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ORIGINAL ARTICLE

Preparation of human milk fat analogue by enzymatic interesterification reaction using palm stearin and fish oil

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Abstract Palm stearin fractionate (PSF), obtained from palm stearin by further fractionation with solvents and n-3 polyunsaturated fatty acids (n-3 PUFA) rich fish oil (FO) were subjected to interesterification at 1:1, 1:2, 1:3, 2:1 and 3:1 substrate molar ratio and catalyzed by lipase from *Thermomyces lanuginosa* for obtaining a product with triacylglycerol (TAG) structure similar to that of human milk fat (HMF). The parameters (molar ratio and time) of the interesterification reaction were standardized. The temperature of 60 °C and enzyme concentration of 10 % (*w*/w) were kept fixed as these parameters were previously optimized. The reactions were carried out in a stirred tank reactor equipped with a magnetic stirrer for 6, 12, 18 and 24 h. The blends were analyzed for fatty acid (FA) composition of both total FAs and those at the *sn*-2 position after pancreatic lipase hydrolysis. All the blended products were subjected to melting point determination and free fatty acid content. Finally, blend of PSF and FO at 2:1 molar ratio with 69.70 % palmitic acid (PA) content and 12 h of reaction produced the desired product with 75.98 % of PA at *sn*-2 position, 0.27 % arachidonic acid (AA), 3.43 % eicosapentaenoic acid (EPA) and 4.25 % docosahexaenoic acid (DHA) and with melting point of 42 °C. This study portrayed a successful preparation of TAG containing unique FA composition i.e. ≥ 70 % of the PA, by weight, were esterified at the *sn*-2 position which could be used in infant formulation with health benefits of n-3 PUFAs.

Keywords Human milk fat analogue · Palm stearin fractionate · Fish oil · Lipase · Fatty acids

Research Highlights:
1. Human milk fat has unique composition of *sn-2* position which is enriched with palmitic acid. Various attempts to produce human milk fat analogue are taking place world wide.
2. Enzymatic reactions are essentially suitable for this reactions which deals with heat sensitive starting materials.
3. Palm stearin, a by product of palm oil industry, was utilised in this study to prepare human milk fat analogue. To provide the essential fatty acids, especially DHA, fish oil was also used.
4. On enzymatic interesterification, specific blends of palm stearin fractionate and fish oil produced human milk fat analogue with desired fatty acid composition and melting point.
5. The study highlighted the utilisation of one by product to produce value added product.

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Introduction

Human milk is regarded as a natural and nutritious food for infants, containing optimal composition to meet their nutritional needs in early life, and providing essential PUFAs to support the growth and development of the breastfed infant. Carbohydrates, proteins (lactalbumin, lactoglobulin), lipids, various growth factors and immune factors, enzymes, acids, vitamins, lactoferrin etc. are presented in mother's milk. Human milk contains 3–5 % lipid termed as human milk fat (HMF). After protein, HMF is the second most abundant component of human milk. It is found in the form of fat globule which is called milk fat globule (MFG). MFG is highly structured and contains unique polar lipids and membrane-specific proteins (El-Loly 2011). Although the FA composition and distribution of milk fat have a minor impact on the microstructure of MFG, they are of great significance to infant nutrition (Zou et al. 2012b). The infant consumes fats largely as TAG

(98 %), which needs to be broken down by the consecutive actions of several lipases in the upper gastrointestinal tract before absorption. The digestion of lipids in the infant gut is fully dependent on the distribution of FA in the TAG molecule. Many studies regarding the effects of FA distribution in HMF on the digestion and absorption of infants have proved the beneficial function of the location of saturated FA at the *sn*-2 position of TAG (Akoh 2006; Innis et al. 1995). The metabolic importance of the location of PA at the *sn*-2 position was further highlighted and that the overall absorption and transport rate of the oleic -palmitic -oleic (OPO) group was higher than that of the oleic -oleic -palmitic (OOP) group, which again indicated the beneficial functions of the intramolecular structure of HMF (Aoe et al. 1997; Xu 2000). The long chain polyunsaturated fatty acids (LCPUFAs) present in HMF play a major role in infant nutrition. Amongst them most obtained LCPUFA is DHA ($C_{22:6n-3}$), but it varies from mother to mother. Both nervonic acid (NA) and DHA are important for intrauterine and extrauterine neurodevelopment of both fetus and infant as these FAs are incorporated in large amounts in structural lipids of central nervous system of the baby. Also DHA and arachidonic acid (AA) are beneficial for neonate as they are primary components of retinal rod cells and phospholipids (Guesnet and Alessandri 2011; Wang et al. 2006).

In some unusual cases infants cannot be nourished by this unique gift of nature. Hence the importance of preparing HMF analogue arises. Many workers from different parts of country tried to prepare human milk fat analogue (HMFA) using various starting materials (Zou et al. 2011; Teichert and Akoh 2011; Turan et al. 2012; Li et al 2014). Among them most of the workers adopted the enzymatic reactions due to its mild reaction conditions to protect the PUFA from deterioration during reaction and also to utilize the specificity of enzymes. Zou et al. (2012a, c) was first tried to produce HMFA from palm stearin by combining enzymatic and physical method. In this study HMFA were prepared by two step process namely, acidolysis of interesterified high-melting palm stearin with fatty acids from rapeseed oil by using lipozyme RM IM and blending of the enzymatic product with the selected oils.

