma-Aldrich (Shanghai, China). Palm stearin (PS), coconut oil (CO) and soybean oil (SO) were donated by Shanghai Kerry Oils & Grains Industries Co., Ltd. (Shanghai, China). Algal Oil (AO) and microbial oil (MO) were purchased from Fuxing Biotechnology Co., Ltd. (Wuhan, Hubei). The fatty acid profiles of CO, SO, MO and AO are shown in Table 1. Analytical or chromatographic-grade reagents were from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Other reagents were purchased from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China).

2.2. Preparation of PPP-rich fraction

Palm stearin was mixed with acetone (1:5 w/v) and placed in a vacuum drying oven at 30 °C for 4 h. The PPP-rich fraction was collected after filtration and the removal of acetone was carried out in a vacuum-rotary evaporator at 60 °C and 1.33 × 103 Pa for 1 h (Zou *et al*., 2012b). The percentage of PPP in the sample increased from 52.8 to 85.4%.

2.3. Lipase-catalyzed synthesis of OPO

The synthesis of OPO was carried out in a solvent-free system. The PPP-rich fraction was mixed acyl (oleic acid or methyl oleate or ethyl oleate) donors at substrate molar ratios of 1:8 and then lipases (10% based on the weight of the reaction mixture) were add-

Table 1. Fatty acid profiles (%) of coconut, soybean, algal, microbial oil

Fatty acid	CO		SO		AO		MO	
	total	sn-2	total	sn-2	total	sn-2	total	sn-2
C8:0	7.9±0.4	1.6±0.1	-	-	-	-	-	-
C10:0	8.1±0.3	3.0±0.1	-	-	-	-	-	-
C12:0	48.9±0.7	80.5±0.9	-	-	-	-	-	-
C14:0	18.4±0.6	8.2±0.6	-	-	8.7±0.4	15.1±0.6	-	-
C16:0	8.3±0.6	1.1±0.1	11.8±0.2	1.4±0.1	32.7±0.6	19.1±0.4	9.2±0.1	6.5±0.5
C18:0	2.2±0.1	-	3.3±0.1	-	0.7±0.5	1.1±0.2	5.1±0.3	4.4±0.5
C18:1	4.7±0.2	4.4±0.2	21.3±0.4	22.8±0.5	1.4±0.3	4.7±0.2	11.7±0.4	17.9±0.6
C18:2	1.5±0.1	1.2±0.1	54.8±0.3	70.6±0.8	0.4±0.1	-	9.4±0.4	15.6±0.6
C18:3	-	-	7.7±0.2	5.2±0.2	0.3±0.1	0.3±0.1	5.1±0.3	6.7±0.2
C20:0	-	-	0.3±0.1	-	0.4±0.2	-	6.6±0.1	-
C20:4n-6	-	-	-	-	-	-	48.1±0.6	45.6±0.8
C22:0	-	-	0.8±0.1	-	-	-	4.8±0.2	3.3±0.1
C22:5n-3	-	-	-	-	7.1±0.2	8.1±0.2	-	-
C22:6n-3	-	-	-	-	48.3±0.7	51.6±0.9	-	-

-: compound not detected because its content was too low. CO: Coconut Oil; SO: Soybean Oil; AO: Algal Oil; MO: Microbial Oil. Results are expressed as mean ± standard deviation (n = 2)

ed to start the reaction in a shaking water bath at 55 °C and stirred at 200 rpm. The synthesis conditions were selected on the basis of previous research presented in this paper.

2.4. Fatty acid composition analysis

The fatty acid composition of the products was analyzed through GC-MS using a Shimadzu GC2010A (Kyoto, Japan) gas chromatography instrument coupled with a GCMS-QP2010 quadrupole mass spectrometer (Shimadzu) and a Rtx®-Wax capillary column, 30 m length, 0.25 mm i.d and 0.25 μm film, consisting of cross-bond polyethylene glycol (Restek). The GC analysis method was reported by Zou *et al*. (2012a).

2.5. Analysis of C52 content by GC-FID

C52, PPP and diacylglycerols (DAGs) in the reaction products were detected through GC using an Agilent 8890A GC platform equipped with a flame ionization detector and a fused silica capillary column DB-1HT (15 m length × 0.25 mm internal diameter × 0.25 μm film thickness, Agilent, Santa Clara, USA). The GC analysis method was reported by Liu *et al*. (2020).

2.6. Sn-2 Positional analysis of TAGs by pancreatic lipase

The hydrolysis of TAGs and determination of FA composition and sn-2 FA composition in TAGs were carried out according to the method described by Lee *et al*. (2010).

2.7. Molecular distillation

KDL1, UIC GmbH (Alzenau, Germany) was used to purify TAGs in the products under the following conditions: the operating pressure was set at 1×10^{-3} mbar, the rotor velocity at 300 min^{-1}, and the feed flow rate at 1.5 mL/min (feed temperature was 60 °C). When the heating oil temperature was increased to 210 °C, ethyl oleate, DAGs and free fatty acids were separated from the product.

