Palm-based medium-and-long-chain triacylglycerol (P-MLCT): production via enzymatic interesterification and optimization using response surface methodology (RSM)

Yee-Ying Lee · Teck-Kim Tang · Eng-Tong Phuah ·
Nur Azwani Ab Karim · Siti Maslina Mohd Alwi ·
Oi-Ming Lai

Revised: 5 June 2013 / Accepted: 10 June 2013 / Published online: 27 June 2013
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Abstract Structured lipid such as medium-and long-chain triacylglycerol (MLCT) is claimed to be able to suppress body fat accumulation and be used to manage obesity. Response surface methodology (RSM) with four factors and three levels (+1,0,−1) faced centered composite design (FCCD) was employed for optimization of the enzymatic interesterification conditions of palm-based MLCT (P-MLCT) production. The effect of the four variables namely: substrate ratio palm kernel oil: palm oil, PKO:PO (40:60–100:0 w/w), temperature (50–70 °C), reaction time (0.5–7.5 h) and enzyme load (5–15 % w/w) on the P-MLCT yield (%) and by products (%) produced were investigated. The responses were determined via acylglycerol composition obtained from high performance liquid chromatography. Well-fitted models were successfully established for both responses: P-MLCT yield ($R^2=0.9979$) and by-products ($R^2=0.9892$). The P-MLCT yield was significantly ($P<0.05$) affected by substrate ratio, reaction time and reaction temperature but not enzyme load ($P>0.05$). Substrate ratio PKO: PO (100:0 w/w) gave the highest yield of P-MLCT (61 %). Nonetheless, substrate ratio of PKO: PO (90:10w/w) was chosen to improve the fatty acid composition of the P-MLCT. The optimized conditions for substrate ratio PKO: PO

(90:10 w/w) was 7.26 h, 50 °C and 5 % (w/w) Lipozyme TLIM lipase, which managed to give 60 % yields of P-MLCT. Up scaled results in stirred tank batch reactor gave similar yields as lab scale. A 20 % increase in P-MLCT yield was obtained via RSM. The effect of enzymatic interesterification on the physicochemical properties of PKO:PO (90:10 w/w) were also studied. Thermoprofile showed that the P-MLCT oil melted below body temperature of 37 °C.

Keywords Medium-and long-chain triacylglycerol (MLCT) · Response surface methodology (RSM) · TLIM lipase · Palm oil · Palm kernel oil · Interesterification

Introduction

Medium-and-long chain triacylglycerol (MLCT) is a type of modified lipid where the individual triacylglycerol molecule consist of both medium chain fatty acid (MCFA) and long chain fatty acid (LCFA) attached in a glycerol backbone. Results from both preclinical and clinical studies showed that when 10–12 % of MCFA is present in the MLCT, ingestion of it showed a remarkable reduction in body weight gained by suppressing the body fat accumulation as well improve blood lipid profile especially in reducing cholesterol level (Kasai et al. 2003; Zhang 2007; Liu et al. 2009; Xue et al. 2009). Rats fed with MLCT had higher diet-induced thermogenesis and hepatic fatty acid oxidation enzymes that were activated (Shinohara et al. 2002; Ogawa et al. 2007). This showed that MLCT possesses the ability to act as potential anti-obesity functional oil to used to manage obesity problem through diet.

The ability of MLCT oil to reduce body weight and fat accumulation is mainly due to the presence of MCFA. Unlike LCFA, which are further incorporated into chylomicrons and

Y.-Y. Lee · T.-K. Tang · E.-T. Phuah · O.-M. Lai (✉)
Institute of Bioscience, Universiti Putra Malaysia, 43400 Serdang,
Selangor, Malaysia
e-mail: omlai@biotech.upm.edu.my

N. A. A. Karim · S. M. M. Alwi
Sime Darby Research Sdn Bhd, R&D Carey Island-Upstream, Lot
2664 Jln Pulau Carey, 42960 Carey Island, Selangor, Malaysia

O.-M. Lai
Department of Bioprocess Technology, Faculty of Biotechnology
and Biomolecular Sciences, Universiti Putra Malaysia,
43400 Serdang, Selangor, Malaysia

transported to the lymph and end up deposited in the body, MCFA are more rapidly metabolized due to its small molecular size which are relatively soluble in the body fluid and easier for pancreatic lipase to react (Bach and Babayan 1982; Papamandjaris et al. 1998). Besides, MCFA are absorbed directly via portal vein to the liver giving a rapid source of energy without undergoing the carnitine transport system (Papamandjaris et al. 1998).

The idea of MLCT is borned from MCT. MCT is commercially available in the market since 1955 where it has been used as endurance sports drink, to treat malabsorption, hyperlipidemia cases as well as preterm infant (Bach and Babayan 1982; Heydinger and Nakhasi 1996; St-Onge and Jones 2002) as it can provide rapid source of energy to these patient easily. Nonetheless, MCT lack the essential fatty acid which is present in LCT. Studies also demonstrated that consumption of MCT can cause remarkable rise in serum cholesterol and induced atherosclerosis (Malmros et al. 1972). The low smoke point of MLCT caused foaming easily during frying which limit its applications. As such, MCT is incorporated with LCFA to improve its functional properties. Incorporation of LCFA will provide the essential fatty acid to the body as well as increase the smoke point of the MCT.