Presently a huge amount of palm oils are imported to India and further fractionated for producing palm olein i.e. a stable frying oil. The oil industries are trying to find out a proper pathway to utilize the high melting stearin part. In the present study attempts have been made to prepare HMFA utilizing this stearin fraction by further fractionating it to obtain PSF (mp = 58 °C) with PA content of 88.57 %. This fractionate was interesterified with FO containing the omega-3 FAs especially EPA and DHA. The regio-specific lipase Lipozyme TLIM was used as biocatalyst so as to protect the PA present at *sn*-2 position. Instead of blending of FO with PSF we opted for interesterification to obtain the uniform desired triglyceride composition and melting point.

Materials and methods

Materials

Palm stearin, that was fractionated, was provided by Budge Budge Refineries LTD, India. FO capsule Mega-Shel Cal from Elder Pharmaceuticals, India was purchased from local medical shop. Lipozyme TLIM (*Thermomyces lanuginose*) was obtained from Novozyme India Ltd., Bangalore, India, as a gift. The enzyme has an activity of 250 IU/g as per the literature provided by the manufacturer. The EC number of the enzyme is 3.1.1.3. Pancreatic lipase was procured from Sigma, Aldrich, Mont. Luis, MO. All other chemicals were supplied by Merck India Ltd. Mumbai.

Methods

Productions of PSF

PSF was selected as starting material due to its adequacy of PA which is suitable for use in infant formula. PSF was produced from palm stearin by solvent fractionation process using acetone in a ratio of 1:5 *v*/v (palm stearin: acetone) for 3½ hr. at a temperature of 20 °C. The solid fraction was collected by vacuum filtration, then liquefied and vacuum dried.

Interesterification

The enzymatic interesterification reactions were performed in a stirred tank reactor equipped with a magnetic stirrer cum hot plate. PSF was mixed with FO at five different substrate molar ratios (1:1, 2:1, 3:1, 1:2 and 1:3) at a constant temperature of 60 °C. These ratios were selected to provide FA composition similar to that of HMF. 10 % (*w*/w) enzyme (based on total substrate weight) Lipozyme TLIM was added to the reaction as the biocatalyst. The reaction was carried out for different course of time (6, 12, or 18, 24 h) with continuous stirring at 200 rpm under complete vacuum. After the reaction, the mixtures were filtered to remove the enzyme. The products were used in subsequent analysis.

Isolation of TAG

A portion of the product was dissolved in hexane (1:2, by volume) and then applied in a band on a silica gel plate (SRL, Mumbai, India). Plates were developed in hexane/diethyl ether/acetic acid (80:20:1, by volume), dried, and sprayed with 1 % 2,7-dichloroflourescein in methanol. To determine relative migration of the neutral glycerides, glyceride standards (monoglycerides, triglyceride and fatty acids) were used. The bands were visualized in iodine vapour. The band corresponding to TAGs was scraped and extracted with diethyl ether, then the solvent was removed under a stream of

nitrogen gas. The isolated TAG was used for its total FA composition determination and those at the *sn*-2 position.

FA composition

FA compositions of the TAG samples were analyzed by gas chromatography (GC). Fatty acid methyl esters (FAME) were prepared by the method described by Metcalfe and Schmitz (1961). TGs were converted to their methyl esters by adding 0.5 (N) methanolic KOH shaken vigorously for 10 min. To neutralize the alkali, 1 ml of 1 (N) HCL was added into the sample solution and shaken for a while. Fatty acid methyl esters (FAME) were then extracted with petroleum ether (boiling point 40–608C). FAMEs were analyzed by an Agilent 6890 N computerized gas chromatograph (network GC system – G 1530 N). The GC instrument used was equipped with FID detector and capillary DB-Wax column (30 m L, 0.32 mm I.D, 0.25 μm FT). N_2, H_2 and airflow rate was maintained at 1 mL/min, 30 mL/min and 300 mL/min respectively. Inlet & detector temperature was kept at 250 °C and the oven temperature was programmed as 150–190-230 °C with increase rate of 15 °C/min and 5 min hold up to 150 °C and 4 °C/min with 10 min hold up to 230 °C. Results were expressed as percent weight by weight (% *w*/w) basis. The gas chromatograph was calibrated prior to sample injection on each day, and all chemical methods were validated before sample analysis.

Analysis of sn-2 position of structured TAG prepared

The *sn*-2 positional fatty acid composition was determined following the method described by Luddy et al. (1963). 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 9 mg of pancreatic lipase were added to the TAG. The mixture was incubated in a water bath at 40 °C for 2 min with vigorous shaking, and then, 0.5 ml of 6 N HCl and 2 ml of diethyl ether were added and centrifuged. Diethyl ether was dried by anhydrous sodium sulfate and evaporated by vacuum. The hydrolytic products were separated on silica gel G TLC plates, and the developing solvent system was hexane/diethyl ether/acetic acid (60:40:1, vol/vol/vol). The band corresponding to *sn*-2 MAG was scraped off, methylated, and analyzed in GC by the same method mentioned above.

