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Lipase-catalyzed acidolysis of palm olein and caprylic acid in a continuous bench-scale packed bed bioreactor

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Abstract

Enzymatic acidolysis of refined, bleached and deodorized (RBD) palm olein with caprylic acid was carried out in a continuous packed bed bioreactor to produce structured lipid (SL) that can confer metabolic benefits when consumed. Lipozyme® IM 60 from *Rhizomucor miehei*, a 1,3-specific lipase, was used as the biocatalyst in this study. After 24 h of reaction, 30.5% of the total fatty acid content of the modified oil was found to be caprylic acid, indicating its incorporation into the palm olein. The triacylglycerols (TAGs) of palm olein after acidolysis were separated and were characterized by seven clusters of TAG species with equivalent carbon number (ECN), C28, C30, C32, C34, C36, C38 and C40. Caprylic–oleic–caprylic TAGs were predicted in cluster C32, which recorded the highest amount, with 35.3% of the total TAG. Fatty acid composition at the *sn*-2 position was determined, by pancreatic lipolysis, as C8:0, 9.2%; C12:0, 2.3%; C14:0, 1.8%; C16:0, 21.3%; C18:0, 4.7%; C18:1, 60.7%. Iodine value (IV), slip melting point (SMP) and differential scanning calorimetric (DSC) analyses of SL were also performed. In IV analysis, SL recorded a drop of value from 60.4 to 48.2 while SMP was reduced from 13 to 4.2 °C, in comparison to RBD palm olein. DSC analysis of SL gave a melting profile with two low melting peaks of −15.97 and −11.78 °C and onset temperatures of −18.43 and −14.03 °C, respectively.

Keywords: Acidolysis; Caprylic acid; RBD palm olein; Enzymatic synthesis; Lipozyme® IM 60; Packed bed bioreactor; Structured lipids

1. Introduction

Today, structurally defined lipids have been developed to meet the demand of health conscious consumers. These oils contain structured lipids (SLs), i.e., lipids that have been restructured to change the positions and composition of fatty acids from the native state. In this context, SLs are triacylglycerols containing short-chain or medium-chain, or both, and long chain fatty acids, preferably on the same glycerol molecule to exhibit maximum efficiency (Akoh, 1995). SLs of such kind are not available in nature and cannot be produced by chemical reactions. They can only be synthesized by enzymatic methods using *sn*-1,3 specific lipase (Fomuso & Akoh, 1997, 1998). The applications of SL have attracted much attention and this has resulted in the increase of interest in producing modified lipid (Akoh, 2000; Mu, Xu, & Hoy, 1998; Rhee & Kim, 1991; Schmid, 1998; Xu, Balchen, Jonsson, & Adler-Nissen, 2000a, Xu, Fomuso, & Akoh, 2000b).

Medium chain triacylglycerols (MCT) belong to the family of triacylglycerols that consists of caprylic acid (C_8) and capric acid (C_{10}), with caproic acid (C_6) and lauric acid (C_{12}) in minor amounts (Bach & Babayan, 1982). Compared to long chain triacylglycerols (LCT), MCTs are less likely to be deposited as body fat during metabolism because they are not readily re-esterified into triacylglycerol (TAG) (Geleibter, Torbay, Bracco, Hashim, & Van Itallie, 1983). Also, MCTs are metabo-

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lized as fast as glucose and have caloric densities two twice higher than protein or carbohydrate (Babayan, 1987). This finding has contributed to the utilization of MCTs as rapid energy sources due to their quick absorption. It is also used as an alternative solution for consumers with digestive problems.

In the case of SL, medium chain fatty acids are located at the *sn*-1,3 positions and an essential long chain (LC) fatty acid is at the *sn*-2 position of the glycerol backbone. This unique combination gives a quick energy effect and other health benefits. During metabolism, 2-monoacylglycerol (MAG) is produced by pancreatic lipase digestion and is the main carrier of fatty acids through the intestinal wall. Therefore, the absorbency of long chain fatty acid esterified at the *sn*-2 position is more efficient than those esterified at *sn*-1 and *sn*-3 positions.

In our work, refined, bleached and deodorized (RBD) palm olein was chosen as the acidolysis substrate because of its high oleic acid content. Research has shown links between high dietary intakes of oleic acid with reduction in the levels of low density lipoprotein (LDL) and blood cholesterol, leading to the prevention of various cardiovascular diseases. By using a 1,3-specific lipase, caprylic acid can be inserted at the *sn*-1,3 position of RBD palm olein, while retaining oleic acid at the *sn*-2 position for more efficient absorptivity during metabolism.