Oil palm (*Elaeis guineensis*) produce two types of oil which is palm oil (PO) from the mesocarp and palm kernel oil (PKO) from the seed of the fruit. Both oils have different physical and chemical properties (Rossell et al. 1985). Today, palm oil has been the leading oil traded in the world. It has gained popularity as frying oil in the Southeast Asian countries due to its cheaper price, stability towards oxidation due to the presence of tocotrienol and beta carotene (Matthäus 2007). Studies have found that *trans* fat are associated with several uncertain health diseases (Ascherio and Willett 1997). The aforementioned issue has caused the USA and Denmark to control the composition of *trans* fat in food products by introducing the regulation of labeling *trans* fat and phasing out the use of *trans* fat in restaurants (Hunter 2006; Angell et al. 2009). Semisolid property possessed by palm oil give palm oil its reputability today as an alternative to hydrogenated fat for margarine and shortening making to produce *trans* free fat, replacing the conventional ways of margarine and shortening making which involved hydrogenation process (Lai et al. 1999; Farmani et al. 2006; Nor Aini and Miskandar 2007; Reshma et al. 2008). PKO is a lauric rich oil (Rossell et al. 1985). It is normally used in confectionary industries, cream or biscuit filling, and ice cream coating as it melts below body temperature without giving a waxy feeling in the mouth. It also gives a cooling sensation when melt in the mouth (Young 1983).

From the health impact point of view, people are still skeptical about PO and PKO. Recent study has showed that PO was able to reduce the risk of cardiovascular disease due to the close unity of saturated and non saturated fatty acids in

ratio of 1:1 (Edem 2002). As for PKO which contained saturated fatty acid mostly of lauric (48.2 %) and myristic acid (16.2 %), studies have also shown that PKO has a cholesterol level rising effect mainly due to the presence of myristic acid. However, these studies only focused on the synthetic trimyristic acid. Composition of trimyristic acid is not high in PKO. In fact, myristic acid is attached in the glycerol backbone along with others fatty acid in the individual triacylglycerol molecule.

Response surface methodology (RSM) is a collection of mathematical and statistical model used to determine the interaction between reaction parameters, to obtain the “sweet spot” for a reaction with just minimum amount of experimental run compared to the conventional way. It has also been used to develop, improve and optimized a product and process (Baş and Boyacı 2007). Various lipase-catalyzed reaction for fats and oils modification also utilized RSM for optimization purposes.

The objective of the present study is to optimize the enzymatic interesterification reaction of PKO and PO blends for the P-MLCT production using faced central composite design (FCCD). No studies have been carried out so far to utilize and to determine the optimize conditions for this P-MLCT yield from blend of both the palm-based oil. Most studies so far only focused on the melting properties of the different ratio of blends of PKO and PO for application purposes. For the P-MLCT production, enzymatic interesterification method is chosen as it is much lower in cost compared to esterification and acidolysis method. MCFA and LCFA fractions in this experiment will be contributed by PKO and PO, respectively. The independent variables studied were namely substrate ratio PKO: PO (Sb), reaction time (Ti), reaction temperature (Te) and enzyme load (En). Lipozyme TLIM lipase is selected in the present study as it is commonly used for fats and oils enzymatic modification to produce margarine and shortening and is food grade. It is also cheaper in price and reportedly gives a higher interesterification activity in batch reactor system (Zhang 2007). The changes in the physicochemical properties such as fatty acid composition and thermo-profile of the PKO-PO blend before and after interesterification will also be investigated.

Materials and methods

Materials

Refined, bleached and deodorized palm oil (RBD PO) and palm kernel oil (RBD PKO) was obtained from Golden Jomalina Industries Sdn Bhd (Banting, Malaysia). Commercial 1,3 specific Lipozyme TLIM lipase was purchased from Novozymes A/S (Bagsvaerd, Denmark). Standards used for determination of triacylglycerol species were bought from Sigma Aldrich Inc

(Sigma Chemical Co., USA). All solvents used were of high performance liquid chromatography (HPLC) grade.

Experiment design

Design Expert 7.0.0 software was used to optimize the P-MLCT yield from interesterification of RBD PKO and RBD PO with Lipozyme TLIM lipase. A four factors and three levels Face Centre Composite Design (FCCD) consisting of 30 experiment runs with six replicates at the centre point was employed in this study. The independent variables investigated were: substrate ratio PKO: PO (Sb), reaction time (Ti), reaction temperature (T), and enzyme load (En). Table 1 showed the experiment variables in coded and actual units. The response variables determined were: P-MLCT yield and by products (FFA, MAG and DAG) produced. P-MLCT yield, MAG and DAG were quantified using High Performance Liquid Chromatography with Evaporative Light Scattering Detector (ELSD) while FFA was determined using AOCS official method Ca 5a-40 (AOCS 1993).

The 30 experiments run were conducted randomly. Data obtained from the experiment were analyzed by using ANNOVA, multiple regressions analysis with backward elimination at a 95 % significance level ($P < 0.05$). The experiment data were fitted with the second-order regression equation:

$$Y = \beta_0 + \sum_{i=1}^3 \beta_i X_i + \sum_{i=1}^3 \beta_{ii} X_i^2 + \sum_{i=1}^2 \sum_{j=i+1}^3 \beta_{ij} X_i X_j$$

where Y is the responses (P-MLCT Yield and by-products), β_0 is the intercept, β_i β_j β_{ij} are the linear, quadratic, and the interaction coefficients respectively. X_i and X_j are the levels of independent variables. A three dimensional response surface plots were generated showing the relationship between the response and independent variable. Subsequently, numerical optimization was used to optimize the responses of interest.