Slip melting point

Slip melting point of the structured TAGs was measured by the method described in the AOCS (1989a) Official Method Cc 1–25.

Free fatty acid (FFA) content

FFA contents defined as the percentage by weight of FFA groups existing in oils and fats were measured by AOCS (1989b) Official Method Te 1a - 64. Approximately 1 to 2 g of sample and 25 ml of neutral alcohol was taken in a conical and boiled for few minutes in water bath and then titrated with 0.1 (N) NaOH solution using phenolphthalein as indicator until pink color end point was not found.

Statistical analysis

All data were analysed in triplicate and the results were expressed as mean ± standard deviation (SD). A normality test (one-way ANOVA) was done for all samples and the p value was determined $p < 0.05$.

Results and discussion

The organic solvent containing system is effective in the interesterification reaction, especially in case of solid state substrates. However, in case of liquid state substrates at the reaction temperature, the reaction progresses efficiently even in a solvent free system (Shimada et al. 2000). Therefore, we choose solvent free system in our experiments.

Palm stearin is one of the major byproduct of palm oil industry and is a rich source of PA. On further fractionation it can yield PSF with melting point 58 °C, can be used as a suitable starting material instead of palm stearin, i.e. normally obtained in single step fractionation. PSF (PA = 88.57 %), after solvent fractionation, was thus used as the starting TGs for its suitable PA content. The FA compositions of the PSF and FO have been shown in Table 1. From this table, it was observed that, the major FA in PSF was PA (88.57 %) followed by stearic acid (7.53 %) and oleic acid (3.90 %). The enhanced PA content (70 % PA) especially at the *sn*-2 position, which was advantageous upon other oils. On the other hand, docosahexaenoic acid (50.76 %) followed by eicosapentaenoic acid (21.87 %) were present in FO primarily. Some amount of palmitic (2.05 %), palmitoleic (0.64 %), stearic (1.73 %), oleic (4.19 %), linoleic (1.64 %), eicosadienoic (1.16 %), eicosatrienoic (3.32 %), arachidonic (1.63 %), erucic (0.98 %), docosapentaenoic (2.82 %) and nervonic acid (7.21 %) were also present in FO. The LCPUFAs of fish oil have positive effects on the growth and development of infants and ameliorates the visual and cognitive functions especially in preterm infants (Birch et al. 2005; Innis et al. 2001). On the basis of these characteristics, it was possible to prepare structured TAGs with similar FA composition and distribution to HMF by enzymatic interesterification, the fractionated palm stearin with LCPUFAs rich FO catalyzed by lipozyme TLIM.

Table 1 Fatty acid profile of PSF and FO

Serial No.	Fatty acids	Common names	Fatty Acids (%*w*/w)	
			PSF	FO
1.	$C_{16:0}$	Palmitic acid	88.57 ± 0.06	2.05 ± 0.05
2.	$C_{16:1}$	Palmitoleic acid	-	0.64 ± 0.06
3.	$C_{18:0}$	Stearic acid	7.53 ± 0.03	1.73 ± 0.07
4.	$C_{18:1}$	Oleic acid	3.90 ± 0.06	4.19 ± 0.19
5.	$C_{18:2}$	Linoleic acid	-	1.64 ± 0.08
6.	$C_{20:2}$	Eicosadienoic acid	-	1.16 ± 0.13
7.	$C_{20:3}$	Eicosatrienoic acid	-	3.32 ± 0.25
8.	$C_{20:4}$	Arachidonic acid	-	1.63 ± 0.04
9.	$C_{20:5}$	Eicosapentaenoic acid	-	21.87 ± 0.27
10.	$C_{22:1}$	Erucic acid	-	0.98 ± 0.12
11.	$C_{22:5}$	Docosapentaenoic acid	-	2.82 ± 0.07
12.	$C_{24:1}$	Nervonic acid	-	7.21 ± 0.11
13.	$C_{22:6}$	Docosahexainoic acid	-	50.76 ± 0.24