2.8. OPO-HMFS blending with selected oils to prepare Final-HMFS

OPO-HMFS TAGs are the main components in the fat in infant formula, and are rich in OA and PA. Nevertheless, the contents in other major fatty acids, such as linoleic acid, lauric acid and some LC-PUFAs should also be within the ranges of their corresponding fatty acids in HMF. According to the fatty acid composition and distribution of typical human milk fat, a linear restraint function for the development of HMFS formula was established as follows:

$$\sum_{i=1}^{5} x_i = 1 \quad (i = 1, 2, 3, 4, 5) \quad (1)$$

$$\sum_{i=1}^{5} a_{i1} x_i < 20 \quad (2)$$

$$19.6 < \sum_{i=1}^{5} a_{i2} x_i < 29.0 \quad (3)$$

$$26 < \sum_{i=1}^{5} a_{i3} x_i < 40.6 \quad (4)$$

$$7.1 < \sum_{i=1}^{5} a_{i4} x_i < 20.1 \quad (5)$$

$$0.5 < \sum_{i=1}^{5} a_{i5} x_i < 3 \quad (6)$$

$$0.2 < \sum_{i=1}^{5} a_{i6} x_i < 0.4 \quad (7)$$

$$0.35 < \sum_{i=1}^{65} a_{i7} x_i < 0.6 \quad (8)$$

$$5 < \left[\sum_{i=1}^{5} a_{i4} x_i\right] : \left[\sum_{i=1}^{5} a_{i5} x_i\right] < 15 \quad (9)$$

$$y = c_2 x_2 + c_3 x_3 + c_4 x_4 + c_5 x_5 \quad (10)$$

i is the serial number of the selected oils and x_1, x_2, x_3, x_4 and x_5 represent the OPO-HMFS, coconut oil, soybean oil, algal oil and microbial oil, respectively; a_{i1}, a_{i2}, a_{i3}, a_{i4}, a_{i5}, a_{i6} and a_{i7} are the contents in lauric acid (C12:0)+myristic acid (C14:0), palmitic acid (C16:0), oleic acid (C18:1n-9), linoleic acid (C18:2n-6) , Linolenic acid (C18:3n-3), docosahexaenoic acid (DHA) and arachidonic acid (ARA) in the selected oils, respectively. c_2, c_3, c_4 and c_5 represent the marketing cost of coconut, soybean, algal and microbial oils, respectively.

2.9. Oil properties analysis

2.9.1 DSC melting profile

The melting profiles of oil samples were determined by differential scanning calorimetry (DSC) using a DSC Q2500 according to the method Cj1-94 (AOCS, 2009).

*2.9.2 Acid value (AV), peroxide value (PV) and p-anisidine value (*p*-AV)*

The AV, PV and *p*-AV were determined according to methods 5a-40 Cd, 8b-90 and p 2.4 (AOCS, 1997), respectively.

2.9.3 Induction period (IP)

The oxidative stability was determined in terms of induction period (IP) using the Rancimat method (Olajide *et al*., 2020). 3 Grams of sample with and without added antioxidants were carefully weighed into Rancimat tubes and subjected to accelerated oxidation at 100 °C under an air flow of 20 L/h.

2.9.4 Emulsion preparation and oven test

For convenience, infant formula liquid milk is also popular in the market. Most papers only tested the oxidative stability of HMFS itself, but did not evaluate the oxidation stability of HMFS in emulsion. HMFS-in-water emulsions (500 g) were prepared as mixtures of purified HMFS, Tween 80 and a phosphate buffer solution (pH 7.0) according to methods described by Chen *et al*. (2020). These samples were divided into experimental groups (0.02% antioxidants added) and a control group (no antioxidant added). Particle size distributions of the emulsions were measured by Mastersizer 2000 (triplicate, at room temperature) according to the method by Zou *et al*., (2012a). The average emulsion droplet size 14.90±0.76 nm was similar to HMF, and there was no visible significant change in the state of the emulsions over the course of the oven test. The emulsions were held in an oven at 63 ± 1 °C and the induction period of the samples was dependent on the oil reaching a peroxide value of 80 meq O_2/kg oil (Shi *et al*., 2017; Olajide *et al*., 2020).

2.10. Statistical analysis

All assays were carried out in duplicate or triplicate and the data are presented as mean ± standard deviation (SD). Analysis of variance (ANOVA) was carried out using IBM SPSS 22.0, followed by Duncan's multiple range test ($P < 0.05$).

3. RESULTS AND DISCUSSION

3.1. Influence of reaction time and acyl donors on the contents in C52 and *sn*-2 PA

The whole process for obtaining OPO consisted of two reactions: first, obtaining TAGs rich in tripalmitin (PPP) from palm stearin and second, obtaining OPO by interesterification or acidolysis of TAGs enriched in PA at position 2 and acyl (oleic acid or methyl oleate or ethyl oleate) donors, catalyzed by sn-1, 3 specific lipases.