Table 1 Experiment variables in coded and actual unit

Independent variables	Symbol	Coded variable		
		−1	0	1
Substrate ratio PKO:PO (w/w)	Sb	40:60	70:30	100:0
Reaction time (hr)	Ti	0.5	4.0	7.5
Reaction temperature (°C)	T	50.0	62.5	75.0
Enzyme load (%)	En	5.0	10.0	15.0

P-MLCT production through enzymatic interesterification

Enzymatic interesterification of the blend RBD PO and RBD PKO took place in a 250 ml conical flask with stopper in solvent-free media. The blend was stirred at 350 rpm with magnetic stirrer bar and temperature was maintained with water bath. The reaction started with the addition of Lipozyme TLIM lipase once the desired temperature was achieved. At the end of the reaction, the interesterified RBD PKO and RBD PO oil was centrifuged and filtered with 0.45 μ m PTFE membrane filter to separate the enzyme. Samples were stored at -20°C for further analysis.

Large scale production of P-MLCT

Pilot scale production of P-MLCT was produced using a 10 kg stirred tank batch reactor, stirring at 350 rpm. The optimized conditions obtained through RSM which is: substrate ratio of PKO: PO (90:10 w/w), 50°C , 7.26 h and 5 % (w/w) Lipozyme TLIM lipases was employed in the large scale production. Physical refiner was used to remove free fatty acid from the enzymatically modified MLCT. After physical refining, FFA of P-MLCT was reduced to 0.1 % (AOCS official method Ca 5a-40) and peroxide value to 0 % (AOCS official method Cd 8-53).

Analysis of triacylglycerol (TAG) species by high performance liquid chromatography (HPLC)

The triacylglycerol profile was analyzed using Waters HPLC e2695 (Milford Massachusetts, USA) separation module with Evaporative Light Scattering Detector (ELSD). The stationary phase was a pre-coated silica reversed phase C18 HPLC column, Lichro CART 5 μ m (4 mm $\times$ 25 cm) from Merck (Darmstadt, Germany). Gradient mobile phase of acetone (A) and acetonitrile (B) was used to elute the sample at a flow rate of 1 mL/min with a total run time of 65 min. The gradient phase was as follows: 0–8 min (100%B), 8–15 min (25%A, 75%B), 15–35 min (25%A, 75%B), 35–40 min (100%B), 40–65 min (100%B). Column temperature was set at 35°C . For analysis, 100 mL of the sample was dissolved in 900 mL of acetone and 10 μ l aliquots were injected for acylglycerol analysis. Peak identification was done by calculating the ECN value. The calculation of ECN value was (Equivalent Carbon Number) = CN (carbon number) - 2DB (double bond). Tridodecanoate, tripalmitin and trioleate standard were used as reference standard for peak identification from chromatogram.

P-MLCT yield is the individual triacylglycerol containing both the medium (C8-C12) and long chain fatty acid (C12-

C20) attached to glycerol backbone. This includes triacylglycerol of structure: MLM, MML, LMM, LLM,

LML, MLL where M represents MCFA and L represents LCFA. The P-MLCT yield was calculated based on:

$$\text{P-MLCT Yield} = \left[(\text{MLM} + \text{MML} + \text{LMM} + \text{LLM} + \text{LML} + \text{MLL}) / \text{Total Acylglycerol} \right] \times 100\%$$

Determination of fatty acid composition (FAC) using gas chromatography

FAME was prepared based on (O'Fallon et al. 2007) method. Oil sample (20ul) was placed in 15 ml centrifuge tube together with 0.7 ml 10 N KOH in water and 5.3 ml methanol. The tube was then incubated at 55 °C water bath for 1.5 h with vigorous shaking every 20 min to properly permeate, dissolve and hydrolyze the sample. After cooling to room temperature under cold tap water, 0.58 ml of 24 N H₂SO₄ was added. The tube was mixed by inversion and with precipitated K₂SO₄ present, was incubated again at 55 °C water bath for 1.5 h with 5 s hand shaking every 20 min. The tube was cooled under tap water to room temperature. Three ml of hexane was added and vortexed for 5 min. The hexane layer containing FAME was placed in chromatography vial to be analyzed in gas chromatography. The derivatized FAME was analyzed with Agilent (Santa Clara California, USA), gas chromatography A2890 equipped with Flame Ionization Detector (FID). Separation was carried out using capillary column BPX70 70 % Cynopropyl Polysilphenylene-siloxane (SGE Analytical Science, Ridgewood Victoria, Australia), 30 m in length, with internal diameter of 0.32 um and nitrogen as carrier gas. The injector and detector temperature were set at 250 and 280 °C respectively and split ratio of 15:1 was used. Oven temperature was programmed as follows: holding at 150 °C for 0.5 min, 150 to 180 °C at rate of 10 °C/min, 180 to 220 °C at rate of 1.5 °C/min, 220 to 260 °C at rate of 30 °C/min and hold at 260 °C for 5 min. Duplicate analysis was performed on each of the sample. Results were expressed as mean ± standard deviation.