Palm stearin (PS), obtained as a solid fraction from palm oil by partial crystallization at controlled temperature, is a useful source of natural hard vegetable fat for bakery products due to its β crystals. Further, PSF contains significant amount of PA at the *sn*-2 position, which would be adequate for use in infant formula. The aim of this study was to prepare TAG utilizing the unique content of PA in PSF along with incorporation of LCPUFAs to meet the FA requirements for HMF. The FA composition of different blends prepared at different substrate molar ratio of PSF and FO is presented in Table 2. Enzymatic interesterification is more spatially selective for producing more specific TAGs (Gunstone 2001). Enzymatic interesterification reactions were carried out at 60 °C for 6, 12, 18 and 24 h with continuous stirring (300 rpm) under complete vacuum condition to reduce the chances of free fatty acid formation in the present study. All the reactions were performed in triplicates, and average results with standard deviations were calculated. Lipozyme TLIM enzyme was used in this interesterification reaction due to its *sn*-1, 3 specificity that would result in suitable incorporation of PA, EPA and DHA at the specific positions of the glycerol moieties (Xu 2000; Innis et al. 1995). It can be seen from the table that the highest value of PA was 70.30 % in 3:1 (PSF: FO) molar ratio and the lowest was 11.23 % in 1:3 substrate molar ratio of PSF:FO. It was observed that the PA content was gradually increased with increasing quantities of PSF in the blend for obvious reason (Sellappan and Akoh 2001). Recently, palm stearin is mainly used as a material for production of various fats. HMFA preparation by utilization of palm stearin can not only increase the added value of palm oil but also decrease the production cost. Meanwhile, physical blend of palm stearin with vegetable oils to prepare HMFAs were used in some studies. Those products had similar FA compositions to that of HMF, but the distribution of FA was quite different, i.e., the saturated FAs were evenly distributed at three positions. The blend of 2:1 substrate molar ratio of PSF to FO contained optimum amount of PA (63.33 %). EPA and DHA, the two most imperative LCPUFAs for intrauterine and extrauterine neurodevelopment of both fetus and infant (Fleith and Clandinin 2005), were also present in significant amount in all the blends and their amount was directly proportional with increased content of FO. NA (Nervonic acid), a monounsaturated ω-9 FA which also plays an important role in functioning of nervous system was also present in FO as well as in the PSF: FO blends. The highest value achieved was 5.63 % in the blend of 1:3 ratio of PSF: FO. AA another important essential fatty acid vital for brain growth was present in minor quantity in the blends. The highest value achieved was 1.32 % in the blend of 1:3 ratio of PSF: FO. The amount of total unsaturated FA including LCPUFA in the HMFAs was nearly identical with HMF. Unsaturated FA rich diets are advantageous in infant feeding, since they are absorbed better than SFAs due to less interference with calcium absorption (Karabulut et al. 2007). From Table 2 it could be concluded that the blend of 2:1 PSF:FO provided the optimum properties of HMF as they contain high amount of PA in combination with significant quantity of other FAs in terms of stearic, oleic, linoleic acid and also LCPUFAs in terms of EPA, DHA and NA. The total FA composition of the blends remained similar after the interesterification reaction as there was only rearrangement of acyl groups within the glycerol backbone. However, the acidolysis and the concentrated LCPUFA as acyl donors, were used as the reaction method in most studies which led to huge loss of LCPUFA and thus enhanced the production cost. With respect to LCPUFA, considering their high value and low content of LCPUFA in HMF, physical addition by the precise calculation could be a favored alternative method. It is supposed that this type of structured lipid, prepared in the present study, provide higher FA and calcium absorption and thus efficient use of dietary energy (Xu 2000; Innis et al. 1995).

The melting point profiles of the blends and interesterified samples are given in Fig 1. The melting point of all samples were within the range of 42 °C which is suitable for optimum absorption of lipid in human body as the activation temperature of pancreatic lipase is approximately at 40 °C (Squire et al. 2003). It was observed that the melting points of the products initially in case of all the different molar ratios are high but as interesterification reaction proceeded their melting points gradually reduced. This phenomenon can be justified by the fact that during the course of interesterification reaction, the FA was rearranged and new TAG with altered composition was formed which had decreased melting point. It was obvious that blends having higher amount of PSF than FO had higher melting point. The melting point of interesterified

Table 2 Fatty acid compositions of blends prepared at different substrate molar ratio of PSF and FO

Serial No.	Fatty acid	Fatty Acids (% *w*/w)				
		1:1	2:1	3:1	1:2	1:3
1.	$C_{16:0}$	43.94 ± 0.06^{bcde}	63.33 ± 0.04^{acde}	70.30 ± 0.43^{abde}	33.11 ± 0.14^{abce}	11.23 ± 0.18^{abcd}
2.	$C_{18:0}$	4.09 ± 0.13^{bcde}	5.12 ± 0.03^{acd}	$1.30 \pm 0.08a^{bce}$	3.38 ± 0.28^{abc}	3.87 ± 0.04^{ace}
3.	$C_{18:1}$	4.05 ± 0.14^{bcde}	4.77 ± 0.10^{a}	2.40 ± 0.19^{ade}	4.20 ± 0.70^{ac}	4.37 ± 1.39^{ac}
4.	$C_{18:2}$	1.01 ± 0.16^{bcde}	3.36 ± 0.08^{acde}	4.06 ± 0.08^{abde}	1.10 ± 0.70^{abc}	1.64 ± 0.10^{abc}
5.	$C_{20:2}$	-	0.71 ± 0.07^{cde}	0.67 ± 0.07^{bd}	1.67 ± 0.03^{bce}	0.59 ± 0.10^{bd}
6.	$C_{20:3}$	-	0.04 ± 0.03^{de}	-	0.24 ± 0.06^{be}	2.94 ± 0.03^{bd}
7.	$C_{20:4}$	0.72 ± 0.32^{bcde}	0.46 ± 0.01^{acde}	0.70 ± 0.07^{abde}	1.03 ± 0.04^{abce}	1.32 ± 0.03^{abcd}
8.	$C_{20:5}$	11.3 ± 0.82^{bcde}	5.78 ± 0.11^{acde}	5.27 ± 0.22^{abde}	13.48 ± 0.41^{abce}	18.63 ± 0.10^{abcd}
9.	$C_{22:1}$	1.79 ± 0.13^{bcde}	2.66 ± 0.14^{acde}	3.76 ± 0.06^{abe}	3.68 ± 0.17^{abe}	3.57 ± 0.10^{abcd}
10.	$C_{22:5}$	1.78 ± 0.11^{bce}	0.84 ± 0.01^{acde}	0.76 ± 0.11^{abde}	1.88 ± 0.07^{bce}	2.47 ± 0.10^{abcd}
11.	$C_{24:1}$	4.07 ± 0.07^{bcde}	2.05 ± 0.01^{acde}	1.08 ± 0.03^{abde}	4.73 ± 0.04^{abce}	5.63 ± 0.04^{abcd}
12.	$C_{22:6}$	27.25 ± 0.07^{bcde}	10.88 ± 0.03^{acde}	9.70 ± 0.16^{abde}	31.50 ± 0.21^{abce}	43.74 ± 0.10^{abcd}