Acyl donors are important raw materials for the preparation of OPO. Srivastava, *et al*., (2006) reported that methyl ester is a better acyl donor than free acid for producing HMFS. Three kinds of oleic acyl donors (oleic acid, methyl and ethyl oleate) were compared to investigate their influence on the contents in C52 and sn-2 PA in our study. The initial rate of C52 synthesis using methyl or ethyl oleate with TAGs rich in PPP was higher than the oleic acid tested (Figure 1A). This was mainly due to better solubility of methyl or ethyl oleates in solvent-free systems than oleic acid. It should be noted that PPP with a high melting point (about 60 °C) hardly melt into liquid at the optimal reaction temperature of lipase, so organic solvent must always be added to the reaction system to reduce the PPP dissolution temperature and the viscosity of the reaction system so as to ensure the high activity of the lipase. However, large scale use of solvents may bring potential safety hazards. Methyl or ethyl oleates can act as both substrate and solvent to accelerate the dissolution of PPP at appropriate temperatures. There

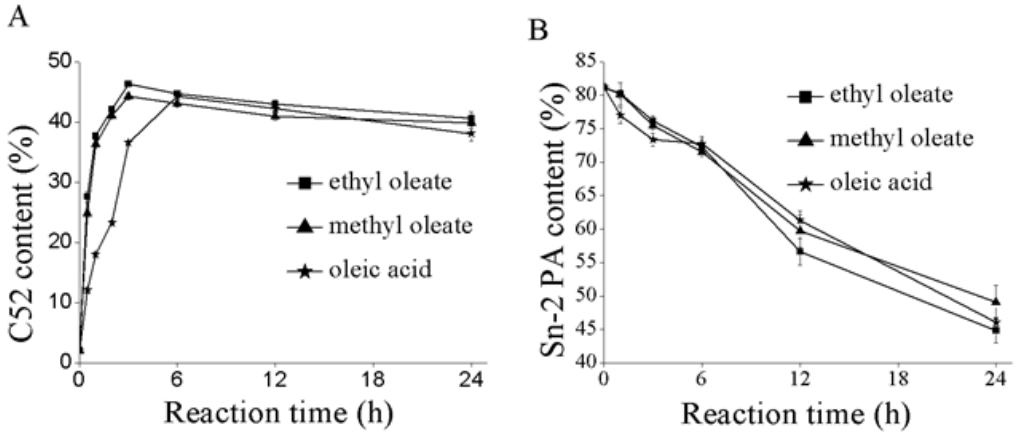

FIGURE 1. Influences of reaction time and acyl donors on the content in C52 (A) and sn-2 PA (B) using sn-1,3 specific lipases. Values are mean ± standard deviation (n=2).

was no significant difference in the initial rate of C52 synthesis between methyl or ethyl oleate use. Ethyl oleate was eventually selected because ethanol hydrolyzed by ethyl oleate in the interesterification reaction is much safer compared to methanol. Moreover, the boiling point of ethyl oleate (220 °C) is lower than that of oleic acid (241 °C) at the same pressure, hence easier to remove during the purification of OPO. When the heating oil temperature of molecular distillation was increased to 210 °C, the by-products of interesterification reaction including DAGs, MAGs and ethyl oleate were almost completely removed. The purified product did not contain ethyl oleate and only a small amount of DAGs (1.6 ± 0.2%, Table 2). The C52 content increased with time, but was practically constant from 6 h and its maximum content of over 40% was similar to all the acyl donors tested.

The content in PA at the sn-2 position decreased with the extension of reaction time (Figure 1B). Although Lipozyme RM IM exhibited the sn-1, 3-specificity, TAG with OA at the sn-2 position was possible to produce due to acyl-migration in the form of DAGs. DAGs are inevitable intermediates in lipase-catalyzed interesterification and they could be converted from 1,2-DAGs and 1,3-DAGs (Xu *et al*., 1998). The thermal instability of these DAGs may lead to acyl transfer as reaction time increases, reducing the yield of OPO (Xu *et al*., 1998). The percentage content of PA in the sn-2 position, determined by GC analyses, represents acyl migration. The acyl migration rates of oleic acid, methyl oleate and ethyl oleate in 3 h were 7.8, 5.7 and 5.1%, respectively. Compared to oleic acid, the PA content at the sn-2 position decreased more slowly in a short reaction time (3 h) with ethyl oleate tested, thus showing a lower acyl migration rate, which may be related to the polarity difference between oleic acid and ethyl oleate (Figure 1B). Li *et al*. (2010) have reported that organic solvents with low polarity could inhibit the acyl-migration of sn-2 fatty acids in TAGs. To obtain a higher C52 and sn-2 PA contents in short reaction times, ethyl oleate was selected to be the most ideal acyl donor.

TABLE 2. The composition of fatty acids and acylglycerols of the OPO-HMFS under optimum conditions

Fatty acid (area%)	Total	Sn-2 position	Sn-1,3 position
C16:0	33.6±1.1	75.3±1.5	12.8
C18:0	6.9±1.3	11.1±1.1	4.7
C18:1	53.3±0.7	9.5±0.2	75.2
C18:2	6.2±1.2	4.1±1.1	7.3
Acylglycerols (area%)	C52	PPP	DAGs
	46.7±0.8	2.9±1.2	1.6±0.2

Results are expressed as mean ± standard deviation (n = 3). Operational conditions: temperature, 55 °C; substrate molar ratio, 1:10 (PPP-rich fraction/ ethyl oleate); enzyme load, 10 wt %; water content, none; reaction time, 3 h. C52: primarily OPO and its isomeric compounds; PPP: tripalmitin; DAG: diacylglycerols. Fatty acid content at sn-1,3 positions of TAGs is determined by equation:

$$\text{FA at sn} - 1,3 \text{ positions (mol\%)} = \frac{3 * \%\text{total FA} - \%\text{FA at sn} - 2 \text{ position}}{2}$$