Analysis of thermoprofile by differential scanning calorimeter (DSC)

The crystallization and melting thermograms of the P-MLCT oil before and after interesterification was determined using Perkin Elmer DSC 8000 (Waltham Massachusetts, USA). Nitrogen gas was purged at flow rate of 20 min/mL. An empty aluminium pan was used as reference. 5.0–6.0 mg of sample was accurately weighed in aluminium pan and hermetically sealed with cover. Sample was subjected to the following temperature programme. Firstly, the sample was heated to 80 °C and held for 5 min. This was to destroy any crystal

memory contained in sample. Thereafter, it was cooled to –50 °C at rate of 5 °C/s and held at –50 °C for 10 min to obtain the crystallization thermogram. The sample was then heated from –50 °C to 80 °C at a rate of 5 min/s and held at 80 °C for 10 min. An effort was made to ensure that all samples were of the same weight but not identical. All determinations were carried out with duplicate analysis.

Results and discussion

Screening of parameter range

Prior to conducting RSM, a screening process was carried out on the parameters which were important for interesterification reaction in order to determine the operational and range of interest for P-MLCT yield. This include: substrate ratio PKO: PO (20:80–100:0 w/w), reaction time (0–10 h), reaction temperature (50–75 °C) and Lipozyme TLIM lipase load (5–15 % w/w). Substrate ratio PKO: PO of 20:80, 40:60, 50:50, 60:40, 80:20 and 100:0 (w/w) gave P-MLCT yield of 14.40 %±0, 34.15 %±0.39, 42.22 %±0.49, 51.84 %±0.30, 59.08 %±0.04, and 54.51 %±0.57, respectively. The substrate ratio ranges of 40:60 (w/w) to 100:0 (w/w) were chosen for the RSM, eliminating the substrate ratio of PKO: PO 20:80. Reaction time of 0.5 to 7.5 h were chosen for RSM as drastic changes in the P-MLCT yield can be seen from 0 h to 3rd hr (48.62 %±0.58, 59.29 %±1.46, 62.03 %±0.52, 59.50 %±0.81) of the reaction and become stable from 4th hr onwards (≈60 %). Reaction time of 7.5 h was chosen as most enzymatic interesterification completed around this period. Lipozyme TLIM lipase load and reaction temperature did not affect much of the P-MLCT yield but it was included as well in RSM to see their interaction, with other factors. The Lipozyme TLIM lipase load and reaction temperature chosen were 5–15 % (w/w) and 50–75 °C, respectively. Temperatures and enzyme loads that were too high were not considered during the scaling up process as it will incur higher operational cost.

Model fitting

In this study, FCCD of three levels and four factors was employed to determine P-MLCT yield and by-products (FFA, MAG, DAG) produced. Similar studies had being

conducted using FCCD for enzyme TLIM-catalysis of acidolysis reaction (Hamam and Budge 2010; Öztürk et al. 2010; Elibal et al. 2011). FCCD has only three levels compared to rotatable central composite design (RCCD) which has five levels, making the experiment easier to conduct and reduced the experimental error caused by setup and operation. FCCD was selected when the region of interest falls under the region of operation. The 30 experimental runs of three levels and four variables are shown in Table 2. The best fitting models were determined through multiple linear regression with backward elimination where insignificant factors and interactions were removed from the models. Among the various models (linear, 2 level factorial, quadratic, cubic), enzymatic interesterification for P-MLCT production fitted best with quadratic model (modified model). The goodness of fit model

was determined by coefficient determination, R^2 . A good model will have higher value of R^2 , but by evaluating only the value of R^2 can lead to poor prediction. R^2 value will increase when variables are added to the model regardless of whether the variable is statistically significant or not. Thus, it is more reliable to look into the value of adjusted R^2 and predicted R^2 value. In this model the value of adjusted R^2 and predicted R^2 value for P-MLCT yield and by products (FFA, MAG, DAG) were 0.9979, 0.9948 and 0.9892, 0.9784, respectively. ANOVA table for both responses are shown in Table 3. The model had insignificant lack of fit ($P>0.05$). This showed that the quadratic model with backward elimination was highly significant and sufficient to show the relationship between both responses and the variables in this study. Second order equation for P-MLCT yield and by products were as follows:

$$Y_{\text{P-MLCT yield}} = +53.89 + 12.01A + 2.10B + 0.20C + 0.77D - 0.47AB - 1.29BC - 0.91BD - 6.44A^2 - 2.38B^2$$

$$Y_{\text{by-products}} = +6.82 + 3.19A + 0.032B + 1.43C + 0.064D - 0.34AC + 0.55BC + 0.25BD + 1.42A^2 + 0.68C^2$$

The degree of four parameters on EIE reaction for MLCT production

Table 3 shows the regression coefficient and P value for P-MLCT production with backward elimination of P-MLCT yield. All the four factors positively affected the P-MLCT yield. Based on the regression coefficient, P-MLCT production was greatly affected by the substrate ratio of PKO: PO, followed by reaction time, reaction temperature, and enzyme load. Enzyme load was the only parameter that had insignificant effect with p-value of 0.1992 ($P>0.05$). This finding was similar to the study on incorporation of CA in corn oil using TLIM lipase, showing the yield was strongly affected by substrate ratio followed by reaction time and less affected by enzyme load (Öztürk et al. 2010). Enzyme only speed up reaction but does not affect the yield. From the 30 experimental runs, the highest P-MLCT yield obtained was 60.58 and 60.53 %, respectively while lowest yield was 25 %. P-MLCT (≈ 61 %) can be obtained at the conditions of substrate ratio PKO:PO (100:0), 50 °C, 5 % enzyme load and reaction time of 7.5 h. As for the by-products' response, it was significantly affected by both substrate ratio and enzyme load.