[a] comparison between 1:1 PSF:FO group with other groups ($p < 0.05$), [b] comparison between 2:1 PSF:FO group with other groups ($p < 0.05$), [c] comparison between 3:1 PSF:FO group with 1:2 groups ($p < 0.05$), [d] comparison between 1:2 PSF:FO group with other groups ($p < 0.05$), [e] comparison between 1:3 PSF:FO group with other groups ($p < 0.05$)

product of 2:1 PSF:FO blend showed optimum melting point of 42 °C after 12 h reaction.

Free fatty acid (FFA) is one of the products formed as a result of lipase-catalysed interesterification reaction because interesterification is a two step process; the first being the hydrolysis process and the second the re-esterification process (Reyes and Hill 1994). If water is available in the system, the first step predominates and generation of FFA increases. Fig 2 shows the formations of FFA of the interesterified sample at different time by using different molar ratios of PSF and FO. From this figure it was observed that the formation of FFA increased as the interesterification reaction progressed. This may be due to presence of water in the enzyme for maintaining its activity. The enzyme could be an available source for the formed water which in addition was not fully removed by vacuum (Koletzko et al. 1998). However this is not of much concern as the FFA content of the final product was within the desired limit.

Synthesis of triglycerides involves, precise positioning of FAs at the outer *sn*-1 and *sn*-3, and center *sn*-2 positions of the TAG, rather than involving random esterification of three FA to glycerol. The mammary glands of human system has

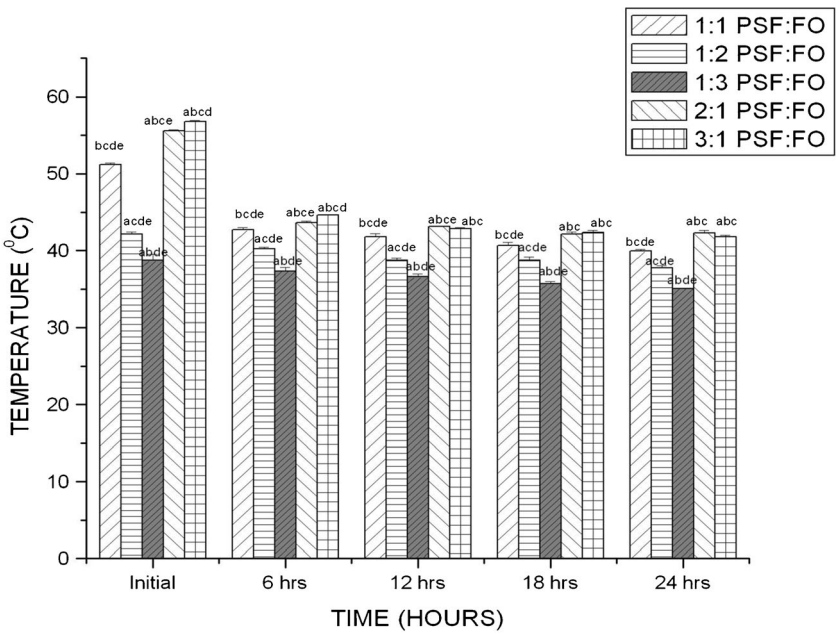

Fig. 1 Melting point profile of Human Milk Fat Analogues, *a* comparison between 1:1 PSF:FO group with other groups ($p < 0.05$), *b* comparison between 1:2 PSF:FO group with other groups ($p < 0.05$), *c* comparison between 1:3 PSF:FO group with other groups ($p < 0.05$), *d* comparison between 2:1 PSF:FO group with other groups ($p < 0.05$), *e* comparison between 3:1 PSF:FO group with other groups ($p < 0.05$)