3.2. Optimization of interesterification conditions

3.2.1. Effect of temperature on the contents of C52 and sn*-2 PA*

Higher temperature decreases the viscosity of substrates and increase mass transfer, which in turn reduce reaction time to equilibrium, but also enhance the rate of occurrence of acyl-migration and other negative impacts, such as fat oxidation. Previous studies show that substrate could be dissolved at low temperature in solvent system, which partitions in a more stable environment (Esteban *et al*., 2011; Wei *et al*., 2015). However, the addition of solvents is harmful to the environment and/or economy. Solvent-free is a much safer, much more environmentally-friendly and economical industrial application. Thus, all the experiments in this study were based on a solvent-free system.

The reaction accelerated as temperature increased from 50 to 55 °C and the C52 content increased with it (Figure 2A). However, the C52 content, when above 55 °C, changed only slightly. At 55 °C, the C52 content was 45.4% and PA content at the sn-2 position was 75.2% at the end of the 3-h reaction. Compared to other temperatures, the products at 50 °C were characterized by higher PA content (i.e. 77.0%) at the sn-2 position; while the C52 content was only 39.8%, which may be due to lower catalytic efficiency. Moreover, higher temperature could decrease the sn-2 PA content significantly and might cause adverse consequences on enzyme properties and so, 55 °C was selected as the most ideal reaction temperature.

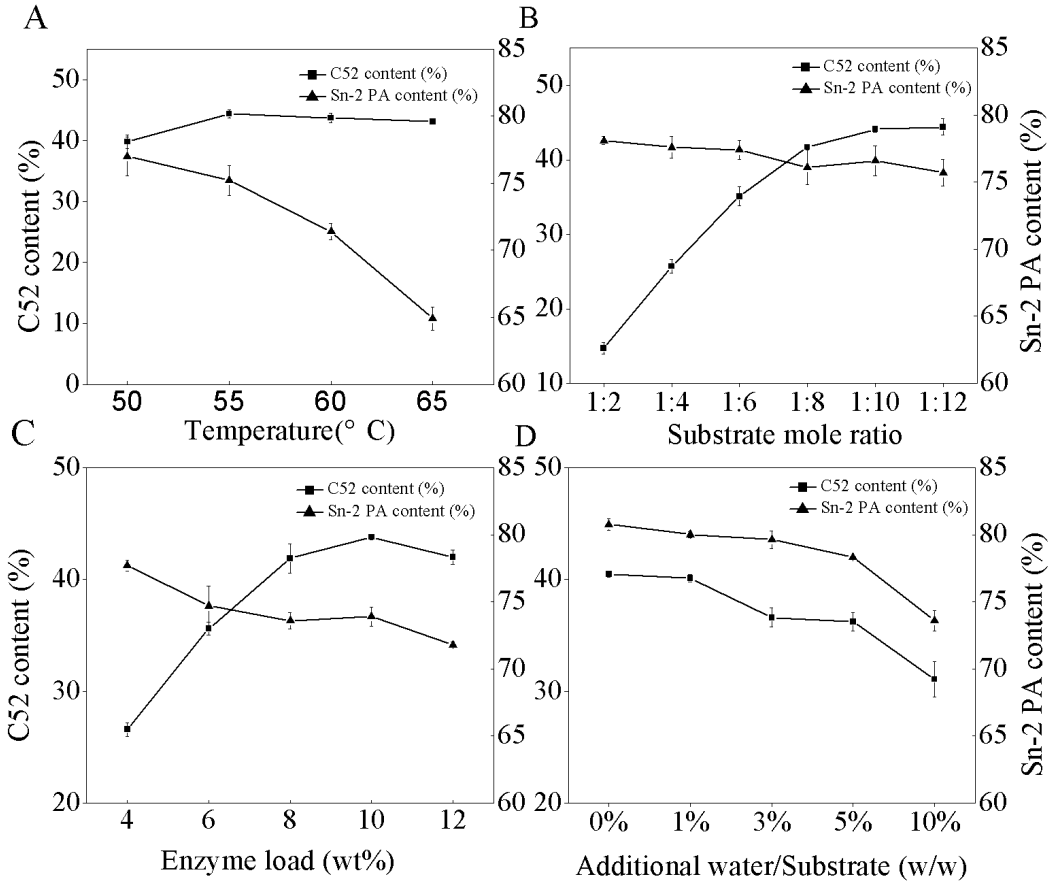

FIGURE 2. Influences of various parameters on the content in C52 and sn-2 PA in the interesterification of PPP-rich fraction with ethyl oleate using Lipozyme RM IM: (A) Effects of temperature. Reaction conditions: substrate molar ratio, 1:10 mol/mol; enzyme load, 10 wt %; 3h; water content, none. (B) Effects of substrate molar ratio. Reaction conditions: enzyme load, 10 wt %; 55 °C; 3 h; water content, none. (C) Effects of enzyme load. Reaction conditions: substrate molar ratio, 1:10 mol/mol; 55 °C; 3 h; water content, none (D) Effects of water content. Reaction conditions: substrate molar ratio, 1:10 mol/mol; enzyme load, 10 wt %; 3h. Values are mean ± standard deviation (n=2).