Single factor response

Figure 1 shows the effect of all the single factors on P-MLCT yield. As the amount of substrate ratio of PKO:PO increased, P-MLCT yield also increased, to a certain point before stabilizing. In this study, PKO was interesterified with PO using Lipozyme TLIM lipase in solvent-free media. Since the fatty acid composition of PKO consists mainly of 60 % MCFA and 40 % LCFA,

an increase in the amount of PO which consists of 99 % of LCFA, will dilute the content of MCFA. Thus, P-MLCT yield will be affected by the substrate ratio of PKO: PO as expected.

For reaction time variable, P-MLCT yield reached its maximum point within 4–5.75 h and further increase in reaction time of more than 5 h has no effect on the P-MLCT yield. This may be because from 4 to 6 h, both interesterification reaction and hydrolysis reaction has reached a steady-state. DAG formation which is the product of TAG hydrolysis and the key component for the FFA to attach to it, forming a new TAG, is at its maximum starting from 6 h to 10 h and thereafter reached an equilibrium state till 24 h (Zhang et al. 2001). Similar results also showed that TLIM catalysed transesterification of vegetable oil reached its maximum yield at 6 h (Zhang et al. 2001; Ihsan et al. 2007; Hamam and Budge 2010). Although some studies conducted their EIE reactions for 24 h, it is not preferable as longer reaction time will lead to randomization of fatty acids in the glycerol backbone (Zhang 2007). Besides, prolonged reaction time will increase the formation of by-products. This is because reaction catalysed by 1,3 specific TLIM lipase will produce 1,3-diacylglycerol and 1-monoacylglycerol due to acyl migration which eventually will be hydrolysed by lipase into free fatty acid and glycerol when reaction time is extended.

Temperature has only slight affect on the P-MLCT yield. An increase in reaction temperature from 50 to 75 °C will only slightly increase the MLCT yield. Similarly, studies also showed that temperature ranging from 50 to 75 °C did not affect the yield of Lipozyme TLIM catalysed palm stearin and coconut oil (75/25w/w) in a batch reactor system. This is

Table 2 FCCD for 30 experiments run and experiment data for the response: P-MLCT yield and by products (FFA, MAG, DAG)

Run	Independent variables ¹				Response (%) ²	
	PKO	Ti	En	T	P-MLCT	FFA + MAG + DAG
1	−1	1	1	−1	33.50	7.78
2	1	1	1	−1	58.94	13.31
3	−1	1	−1	−1	35.5	3.32
4	0	0	0	−1	53.73	6.99
5	1	−1	−1	1	57.01	11.21
6	−1	1	−1	1	35.70	3.51
7	1	1	1	1	58.54	13.91
8	0	0	0	0	53.70	7.12
9	1	1	−1	1	60.53	10.85
10	0	0	1	0	52.93	9.06
11	−1	−1	1	−1	29.31	6.80
12	−1	1	1	1	33.31	8.83
13	0	0	0	0	53.44	6.93
14	0	0	0	0	53.81	7.00
15	1	−1	1	−1	56.64	12.87
16	0	0	0	1	53.91	7.29
17	−1	−1	−1	1	29.74	4.20
18	0	−1	0	0	49.32	6.97
19	0	0	0	0	53.82	7.14
20	0	0	−1	0	53.95	6.09
21	1	0	0	0	60.51	11.20
22	1	−1	−1	−1	53.63	11.86
23	−1	0	0	0	34.98	5.44
24	−1	−1	−1	−1	25.25	4.63
25	1	1	1	1	58.67	12.86
26	1	−1	−1	−1	60.58	10.30
27	−1	1	1	1	33.57	6.36
28	0	0	0	0	54.39	6.10
29	0	0	0	0	54.29	6.52
30	0	0	0	0	54.63	6.03

¹ Independent variables. Substrate ratio palm kernel oil: palm oil (PKO: PO), Reaction time, hr (Ti), Enzyme load % (En), Temperature °C (T)

² Response. MLCT = medium-long-chain-triacylglycerol, FFA = free fatty acid, MAG = monoacylglycerol, DAG = diacylglycerol

because enzyme TLIM act optimumly at this range of temperatures as can be seen in other studies that utilized Lipozyme TLIM lipase for EIE (Zhang et al. 2001; Elibal et al. 2011). In contrast, Hamam and Budge (2010) revealed that an increase in 10 °C in temperature managed to double the yield of CA being incorporated in fish oil in a single packed bed reactor in the presence of RMIM (Mu et al. 1998). This may be due to different types of enzyme used. Temperature higher than 75 °C is not preferable either as it will render the enzyme inactive due to protein denaturation. Besides, higher temperature will easily lead to lipid peroxidation and affect the