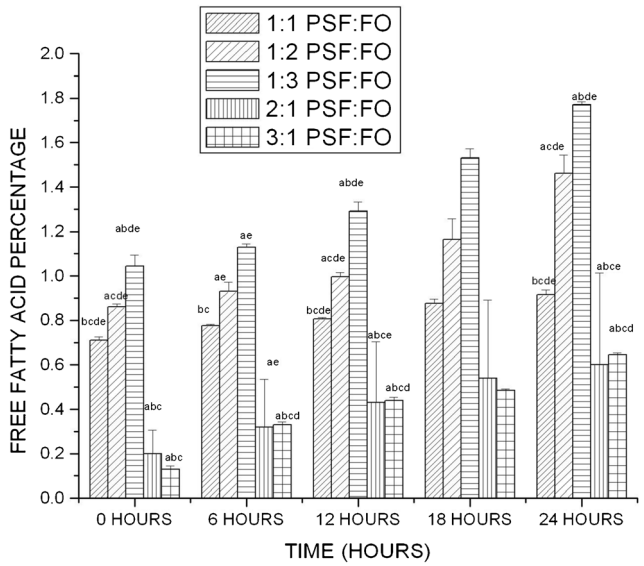

Fig. 2 Free fatty acid formation during interesterification process. *a* comparison between 1:1 PSF:FO group with other groups ($p < 0.05$), *b* comparison between 1:2 PSF:FO group with other groups ($p < 0.05$), *c* comparison between 1:3 PSF:FO group with other groups ($p < 0.05$), *d* comparison between 2:1 PSF:FO group with other groups ($p < 0.05$), *e* comparison between 3:1 PSF:FO group with other groups ($p < 0.05$)

developed with unusual pathways which may result in an exact positioning of FA especially at the *sn-2* position of TAG, which is different from the TAG present in other human tissues and plasma (Breckenridge et al. 1969). This stereo - specific positioning of FA in human milk TAGs involves favored positioning of the saturated FAs especially PA at the *sn*-2 position, rather than at the *sn*- 1,3 positions, as in human tissue and plasma lipids, and vegetable oils common in human diets, and in the fat blends used in the manufacture of infant formula (Jensen 1995). Endogenous lipases catalyzed TAG digestion leads to hydrolysis of FA from the *sn*-1,3 linkages of TAG and then release two un-esterified fatty acids and one *sn*-2 MAG from each triglyceride into the intestinal lumen (Mu and Hoy 2004). However this unique structure of PA on the TAG *sn*-2 position of milk or formula fats improves PA absorption (Lien 1994; Lien et al. 1993) and plasma chylomicron TAG of breastfed infants are high in *sn*-2 PA (Carnielli et al. 1995). Unesterified PA, in addition to low intraluminal solubility, has an increased affinity to merge with divalent cations, such as calcium, to form insoluble soaps, which are malabsorbed (Innis 2011). Several studies have established the better efficiency of fat absorption and softer stools in breastfed infants compared to that of formula (containing PA from saturated vegetables) fed infants (Kennedy et al. 1999; Lopez et al. 2001). The PA content of the various PSF and FO interesterified products as a whole and that at *sn*-2 position at different time intervals are shown in Table 3. For the products prepared from PSF and FO using molar ratios of 2:1 and 3:1, at every time interval, 58 % or more PA in *sn*-2 position were observed in all cases. The PA content in the *sn*-2

Table 3 Amount of palmitic acid as a whole and in *sn-2* position of HMFA samples prepared at different molar ratio of PSF and FO

Sample	Palmitic acid (%*w*/w)									
	HMFA at 1:1 PSF:F0	*sn-2* position 1:1 PSF:F0	HMFA at 2:1 PSF:F0	*sn-2* position 2:1 PSF:F0	HMFA at 3:1 PSF:F0	*sn-2* position 3:1 PSF:FO	HMFA at 1:2 PSF:F0	*sn-2* position 1:2 PSF:FO	HMFA at 1:3 PSF:FO	*sn-2* position 1:3 PSF:FO
Initial	43.94 ± 0.06^{bcde}	48.04 ± 0.06^{bcde}	63.33 ± 0.04^{acde}	60.23 ± 0.07^{acde}	70.30 ± 0.43^{abde}	61.94 ± 0.91^{abde}	33.11 ± 0.14^{abce}	54.77 ± 0.23^{abce}	11.23 ± 0.18^{abcd}	26.99 ± 0.18^{abcd}
6 h	27.07 ± 0.07^{bcde}	36.40 ± 0.07^{bcde}	67.66 ± 0.01^{acde}	64.52 ± 0.04^{ade}	73.25 ± 0.53^{abde}	64.77 ± 0.53^{ade}	27.07 ± 0.07^{abce}	39.55 ± 0.10^{abce}	14.45 ± 0.07^{abcd}	27.01 ± 0.07^{abcd}
12 h	21.83 ± 0.04^{bcde}	34.43 ± 0.04^{bcde}	69.70 ± 0.13^{acde}	75.98 ± 0.14^{acde}	74.59 ± 0.53^{abde}	60.78 ± 0.53^{abde}	21.83 ± 0.04^{abc}	39.04 ± 0.06^{abce}	21.82 ± 0.03^{abc}	22.01 ± 0.03^{abcd}
18 h	20.92 ± 0.10^{bcde}	36.86 ± 0.10^{bcde}	75.26 ± 0.14^{acde}	62.56 ± 0.13^{acde}	74.22 ± 0.43^{abde}	58.07 ± 0.43^{abde}	20.92 ± 0.10^{abce}	38.49 ± 0.07^{abce}	26.63 ± 0.04^{abcd}	23.00 ± 0.04^{abcd}
24 h	19.16 ± 0.23^{bcde}	33.14 ± 0.23^{bcde}	57.80 ± 0.07^{acde}	58.26 ± 0.01^{acde}	70.81 ± 0.91^{abde}	61.03 ± 0.43^{abde}	19.16 ± 0.23^{abce}	35.19 ± 0.04^{abce}	20.65 ± 0.07^{abcd}	24.05 ± 0.07^{abcd}