3.2.2 Effect of substrate ratio on the contents of C52 and sn-*2 PA*

According to the reaction formula, OPO can be synthesized under ideal conditions by maintaining the ratio of the PPP-rich fraction to ethyl oleate at 1:2. However, the interesteri- fication reaction is reversible, and the feedback regulation of the product will inhibit the reaction. To obtain the highest OPO yield, the effect of substrate ratio was compared in this experiment. From Figure 2B, the C52 content in the enzymatic products at the substrate ratios of 1:2, 1:4, 1:6, 1:8, 1:10 and 1:12 were 14.7, 25.7, 35.1, 41.7, 44.1 and 44.4%, respectively. The C52 content between the molar ratios of 1:10 and 1:12 were nearly identical, indicating that increasing the amount of ethyl oleate continuously would not necessarily improve the OPO yield. At the same time, using high amounts of ethyl oleate would mean a limitation for industrial production of OPO-rich HMFS. In addition, no significant difference in the sn-2 PA content with all the molar ratios tested indicated that the amount of ethyl oleate had very little influence on the acyl migration reaction. Thus, to improve catalytic and economic efficiency, an optimum molar ratio of 1:10 between the PPP-rich fraction and ethyl oleate is required.

3.2.3 Effect of enzyme load on the contents in C52 and sn-*2 PA*

Enzyme load is related to reaction rate. Higher enzyme load will accelerate the reaction rate and improve the incorporation rate of the acyl donor in the interesterification reaction. When the enzyme load was less than 10%, the content in C52 increased as enzyme load increased and reached its highest point (43.8%) at 10%, but later decreased as enzyme load increased (Figure 2C). Meanwhile, the content in sn-2 PA decreased slowly and continuously as the enzyme load increased. The sn-2 PA content was only 71.8% when the enzyme load reached 12%. These findings are understandable because the water content in the Lipozyme RM IM may have promoted the amount of DAG produced from TAGs, thereby enlarging the possibility of acyl-migration. An enzyme load of 10% (w/w) generated the C52 content (43.8%) and sn-2 PA content (73.9%) after 3 h, showing preferable yield and catalytic efficiency.

3.2.4 Effect of water content on the contents in C52 and sn-2 PA

Researchers have found that the water content in the synthetic reaction of TAG favors the content in DAG, which is due to hydrolytic side reactions (Zou *et al*., 2012b). Moreover, the presence of water on the surface of lipase is important for maintaining its activity and the flexibility of its protein structure. With increased water content in the system, the yields of C52 and sn-2 PA decreased consistently (Figure 2D). The content in C52 was 44.9% in the system without water, which was 3.6% higher than that in the system with 10% water content. Similarly, the content in sn-2 PA was 77.1% in the system without water, which was 7.8% higher than that in the system with 10% water content. A similar study was also reported in a study by Liu *et al*., (2020). The results indicated that high moisture in the reaction system can accelerate the hydrolysis of TAGs to DAGs and cause more acyl-transfer, reducing the content in sn-2 PA. It has been speculated that there are some hydrophilic groups on the surface of Lipozyme RM IM itself, which maintains its activity. As a result, the reaction was carried out in the absence of water.

Overall, the optimal OPO synthetic conditions were as follows: temperature, 55 °C; substrate molar ratio, 1:10 (PPP-rich fraction/ethyl oleate); enzyme load, 10 wt %; water content, none; reaction time, 3 h. Under these conditions, using molecular distillation, the composition of fatty acid and acyl glycerol of the OPO-HMFS are shown in Table 2. The OA and PPP contents in the OPO-HMFS were 53.3 and 2.9%, respectively, showing that OA was effectively incorporated into the substrate. A relatively small amount of PA on the TAGs migrated from the sn-2 to sn-1, 3 positions, since 75.3% of PA can be observed at the sn-2 position of OPO-HMFS.

3.3. OPO-HMFS blending with selected oils to prepare Final-HMFS.

Compared to the fatty acid composition and location distribution of HMF (PA, 19.6% - 29.0%; OA, 26.0% - 40.6%; linoleic acid, 7.1% - 20.1%; myristic acid+ lauric acid < 20.0%; linoleic acid/ linolenic acid, 5-15; DHA, 0.2%-0.4% and ARA, 0.3%-0.6%), the synthesized OPO-HMFS contained a smaller amount of linoleic acid and no medium-chain fatty acids or LC-PUFAs, but was characterized by much higher PA content (33.6%). In order

Table 3. Theoretical and Determined Values (area %) of the Final Product Obtained under Optimum Blending Conditions and Typical fatty acid composition (mol %) of human milk fat