Table 3 ANOVA table of P-MLCT yield and by-products

Factors ²	Percentage ¹				
	SS	DF	MS	F value	Prob > F
Model	3680.40	9	408.94	1047.44	<0.0001
Residual	7.81	20	0.39	NA	NA
Lack of fit test	6.79	15	0.45	2.23	0.1915
Pure error	1.01	5	0.20	NA	NA
A	3046.94	1	3046.94	7804.42	<0.0001
B	79.17	1	79.17	202.79	<0.0001
C	0.69	1	0.69	1.76	0.1992
D	10.73	1	10.73	27.49	<0.0001
A*B	3.52	1	3.52	9.00	0.0071
B*C	26.47	1	26.47	67.80	<0.0001
B*D	13.32	1	13.32	34.12	<0.0002
A*A	143.01	1	143.01	366.31	<0.0003
B*B	19.57	1	19.57	50.12	<0.0004
By products					
Model	256.30	9	28.48	204.24	<0.0001
Residual	2.79	20	0.14	NA	NA
Lack of fit test	1.47	15	0.098	0.37	0.9375
Pure error	1.32	5	0.26	NA	NA
A	183.68	1	183.68	1317.34	<0.0001
B	0.018	1	0.018	0.13	0.7228
C	37.01	1	37.01	265.42	<0.0001
D	0.075	1	0.075	0.54	0.4725
A*C	1.81	1	1.81	12.97	0.0018
B*C	4.91	1	4.91	35.19	<0.0001
B*D	0.96	1	0.96	6.89	0.0162
A*A	6.96	1	6.96	49.91	<0.0001
C*C	1.58	1	1.58	11.30	0.0031

¹ Statistical value. SS = sum of square, DF = degree of freedom, MS = means of square, NA = non available

² Factors involved. A = substrate ratio PKO: PO (w/w), B = reaction time (hr), C = enzyme load (w/w), D = temperature (°C)

quality of oil as well as incur a higher consumption of energy during scaling up purposes.

There was little change in the P-MLCT yield when enzyme load increased from 5 % (w/w) to 15 % (w/w). When substrate PKO and PO is in limited amount, increase in the amount of enzyme will only speed up the reaction time but has no effect on the yield of P-MLCT. High concentration of enzyme will definitely provide abundant activation site for the substrate to react. However, there is a saturation point for the yield where increase in the enzyme load have no effect on the amount of yield as the amount of substrate is in limited conditions. Thus, since there is no difference on the P-MLCT yield produced using either 5, 10, or 15 % of enzyme load, it is much preferable to use the minimum amount of enzyme during upscaling for economic purposes. This was consistent

Fig. 1 Perturbation graph showing the single factors effect on P-MLCT yield. **a** substrate ratio PKO: PO, **b** reaction time, **c** enzyme load **d** temperature

with Zhang (2007) study showing that 6 % of enzyme load was sufficient to obtain a stable degree of interesterification. Similarly, 8 % of enzyme TLIM lipase gave the highest yield of lard based- biodiesel and no significant increase in the amount of yield even with the presence of extra enzyme (Huang et al. 2010).

FFA, MAG, and DAG were by-products in this study and their amount was significantly ($P < 0.05$) affected by substrate ratio PKO:PO and enzyme load as shown in Table 3. In this experiment, the by-products such as FFA, MAG, and DAG should be kept as low as possible at the end of reaction in order to obtain a purer P-MLCT. By-products produced increased with enzyme load. This may be due to the silica coating of the immobilized TLIM lipase which is hydrophilic in nature. As such, TAG are more prone to hydrolyse in the presence of higher amounts of enzyme TLIM producing either FFA, MAG, or DAG. Medium chain fatty acid (MCFA) which are shorter in chain length and smaller in size compared to long chain fatty acid (LCFA) which are longer in chain length and larger in size undergoes hydrolysis more easily than the latter. As such, when the amount of PKO increased it will indirectly cause a rise in the by-products produced due to the hydrolysis of MCFA contributed by PKO.

Relationship between the factors

Figure 2a shows the relationship between enzyme load and reaction time on P-MLCT yield. The response surface plot demonstrated that enzyme load only speed up the reaction but

not the P-MLCT yield. At higher amounts of enzyme load (15 %), P-MLCT yield reached its maximum point within a shorter period of time (4 h) compared to lower amount of enzyme load (5 %) which required 7.5 h to reach its maximum point. This may be because at higher enzyme load, more activation site are available for the substrate to react, causing a higher rate of TAG hydrolysis which produced more partial glycerides that are consequently used as substrate for P-MLCT production. However, at higher enzyme load, when the reaction proceeded to more than 5 h, MLCT yield started to decrease which may be due to the increase in the formation of by-products caused by hydrolysis reaction in the system. Hydrophilic nature of the silica gel of the enzyme caused an increase in the water activity of the system when enzyme TLIM are present in higher amounts. This condition was not seen at 5 % enzyme load.

Figure 2b showed the response surface plot on the relationship between reaction time and temperature which affected the P-MLCT yield. MLCT yield increased with increase in temperature. This supported the theory of Arrhenius equation, showing the direct effect of temperature on the rate of reaction. Less time was needed to reach equilibrium when a higher

Fig. 2 The effect of interaction between factors on P-MLCT yield. **a** Enzyme load (w/w) and reaction time versus MLCT yield. **b** Reaction temperature and reaction time versus MLCT yield

◀ **Fig. 3** Chromatogram showing PKO: PO (90:10 w/w) before and after enzymatic interesterification reaction. **a** before enzymatic interesterification reaction, **b** after enzymatic interesterification reaction. MAG/DAG = mono and diacylglycerol, C = caprylic, Ca = capric, La = lauric, M = myristic, P = palmitic, O = oleic, L = linoleic

temperature is used. P-MLCT yield reached its maximum point in shorter period of time at higher temperatures compared to lower temperatures.