Thus, the objective of this research was to synthesize and characterize SL produced from acidolysis between palm olein with caprylic acid by using Lipozyme® IM 60 as a biocatalyst in a packed bed bioreactor. The SL was produced under optimal conditions and the product mixture purified by short path distillation. The reaction yield and product characterization were investigated using high performance liquid chromatography (HPLC), gas chromatography (GC), pancreatic lipolysis, iodine value (IV), slip melting point (SMP) and differential scanning calorimetry (DSC) methods.

2. Materials and methods

2.1. Materials

Caprylic acid and fatty acid methyl ester (FAME) standard were obtained from Aldrich Chemical Company (Milwaukee, WI). RBD palm olein was a generous gift from the Malaysian Palm Oil Promotion Council (MPOPC, Kuala Lumpur, Malaysia). Immobilized *sn*-1,3 specific lipase (Lipozyme IM 60) was obtained from Novo Nordisk Biochem North America Inc. (Franklinton, NC). Acetone, acetonitrile, and hexane were purchased from Fisher Scientific (Norcross, GA). All solvents used were of HPLC or analytical grade.

2.2. Bioreactor set-up

The esterification reaction was carried out on the pilot scale once, with the parameters used and the set-up of the bioreactor similar to that previously reported (Akoh and Fomuso, 2002; Akoh et al., 2000; Xu, Balchen, Høy, & Adler-Nissen, 1998). The bioreactor had a jacketed stainless steel column with dimensions of 47 mm (i.d) × 50 cm. An FMI Lab pump (model QV) from Fluid Metering Inc. (New York, NY) was used for substrate feeding. The system temperature was maintained by a circulating water bath. Substrates were preheated to a set temperature by a coiled heater before being pumped into the enzyme bed. The column was packed with immobilized Lipozyme IM 60 and the upper and lower ends of the column were layered with glass wool (3.0 and 7.0 cm in thickness, respectively). Air was removed by passing inert nitrogen through the packed bed. The substrates were then pumped through the enzyme bed to condition it overnight before any sampling was done for analysis.

2.3. Acidolysis reaction

Acidolysis reactions, comprised of RBD palm olein and caprylic acid (C_8), were carried out with a molar ratio of 1:5 (RBD palm olein:caprylic acid). This optimized molar ratio was based on research previously reported (Akoh and Fomuso, 2002). Reaction was carried out with the stirring rate set at 200 rpm. Water content was maintained constant during the reaction at 0.1% of total reactants. Temperature and substrate flow rate were set at 60 °C and 1 ml/min, respectively. Regioselective lipase, *sn*-1,3 Lipozyme® IM 60 was added at 10% of total weight of the reactants. Samplings were carried out once 800 ml of product were eluted after 24 h. The reaction mixture was filtered through a sodium sulphate column to remove enzymes and any residual water.

2.4. Short-path distillation

Short-path distillation was carried out to purify the product mixture with a KDL-4 (UIC Inc., Joliet, IL) under the following conditions: heating oil temperature 185 °C, cooling water temperature 10 °C, pump vacuum below 1 mm Hg and feed rate maintained at 100 ml/h. The reaction product was passed through the system twice to reduce the free fatty acid content to an acceptable level, which is defined as less than 0.1% of manufacturing specifications.

2.5. Triacylglycerol identification by HPLC

Sample preparations were made by dissolving 5% of reactant in an appropriate amount of chloroform. Reac-

tion products after purification were analyzed with a SCL-10A VP HPLC system (Shimadzu; Kyoto, Japan) equipped with a Sil-10AD VP auto injector (Shimadzu; Kyoto, Japan) and an ELSD 2000 evaporative light scattering detector (Alltech; Deerfield, IL) which were connected on-line to a Fujitsu computer. The drift tube temperature for ELSD was set at 55 °C, and nebulizer nitrogen gas pressure at 1.30 l/min. A pre-coated silica reversed phase C_{18} HPLC column, LiChroCART®5μm (4.0 mm × 25 cm) from Merck (Darmstadt, Germany) was used as a stationary phase. The column temperature was set at 30 °C. The isocratic mobile phase consisted of acetonitrile and acetone (36.5:63.5, v/v) with a flow rate of 1.0 ml/min. The total HPLC run time was 50 min. Peak identification was based on the polarity and TAG peak separation in commercial RBD palm olein. All analyses were performed in duplicate and average values were reported.