Fatty acid	Theoretical values		Determined Values		HMF	
	total	sn-2	total	sn-2	total	sn-2
C8:0	1.6	1.3	2.0±0.1	1.2±0.2	0.1~0.6	0.0~0.7
C10:0	1.3	0.5	1.0±0.1	0.8±0.1	1.9~3.8	0.1~2.1
C12:0	8.1	10.8	9.6±0.4	12.5±0.1	1.0~9.4	0.9~13.2
C14:0	3.2	1.5	5.4±0.2	2.8±0.3	2.1~10.4	5.4~16.9
C16:0	26.4	49.0	26.2±0.9	47.9±0.5	19.6~29.0	42.5~59.9
C18:0	5.6	7.8	4.1±0.1	3.4±0.3	4.2~8.7	0.9~2.9
C18:1	39.6	17.2	39.5±0.7	20.0±0.6	26.0~40.6	5.4~18.4
C18:2	12.3	10.6	10.6±0.3	10.5±0.5	7.1~20.1	2.6~15.5
C18:3	1.2	0.7	0.8±0.3	0.3±0.1	0.5~3.0	0.1~1.8
C20:4n-6	0.4	0.3	0.4±0.0	0.3±0.1	0.3~0.6	0.0~1.8
C22:5n-3	0.1	0.1	0.1±0.0	0.1±0.0	0.0~0.3	0.0~0.7
C22:6n-3	0.2	0.2	0.3±0.0	0.2±0.1	0.2~0.4	0.5~1.5

Results are expressed as mean ± standard deviation (n = 3). Theoretical values: Calculated from Matlab R2018b software. Determined values: OPO-HMFS was blended with coconut, soybean, algal and microbial oils at a weight ratio of 0.70:0.18:0.11:0.004:0.007. HMF (human milk fat): Typical fatty acid composition (mol %) of human milk fat was adapted from previous studies (Qin *et al*., 2014, Haddad *et al*., 2012).

to add LC-PUFAs into infant formula, many studies used the acidolysis or interesterification method to prepare structural lipids containing LC-PUFAs, which was not efficient and thus increased production costs (Hoffman *et al*., 2009). Consequently, in our study, OPO-HMFS was physically blended with CO, SO, AO, and MO to adjust the contents in major and important fatty acids (ARA and DHA) to the proper ranges and maintain regional specificity of fatty acids like HMF.

Based on the fatty acid profiles of HMF, OPO-HMFS contained a smaller amount of linoleic acid (6.2±1.2%, Table 2) and no medium chain fatty acids or LC-PUFAs; therefore, CO, SO, AO, and MO, which are rich in lauric acid (48.9%), linoleic acid (54.8%), DHA (48.3%), and ARA (48.1%), respectively, were selected to blend with the OPO-HMFS to supplement the lack in fatty acids in OPO-HMFS (Tables 1 & 2). These selected oils are common in the market with low cost and convenient for industrialized production. To guarantee good quality and lower production costs of the Final-HMFS, Matlab R2018b was used to optimize the blending process. The final desirable formula constituted OPO-HMFS/CO/SO/AO/MO at a weight ratio of 0.70:0.18:0.11:0.004:0.007. The determined and theoretically evaluated fatty acid compositions and positional distributions of the final product under this blending ratio are shown in Table 3. The contents in PA and sn-2 PA were 26.2 and 47.9%, and the contents in LC-PUFA including ARA and DHA were 0.4 and 0.3%, respectively. The main and important fatty acids were almost within the ranges of these in HMF (Table 3), indicating that the fatty acid composition of the final product was highly similar to HMF and may be used as a fat substitute in infant formula. Besides, researches have reported differences between the fatty acid compositions of HMF, especially LC-PUFAs, at different stages of lactation (Sala-Vila *et al*., 2005; Haddad *et al*., 2012) and different proportions of fatty acids can also be formulated through the established model to meet the nutritional needs of infants in different stages of lactation.

3.4. Oil properties analysis

3.4.1. DSC melting curve

The melting curves of OPO-HMFS, Final-HMFS and palm stearin were determined by DSC. A lower melting temperature was observed for the OPO-HMFS product (18.1 °C) compared to palm stearin (57.6 °C), which contributed to the high content in OA and low content in PA in the product. The melting

temperatures of both OPO-HMFS and Final- HMFS (14.3 °C) were lower than the normal human body temperature (about 37.5 °C) depicting that they are suitable to be used as fat base materials in infant formulas. The melting range of HMFS is important for its digestion and absorption. The melting range of Final-HMFS was relatively narrower than that of OPO-HMFS (Figure 3), showing that the content in the high melting point component in OPO-HMFS was higher than that of Final-HMFS.

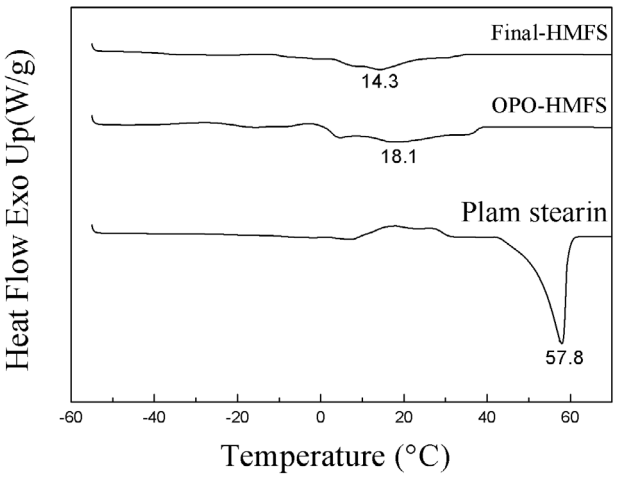

Figure 3. DSC Melting curves of OPO-HMFS, Final-HMFS and Palm stearin.