Optimization of MLCT production

Through numerical optimization using DOE software, among all the PKO: PO substrate ratios, substrate ratios of (100:0 w/w) and (90:10 w/w) gave the highest and almost similar amount of P-MLCT with both the ratios having highest desirability of 0.976 and 0.975. Substrate ratio PKO: PO (100:0 w/w) give 61 % P-MLCT whereas substrate ratio PKO: PO (90:10 w/w) gives 60 % P-MLCT. Though substrate ratio PKO: PO (100:0 w/w) gave similar amount of P-MLCT, PKO:PO (90:10 w/w) was selected because incorporation of PO will produce a much healthier MLCT due to the higher MUFA and PUFA content in PO instead of solely PKO. The optimized reaction conditions selected in this study when substrate ratio PKO: PO (90:10 w/w) was used were: reaction time 7.26 h, reaction temperature 50 °C in the presence of 5 % of enzymes. These conditions manage to produce 60 % P-MLCT. The optimized conditions gave desirability of 0.9986. P-MLCT yield before reaction were initially around 40 %. An increase of 20 % of P-MLCT was obtained through RSM. To further verify the results obtained, confirmation run was carried out prior up scaling up process and it gave similar P-MLCT yield as obtained from RSM study.

Pilot scale production of MLCT

The result obtained from RSM of substrate ratio PKO: PO (90:10 w/w) and its optimum conditions were further used in 10 kg pilot scale in stirred tank batch reactor for large scale production of P-MLCT. Five batches of consecutive pilot scale production of P-MLCT gave 57.61 %±1.27, 58.97 %±1.92, 60.71 %±4.10, 58.51 %±1.72, and 57.91 %±2.31 of P-MLCT, which is almost similar with those determined during optimization in bench scale. The amount of P-MLCT before reaction was initially around 40 %. An increase in 20 % of MLCT was obtained after RSM.

Effect of enzymatic interesterification reaction on physicochemical properties

Acyl glycerol composition

Fats and oils are made up of complex TAG compositions. Figure 3a and b show the TAG chromatogram finger print of

PKO: PO (90:10 w/w) before and after interesterification, respectively. The TAG species is identified based on the retention time and equivalent carbon number (ECN). TAG species that falls under the categories of P-MLCT were those of MLM (LaLaL, LaLaM, LaLaO, LaLaP) and MLL type (LaMM, LaOL/LaPL, LaMO, LaMP, LaOO/LaPO/LaPP). An increase of 20 % in the yield clearly showed that interesterification reaction has taken place. Based on the chromatogram of substrate ratio PKO:PO (90:10 w/w) in Fig. 3a, the major TAG species present before the enzymatic reaction were LaLaLa, LaLaM, LaLaP/LaMM, LaLaO while major TAG species after reaction include LaLaLa, LaLaM, LaLaO, LaLaP/LaMM, LaMO, LaMP, MMO. The enzymatic interesterification caused changes in some of the TAG species. A new peak LaLaL appeared after interesterification. A decrease in TAG species of LaLaLa, LaMM and an increase in the TAG species of LaLaO, LaLaP/LaLaM, LaOL/LaPL, LaMO, LaMP, LaOO/LaPO/LaPP can be seen after the enzymatic reaction. This showed that palmitic acid (C16), oleic acid (C18:1), and linoleic acid (C18:2) contributed by the PO has been successfully interesterified with the major fatty acid of lauric acid (C12) from PKO. Incorporation of PO in the PKO for interesterification is beneficial as it helps to make the P-MLCT oil more nutritious due to the presence of mono-unsaturated fatty acid and at the same time managed to maintain the MCFA composition in the MLCT-rich oil which is important in order to prevent fat accumulation in the body.

Table 4 FAC of PKO, PKO: PO (90:10 w/w) before and after enzymatic interesterification

Type of fatty acid ²	Amount (%) ¹		
	PKO	Blend	IE
C8	2.831±0.00	2.618±0.06	2.278±0.17
C10	3.091±0.02	2.811±0.02	2.653±0.01
C12	47.58±0.01	42.92±0.05	41.49±0.03
C14	16.58±0.01	15.01±0.03	14.89±0.03
C16	8.418±0.01	11.75±0.04	12.40±0.06
C18	2.192±0.00	2.348±0.01	2.412±0.03
C18:1	16.43±0.01	18.85±0.03	19.72±0.10
C18:2	2.858±0.01	3.608±0.02	3.758±0.01
SFA	80.69	77.46	76.12
MUFA/PUFA	19.29	22.46	23.48

¹ Types of fatty acid. SFA = saturated fatty acid, MUFA/PUFA = mono/polyunsaturated fatty acid. Each value in table represents the mean ± standard deviation of sample analysis from duplicate analysis

² Types of oils. PKO: Raw PKO, Blend = before enzymatic interesterification, IE = after enzymatic interesterification

Fatty acid composition (FAC)