2.6. Gas chromatographic analysis

Samples were dissolved in 0.95 ml of hexane in an Eppendorf tube. Then, 0.05 ml of sodium methoxide in methanol was added for the fatty acid methylation process. The mixture was allowed to stand for 1 h before GC analysis, using GC 14A gas chromatography (Shimadzu; Kyoto, Japan) equipped with a flame ionization detector (FID). Nitrogen was used as the carrier gas and the total gas flow rate was 1.4 kg/cm^2. The oven temperature was initially set at 80 °C for 1 min. This temperature was then increased to 160 °C at 15 °C/min and later to 200 °C at 2 °C/min. It was held isothermally for 5 min at 220 °C. Overall running time was 32 min. The amount of FAME, in molar percentage was analyzed using heptadecanoic acid ($C_{17:0}$) as an internal standard. Fatty acid compositions calculated were based on the percentage peak area of the GC chromatogram. All analyses were performed in duplicates and average values were reported.

2.7. Pancreatic lipase-catalysed sn-2 positional analysis

Reactant samples containing SL were first dissolved in hexane. Two ml of Tris buffer (pH 8.0), 0.5 ml of bile salt (0.1% w/v), 0.2 ml of $CaCl_2$ (2.2% w/v) and 1.0 g of pancreatic lipase were added to the reaction mixture (Luddy, Bradford, Herb, Magidman, & Riemenschneider, 1963). The mixture was then incubated at 40 °C for 3–5 min. One millilitre of ethanol, 1 ml of 6 N HCl and 1 ml of diethyl ether were added and the mixture was vortexed. The sample was centrifuged at 5000 rpm for 5 min and the clear phase was spotted onto a silica gel TLC plate which was then developed in hexane:diethyl ether:acetic acid (50:50:1, v/v). The band corresponding to *sn*-2 monoacylglycerol was scraped from the TLC plate and was extracted twice with chloroform:methanol (95:5, v/v). The *sn*-2 monoacylglycerol (*sn*-2 MG) obtained was methylated and analyzed by GC, as previously described. Identification of *sn*-2 MG was based on FAME standard.

2.8. Differential scanning calorimetry

The melting profile of SL was determined by DSC on a Perkin–Elmer (Norwalk, CT) model DSC 7. A sample of 4–6 mg was hermetically sealed in an aluminium pan with an empty pan serving as a reference. American Oil Chemists' Society (AOCS) Cj 1–94 method was followed with minor modifications. Samples were initially heated at 200 °C/min from 50 to 80 °C and held at this temperature for 10 min. This is to destroy any crystal memory contained in the samples. It is then cooled to −30 °C at 10 °C/min and held for 30 min, and finally heated to 80 °C at 5 °C/min to determine the heating profile. Normal standardization of the instrument was performed with gallium (m.p. = 29.78 °C) as reference standard.

2.9. Other analytical method

Free fatty acid (FFA), slip melting point (capillary tube method) and IV (Wijs method) analyses were done according to the American Oil Chemists' Society (AOCS) Official Method Ca 5a-40, Cc. 3.25 (13) and Cd 1b-87, respectively.

3. Results and discussion

3.1. Fatty acid composition

RBD palm olein contains six major types of fatty acids; these are mainly oleic (C18:1), palmitic (C16:0) and linoleic acid (C18:2), which comprise 95% of the total fatty acid in the oil. Table 1 shows the relative fatty acid composition of the oil. Relative fatty acid contents of RBD palm olein before and after acidolysis were different. After 24 h of reaction at a substrate mole ratio of 1:5, 30.5% of caprylic acid was incorporated into the modified oil. Palmitic, oleic and stearic acid were reduced by 22.9%, 5.3% and 1.8%, respectively, in the

Table 1
Fatty acids (mole percent) in refined, bleached and deodorized (RBD) palm olein before and after enzymatic acidolysis