3.4.2. AV

Table 4 summarizes the basic properties of palm stearin, OPO-HMFS and Final-HMFS. TAGs hydrolyze to release free fatty acids during heating or the action of lipase, which influences the stability of oil. AV can be used to indicate the degree of oil hydrolytic rancidity and refining. The undetectable AV of OPO-HMFS showed that molecular distillation was an effective method to remove free fatty acid or fatty acid ethyl ester. The AV of the Final-HMFS was only 0.3 mg KOH/g, indicating that the selected oil blended with OPO-HMFS was of good product quality.

Table 4. AV (mg KOH/g), PV (meq O_2/Kg oil), *p*-AV and IP (h) of samples

Samples	Palm stearin	OPO-HMFS	Final-HMFS
AV	0.4±0.1	-	0.3±0.1
PV	3.8±0.2^{a}	1.1±0.3^{b}	3.4±0.4^{a}
p-AV	3.2±0.3^{a}	0.9±0.1^{b}	3.0±0.4^{a}
IP	16.8±0.7^{a}	4.1±0.9^{c}	6.3±0.6^{b}

-: compound not detected because its content was too low. Results are expressed as mean ± standard deviation (n = 3). Means in the same row with different letters are significantly different according to Duncan's multiple range test ($P < 0.05$). OPO-HMFS was synthesized using palm stearin enriched in tripalmitin and ethyl oleate by enzymatic interesterification under optimum conditions. Final-HMFS was obtained by blending OPO-HMFS with coconut, soybean, algal and microbial oils at a weight ratio of 0.70:0.18:0.11:0.004:0.007.

3.4.3. PV and p-*AV*

PV and *p*-AV were used to evaluate the degree of oil oxidation and deterioration. PV represents the main products of oil oxidation, like hydroperoxides, while *p*-AV represents the small molecular compounds such as aldehydes and ketones produced by further decomposition of peroxides. High PV and *p*-AV indicate high oxidation of oil samples. From the Table 4, the PV and *p*-AV of OPO-HMFS (1.1 meq O_2/kg oil and 0.9, respectively) were quite low compared with palm stearin (3.8 meq O_2/kg oil and 3.2, respectively), thus implying the purification process had also removed the majority of peroxides. The PV (3.4 meq O_2/kg oil) and *p*-AV (3.0) in Final-HMFS was higher than that in OPO-HMFS but it was still within an acceptable range.

3.4.4. Oxidative stability of HMFS

The rancimat assay characterizes the oxidative stability of oils. The oxidative stability indexes of palm stearin, OPO-HMFS and Final-HMFS were measured by the Rancimat test and expressed as IP (Table 4). Higher IP reflects a longer shelf-life of products. The IP value of the OPO-HMFS (4.1 h) and Final-HMFS (6.3 h) were lower compared to palm stearin (16.8 h). For one thing, USFAs in HMFS were more easily oxidized than SFAs in palm stearin; for another thing, the reason may be due to the loss in tocopherols and β-carotene in the process of refining OPO-HMFS by molecular distillation, thus reducing antioxidant capacity. The decrease in oxidative stability after molecular distillation is in agreement with several similar studies (Nielsen *et al*., 2006; Qin *et al*., 2014). Fat oxidative rancidity is due to the long-term storage of HMFS under unfavorable conditions, which usually requires some protection from antioxidants.

The efficacy of β-carotene, L-AP, VE and TBHQ (*tert*-butylhydroquinone) as antioxidants was eval-

uated using the Rancimat test at 100 °C, and concentrations at 0.02% (w/w) under air saturation conditions. The results were expressed as induction period (IP) corresponding to the oxidative stability of OPO-HMFS (Figure 4). The IPs of the blank, β-carotene, L-AP, VE and TBHQ were 3.8, 5.6, 7.8, 8.1, and 12.7 h, respectively. This indicates that the antioxidant activities of the antioxidants decreased as follows: TBHQ > VE ≈ L-AP > β-carotene. Although TBHQ has strong antioxidant activity compared to other antioxidants, it is a synthetic antioxidant. The existence of a double bond in β-carotene can prevent oil from being oxidized, but the effect is not profound. VE, which contains hydroxyl groups, can inhibit lipid oxidation by capturing free radicals, which is a natural antioxidant and a nutritional component contained in vegetable oil. L-AP is the only unnatural antioxidant allowed to be added to baby food in China. In some instances, combined

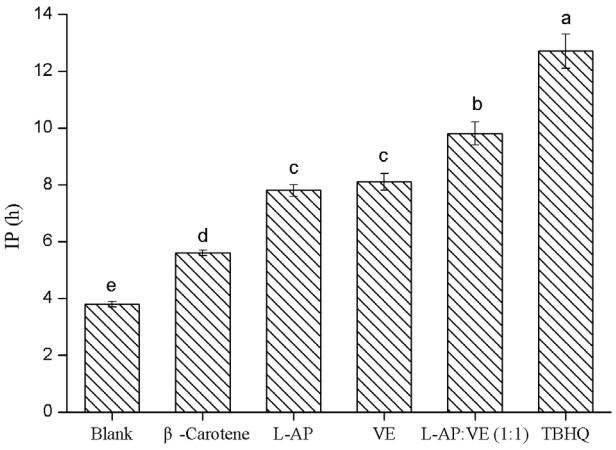

Figure 4. IPs of OPO-HMFS spiked with 0.02% (w/w) of antioxidants and without antioxidant. Values are mean ± standard deviation (n=3). Different letters are significantly different at P < 0.05 according to Duncan's test.