FAC of the PKO: PO (90:10 w/w) before and after interesterification as well as raw PKO is shown in Table 4. From the result, there was no significant difference in the FAC before and after enzymatic interesterification of PKO: PO (90:10w/w). This demonstrated that enzymatic modification does not alter the FAC composition of the oil. When compared to FAC of the raw PKO, it showed an increase in the mono-unsaturated fatty acid (MUFA) and a decrease in the saturated fatty acid (SFA) content. The SFA and MUFA + PUFA content for raw PKO, before (blend) and after enzymatic interesterification (IE) of substrate ratio PKO:PO (90: 10 w/w) were 80.71 %, 77.46 %, 76.12 % and 19.29 %, 22.46 %, and 23.48 %, respectively. The presence of essential fatty acid (MUFA + PUFA) in P-MLCT oil is important as studies had shown that it has blood cholesterol reducing effect. Studies had shown that substituting SFA with MUFA instead

of PUFA was more beneficial to health. High PUFA diet can be harmful due to the susceptibility of PUFA to undergo lipid peroxidation, releasing reactive aldehyde which can cause in vitro oxidation and modification of low density lipoprotein and consequently resulting in atherosclerosis (Reaven et al. 1993). This is consistent with our objective to reduce SFA and increase MUFA and PUFA content. From Table 4, C18:1 composition for raw PKO, before and after interesterification of PO: PKO (90:10 w/w) were 16.42 %, 18.85 %, and 19.72 % whereas for C18:2 composition were 2.862 %, 3.608 %, and 3.758 %, respectively. This shows that blending of PO with PKO manage to increase the MUFA and PUFA content. On the other hand, SFA which consisted mostly of MCFA (C8, C12, C10, and C14) should not be totally eliminated in this study as they are equally important to induce the fatty acid metabolism rate in the body which is vital for reducing the body fat accumulation and body weight gain.

Fig. 4 DSC thermoprote of raw PKO, PKO: PO (90:10 w/w) before and after enzymatic interesterification reaction. Melting profile (*top*), crystallization profile (*bottom*). Blend = before enzymatic interesterification

reaction, IE = after enzymatic interesterification reaction. 100 PKO = Raw PKO, 1&2 = same sample with duplicate run

Thermal profile

The thermal profile of fats and oils is determined by its complexity of the TAG species. Oils do not have a specific melting point but in fact they melt in a temperature range. Figure 4 shows the melting and crystallization thermograms of substrate ratio PKO: PO (90:10 w/w) before and after enzymatic reaction as well as raw PKO. The melting endotherms of the substrate ratio PKO: PO (90:10 w/w) oil before and after enzymatic reaction as well as raw PKO had the similar pattern. There was overlapping peak on the melting transition with smaller lower temperature and larger higher temperature endotherms. The overlapping may be due to the melting of two or more TAG species which occurred simultaneously during heating. The lower melting peak may be due to the SSU content of TAG species (LaLaL, LaLaO, LaMO, LaPO, MMO) and SUU species (LaOL/LaPL, LaOO) whereas higher melting peak consist of SSS content of TAG species (LaLaLa, LaLaM, LaLaP/LaMM and LaMP/MMM, MMP, LaPP) and small melting peak of UUU TAG species (OOO) present in small amount. The endotherms showed a limited endothermic region representing the start of melting point and end of melting point with the former having T_{on} of $-20\text{ }^{\circ}\text{C}$ and T_{off} of $29\text{ }^{\circ}\text{C}$ and later T_{on} $18\text{ }^{\circ}\text{C}$ and T_{off} of $29\text{ }^{\circ}\text{C}$, respectively. This suggested that P-MLCT oil is able to melt completely below body temperature of $37\text{ }^{\circ}\text{C}$. This characteristic is important especially in confectionary industry as it will completely melt in the mouth without giving a waxing after feeling. On the other hand, there is a difference in the crystallization profile of after enzymatic interesterification. The crystallization peak of both PKO: PO (90:10 w/w) was $0.99\text{ }^{\circ}\text{C}\pm 0.33$ and $7.33\text{ }^{\circ}\text{C}\pm 0.21$ (before) and $4.1\text{ }^{\circ}\text{C}\pm 0.31$ and $9.45\text{ }^{\circ}\text{C}\pm 0.36$ (after), respectively. As for melting peak, before enzymatic interesterification reaction, it has three melting peaks of $-14.26\text{ }^{\circ}\text{C}\pm 0.35$, $8.82\text{ }^{\circ}\text{C}\pm 0.32$, $26.28\text{ }^{\circ}\text{C}\pm 0.35$ while enzymatic reaction caused it to have only one melting peak at $25.72\text{ }^{\circ}\text{C}\pm 0.09$.

Conclusions

Palm oil and palm kernel oil can be utilized for the production of structured P-MLCT through enzymatic interesterification process. FCCD model used in the experiment is feasible for the optimization of P-MLCT which produced at least 60 % P-MLCT at the following conditions: substrate ratio PKO: PO (90:10 w/w), reaction temperature of $50\text{ }^{\circ}\text{C}$, 5 % (w/w) TLIM lipase for 7.26 h. Physiochemical characteristics of this P-

MLCT oil suggested that it can be utilized for various applications in food industries such as confectionary, margarine and shortening which are beneficial to health.

Acknowledgments The authors would like to thank Sime Darby Research Sdn Bhd for their financial support.

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