Fatty acid	Before modification (%)	After modification (%)
8:0	0.00	30.5
12:0	0.28	0.17
14:0	0.92	0.44
16:0	38.8	16.0
18:0	3.67	1.85
18:1	45.5	40.2
18:2	10.8	10.9

modified oil, while linoleic acid content remained unchanged. This may be because the fatty acid specificity of Lipozyme® IM 60 does not favour the hydrolysis of linoleic acid from TAG compared to the other fatty acids available in the oil. It has been discovered that lipase originating from *Rhizomucor miehei* is highly against fatty acids that contain the first double bond from the carboxyl end at an even numbered carbon, which include *cis*-4, *cis*-6 and *cis*-8 double bonds (Murkerjee, Kiewitt, & Hills, 1993). Although linoleic acid has double bonds in position *cis*-9 and *cis*-12, the latter double bond may confer selectivity to the lipase against linoleic acid as it is located at an even numbered carbon. As a result, hydrolysis of these fatty acids is much slower than other fatty acids. Selmi, Gontier, Ergan, and Thomas (1998) reported that the rate of TAG synthesis from esterification involving linoleic acid with glycerol by Lipozyme® IM 60 is much lower than these involving oleic and stearic acids. It is clearly shown that unsaturation of fatty acid is responsible for slower hydrolysis and esterification reactions with linoleic acid.

3.2. HPLC peak identification

The TAGs of RBD palm olein were characterized by HPLC. Fig. 1(a) and (b) show the TAG profiles of palm olein before and after acidolysis, respectively. After acidolysis, TAGs of palm olein were separated and identified according to their equivalent carbon number (ECN). The isocratic elution in the HPLC system had an advantage in that a mathematical correlation existed between the retention time (t_R) and the ECN of a molecular species (Fig. 2). It was observed that the logarithm of the elution volume of a TAG was directly proportional to the total number of carbon atoms without glycerol (CN) and inversely proportional to the number of double bonds (X) in the three fatty acyl chains. Hence, higher ECN value is caused by an increase in the respec-

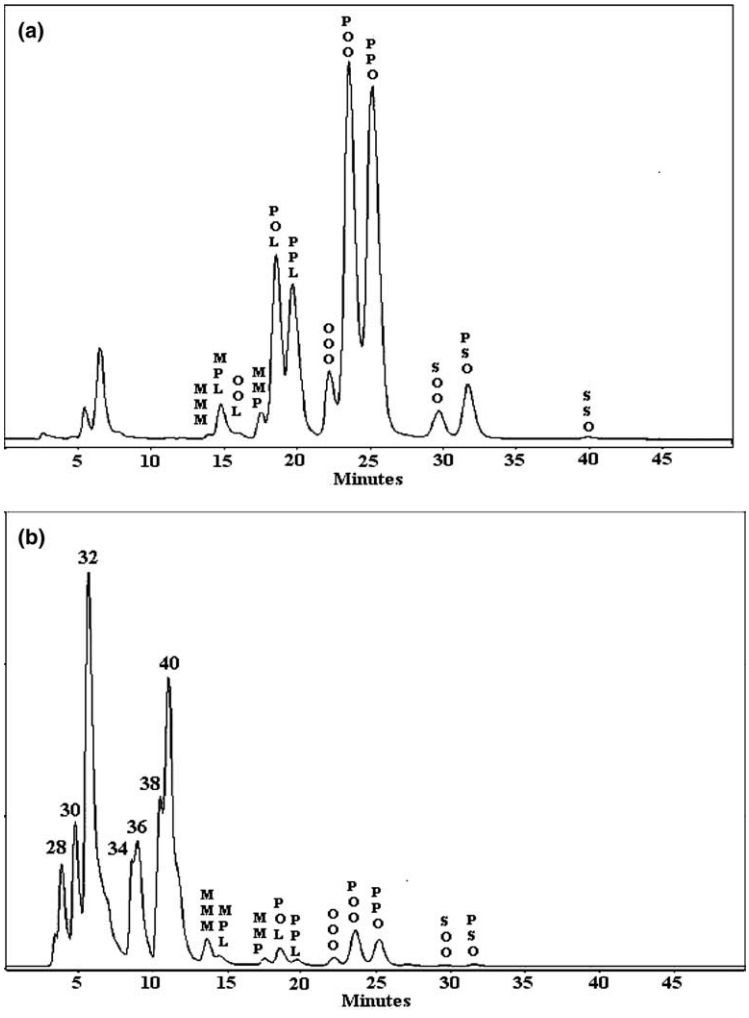

Fig. 1. HPLC separation chromatogram for (a) unmodified RBD palm olein and (b) the modified product mixture from packed bed bioreactor. Peaks are identified according to the published work involving palm olein (Tan, 2001) and equivalent carbon number (ECN), as shown in Table 2. For abbreviation, please refer Table 2.