antioxidants have greater antioxidant activities than their individual effects. The IP of the mixture of VE and L-AP (at a weight ratio of 1:1) is 9.8 h, which is higher than that of VE or L-AP alone, indicating that L-AP has a synergistic effect with VE. When VE and L-AP are used together, L-AP could regenerate the phenoxyl radical of VE back to active VE. The synergistic effect of VE and L-AP cannot only enhance the oxidative stability of OPO-HMFS, but also reduce cost because L-AP is low-priced.

3.4.5. Oxidative stability of OPO-HMFS-Emulsion in oven test

HMFS are widely used in emulsions because of low water solubility. Emulsion can be used as an excellent carrier for wrapping, protecting and transporting HMFS, so that it can reach the designated position in the gastrointestinal tract digestion process (Hu, *et al*., 2003). Nevertheless, the oxidation of HMFS in emulsion is inevitable. In this study, PV was used to evaluate the oxidative stability of OPO-HMFS-Emulsion during the oven test at 63 ± 1, for 21 days. As shown in Figure 5, the PV of the blank group was 2.7±0.5 meq/kg on the first day, which then began to accelerate until it reached a maximum of 118.6±1.9 meq/kg after 21days of storage. In contrast, the PV of HMFS containing the TBHQ, VE, L-AP, mixture of VE and L-AP increased slightly until day 9, and then gradually increase dynamically with storage time. The changes in PV in the samples spiked with TBHQ, VE and L-AP showed that the addition of antioxidants was better at preventing oxidation of the emulsions and they were 20.4, 70.2, and 80.5 meq/kg on the last day, respectively. This indicates that the antioxidant activities of the antioxidants in emulsions decreased in the following order: TBHQ > VE > L-AP. Similarly, the results from Figure 5 showed that the mixture of VE and L-AP resulted in a synergistic antioxidant effect. Besides, the OPO-HMFS-Emulsion oxidative stability of L-AP and VE at a ratio of 2:3 was greater

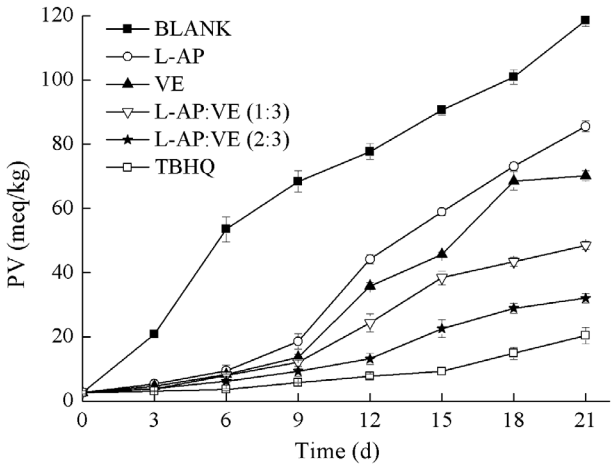

Figure 5. Changes in peroxide value of OPO-HMFS-Emulsion spiked with 0.02% (w/w) of antioxidants and without antioxidant during oven test at 63 ± 1, for 21 days. Values are mean ± standard deviation (n=2).

than that of ratio 1:3. Although increasing the proportion of L-AP in mixed antioxidants can improve the oxidative stability of OPO-HMFS emulsion, the proportion of VE in it should be kept at a higher level because VE also plays an important role in improving immunity and promoting the growth of infants.

4. CONCLUSIONS

This study produced HMFS which contained OPO and LC-PUFAs, whose content and structure of fatty acids are highly similar to HMF. The enzymatic interesterification conditions for OPO-HMFS (enzyme load, substrate molar ratio, temperature, water content) were optimized. A high content in C52 production (46.7%) and a sn-2 PA content of 75.3% were achieved. Moreover, OPO-HMFS was blended with coconut oil, soybean oil, algal oil and microbial oil at weight ratios of 0.70:0.18:0.11:0.004:0.007 to simulate the fatty acid profiles of HMF according to some mathematical models. The main and important fatty acids were closely within the ranges naturally found in HMF. The mixture of L-AP and VE resulted in a synergistic antioxidant effect both in OPO-HMFS and OPO-HMFS-Emulsion.

ACKNOWLEDGEMENT

The authors wish to thank Shanghai Kerry Oils & Grains Industries Co., Ltd. for providing partial oil samples and technical support of molecular distillation equipment for this research and Dr. Wei-Qi Tong at the Instrumental Analysis and Research Center of Shanghai University for the GC-MS of technical assistance. This work was supported by the Open Project Program of State Key Laboratory of Dairy Biotechnology, Bright Dairy & Food Co. Ltd. (SKLDB2018-001).

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