[a] comparison between 1:1 PSF:FO group (for both as original and at sn-2 position) with other groups ($p < 0.05$), [b] comparison between 2:1 PSF:FO group (for both as original and at sn-2 position) with other groups ($p < 0.05$), [c] comparison between 3:1 PSF:FO group (for both as original and at sn-2 position) with 1:2 groups ($p < 0.05$), [d] comparison between 1:2 PSF:FO group (for both as original and at sn-2 position) with other groups ($p < 0.05$), [e] comparison between 1:3 PSF:FO group (for both as original and at sn-2 position) with other groups ($p < 0.05$)

position of the blend was lower than the original in case of HMFA prepared at 2:1 and 3:1 substrate molar ratio of palm stearin fractionate and fish oil. However, the level is higher than that found in most common infant formulas prepared in recent days. The HMFA sample prepared from 2:1 PSF and FO at 12 h contained highest amount of PA at *sn*-2 position i.e. 75.98 %. Lipozyme TLIM is a *sn*-1, 3 specific lipase and therefore, during reaction with TLIM, except acyl migration, the *sn*-2 position of the TAG remains unchanged. In spite of the specificity of the *sn*-1,3-specific lipase, incorporation of FAs into acylglycerols occurred at the *sn*-2 position due to acyl migration during interesterification. There is elevated evidence of this unusual positioning of PA in human milk TAG which has an important role for infant health in different orders, such as absorption of fat and calcium, bone health, intestinal flora and infant comfort and thus the FA composition especially the positioning of palmitic acid at *sn*-2 position of these interesterified products of PSF and FO established their suitability as HMFA.

Thus, HMFs could be successfully produced from high PA rich PSF (≥ 60 %) and fish oil. These kinds of HMF products could be expected to deliver absorption characteristics due to PA at *sn*-2 position with health benefits associated with LCPUFAs of FO. The PA rich PSF (88.57 % PA) and LCPUFAs rich FO (especially 21.87 % EPA and 50.76 % DHA) have proved their suitability as the starting material in this study. The enzymatic interesterification of PSF and FO can produce a wide range of HMFA with varying degree of PA and other FA content. But considering the melting point (42 °C) and PA content of *sn*-2 position (75.98 %), the HMFA prepared from 2:1 blend of PSF and FO by 12 h interesterification reaction seems to be best alternative of natural HMF.

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References

Akoh CC (2006) Handbook of functional lipids. CRC press, Boca Raton

AOCS (1989a) Slip melting point method, Cc 1 - 25. Official methods and recommended practices of the America Oil Chemists Society, 4th edn. AOCS Press, Champaign

AOCS (1989b) Free fatty acids method, Te 1a - 64. Official methods and recommended practices of the America Oil Chemists Society, 4th edn. AOCS Press, Champaign

Aoe S, Yamamura JI, Matsuyama H, Hase M, Shiota M, Miura S (1997) The positional distribution of dioleoyl-palmitoyl glycerol influences lymph chylomicron transport, composition and size in rats. J Nutr 127:1269–1273

Birch EE, Castaneda YS, Wheaton DH, Birch DG, Uauy RD, Hoffman DR (2005) Visual maturation of term infants fed long-chain polyunsaturated fatty acid-supplemented or control formula for 12 mo. Am. J. Clin. Nutr. 81:871–879

Breckenridge WC, Marai L, Kuksis A (1969) Triglyceride structure of human milk fat. Can J Biochem 47(8):761–769

Carnielli VP, Luijendijk IH, Beek RHV, Boerma GJ, Degenhart HJ, Sauer PJ (1995) Effect of dietary triacylglycerol fatty acid positional distribution on plasma lipid classes and their fatty acid composition in preterm infants. Am J Clin Nutr 62(4):776–781

El-Loly MM (2011) Composition, properties and nutritional aspects of milk fat globule membrane – a review. Pol J Food Nutr Sci 61(1):7–32

Fleith M, Clandinin MT (2005) Dietary PUFA for preterm and term infants: review of clinical studies. Crit. Rev. Food Sci 45:205–229

Guesnet P, Alessandri JM (2011) Docosahexaenoic acid (DHA) and the developing central nervous system (CNS) - implications for dietary recommendations. Biochimie 93:7–12

Gunstone FD (2001) Why are structured lipids and new lipids sources required? In: Gunstone FD (ed) Structured and modified lipids. Marcel Dekker, New York, NY, pp. 1–35

Innis SM (2011) Dietary triacylglycerol structure and its role in infant nutrition. Adv Nutr 2(3):275–283

Innis SM, Dyer R, Quinlan P, Diersen-Schade D (1995) Palmitic acid is absorbed as *sn*-2 monopalmitin from milk and formula with rearranged triacylglycerols and results in increased plasma triglyceride s*n*-2 and cholesteryl ester palmitatein piglets. J Nutr 125:73–81

Innis SM, Gilley J, Werker J (2001) Are human milk long-chain polyunsaturated fatty acids related to visual and neural development in breast-fed term infants? J Pediatr 139:532–538

Jensen RG (1995) Comments on the extraction of fat from human milk for analysis of contaminants. Chemosphere 31(9):4197–4200(author reply 200-5)

Karabulut I, Turan S, Vural H, Kayahan M (2007) Human milk fat substitute produced by enzymatic interesterification of vegetable oil blend. Food Technol Biotech. 45(4):434–438

Kennedy K, Fewtrell MS, Morley R (1999) Double-blind, randomized trial of a synthetic triacylglycerol in formula-fed term infants: effects on stool biochemistry, stool characteristics, and bone mineralization. Am. J. Clin. Nutr. 70(5):920–927

Koletzko B, Tangermann R, von Kries R, Stannigel H, Willberg B, Radde I, Schmidt E (1998) Intestinal milk-bolus obstruction in formula fed premature infants given high doses of calcium. J Pediatr Gastr Nutr. 7:548–553

Li R, Pande G, Sabir JSM, Baeshen NA, Akoh CC (2014) Enrichment of refined olive oil with palmitic and docosahexaenoic acids to produce a human milk fat analogue. J Am Oil Chem Soc 91(8):1377–1385

Lien EL (1994) The role of fatty acid composition and positional distribution in fat absorption in infants. J. Pediatr 125(5 Pt. 2):S62–S68.

Lien EL, Yuhas RJ, Boyle FG, Tomarelli RM (1993) Corandomization of fats improves absorption in rats. J Nutr 123(11):1859–1867

Lopez A, Bargallo AIC, Folgoso CC (2001) The influence of dietary palmitic acid triacylglyceride position on the fatty acid, calcium and magnesium contents of at term newborn faeces. Early Hum Dev Suppl 65:S83–S94

Luddy FE, Barford RA, Herb SF, Magidman P, Riemenschneider RW (1963) Pancreatic lipase hydrolysis of triglycerides by a semi micro technique. Presented in part at the AOCS Meeting, Minneapolis

Metcalfe LD, Schmitz AA (1961) The rapid preparation of fatty acid esters for gas chromatographic analysis. J Anal Chem 33(3):363–364

Mu H, Hoy HE (2004) The digestion of dietary triacylglycerols. Prog Lipid Res 43(2):105–133

Reyes HR, Hill CG (1994) Kinetic modeling of interesterification reactions catalyzed by immobilized lipase. Biotechnol Bioeng 43(2): 171–182

Sellappan A, Akoh CC (2001) Synthesis of structured lipids by transesterification of trilinolein catalyzed by lipozyme IM60. J Agric Food Chem 49:2071–2076

Shimada Y, Nagao T, Hamasaki Y, Akimoto K, Sugihara A, Fujikawa S, Komemushi S, Tominaga Y (2000) Enzymatic synthesis of structured lipid containing arachidonic and palmitic acids. J Am Oil Chem Soc 77:89–93

Squire TL, Lowe ME, Bauer VW, Andrews MT (2003) Pancreatic triacylglycerol lipase in a hibernating mammal. II. Cold-adapted function and differential expression. Physiol Genomics Dec 16; 16 (1):131–140.

Teichert SA, Akoh CC (2011) Stearidonic acid soybean oil enriched with palmitic acid at the sn-2 position by enzymatic interesterification for use as human milk fat analogues. J Agric Food Chem 59:5692–5701

Turan D, Yesilcubuk NS, Akoh CC (2012) Production of human milk fat analogue containing docosahexaenoic and arachidonic acids. J Agric Food Chem 60:4402–4407

Wang ZJ, Liang CL, Li GM, Yu CY, Yin M (2006) Neuroprotective effects of arachidonic acid against oxidative stress on rat hippocampal slices. Chem Biol Interact 163(3):207–217

Xu X (2000) Production of specific-structured triacylglycerols by lipase-catalyzed reactions: a review. Eur J Lipid Sci Technol 102:287–303

Zou XQ, Huang JH, Jin QZ, Liu YF, Song ZH, Wang XG (2011) Lipase-catalyzed preparation of human milk fat substitutes from palm stearin in a solvent-free system. J Agric Food Chem 59:6055–6063

Zou XQ, Huang JH, Jin QZ, Liu YF, Tao GJ, Cheong LZ, Wang XG (2012a) Preparation of human milk fat substitutes from palm stearin with arachidonic and docosahexaenoic acid: combination of enzymatic and physical methods. J Agric Food Chem 60:9415–9423

Zou XQ, Guo Z, Huang JH, Jin QZ, Cheong LZ, Wang XG, Xu XB (2012b) Human milk fat globules from different stages of lactation: a lipid composition analysis and microstructure characterization. J Agric Food Chem 60(29):7158–7167

Zou X, Huang J, Jin Q, Liu Y, Song Z, Wang X (2012c) Lipase-catalyzed synthesis of human milk fat substitutes from palm stearin in a continuous packed bed reactor. J Am Oil Chem Soc 89(8):1463–1472