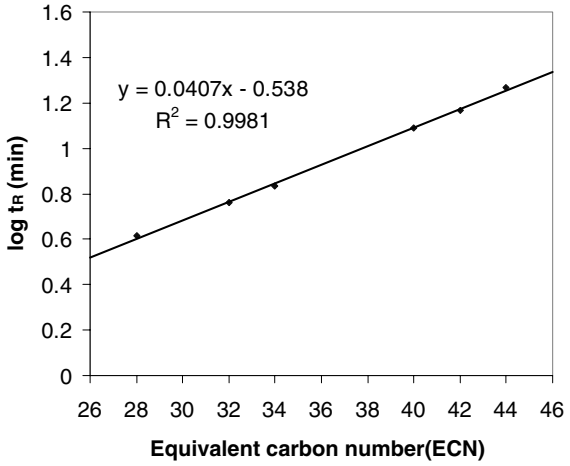

Fig. 2. Correlation between equivalent carbon number (ECN) of triacylglycerols and retention times (t_R) by HPLC.

tive fatty acid carbon chain length in a TAG, while a decrease in both the degree of unsaturation and the polarity of the TAG itself will give a lower ECN value. This can be defined as

$$\mathrm{ECN} = \mathrm{CN} - \mathrm{X}(n),$$

where n is the factor for double bond contribution, normally close to 2. No distinction is made here between TAGs that are positional isomers (Marini, 2000).

The modified palm olein is characterized by seven major clusters of TAG species with ECN, C28, C30, C32, C34, C36, C38 and C40 (Fig. 1(b)). Table 3 shows the possible TAG in each cluster. C32 was found to have the highest content with 35.3% of the total TAG (Fig. 1). The caprylic–oleic–caprylic TAG was predicted to be in this cluster, based on the retention time obtained by relating to the ECNs of TAGs of known molecular species (Fig. 2). The linear line, as shown in Fig. 2, indicates the relationship between retention times in logarithm value to ECN. Tracing of this line from a few known TAGs theoretically permits the prediction of unknown TAG on the chromatograms. The types of TAGs used as standards for peak identification and their retention times are shown in Table 2.

Fig. 1(a) and (b) clearly shows that the amount of unmodified palm olein long chain TAG has been greatly reduced and caprylic acid was successfully incorporated into the oil to form SL. With shorter fatty acid chain length in the TAG structure, most of the peaks for SL formed were eluted earlier due to higher polarity than LC TAG that contain long chain fatty acids in their respective structures.

3.3. Pancreatic lipase-catalyzed sn-2 positional analysis

Pancreatic lipolysis was done to examine the *sn*-2 position fatty acid profile. The compositions of the *sn*-2 position fatty acids after modification were: C8:0, 9.2%; C12:0, 2.3%; C14:0, 1.8%; C16:0, 21.3%; C18:0, 4.7%; C18: 1, 60.7%. The fatty acid composition was obtained from the percentage peak area of the GC chromatogram. Unsaturated fatty acid is preferably located at the *sn*-2 position in the modified oil, as it is easily absorbed during digestion. This aim was achieved with 60.7% of oleic acid, which is a monounsaturated fatty acid preferentially placed at the *sn*-2 position. However,

Table 2
Equivalent carbon numbers and retention times (t_R) of known triacylglycerol standards used in this study

Triacylglycerol	CN	Number of double bond	ECN	t_R (min)
Capric–capric–caprylic (DDC)	28	0	28	4.125
Lauric–capric–capric (LaDD)	32	0	32	5.785
Lauric–lauric–capric (LaLaD)	34	0	34	6.811
Lauric–myristic–myristic (LaMM)	40	0	40	12.25
Lauric–oleic–myristic (LaOM)	44	1	42	14.65
Lauric–palmitic–palmitic (LaPP)	44	0	44	18.53

Abbreviations: CN, carbon number; ECN, equivalent carbon number; t_R, retention time.

Table 3
Peak identification and possible triacylglycerol species in the structured lipid of modified palm olein

ECN	28	30	32	34	36	38	40
Potential TAGs	CCLa CLaC	CMC CCM CCL	CPC CCP COC CCO	CSC CMLa	CMM MCM MCL	CPL MPC CMP MCP COL PCL OCL	POC PPC COO OCO PCO

Abbreviation: C, caprylic acid; La, lauric acid; M, myristic acid; L, linoleic acid; O, oleic acid; P, palmitic acid.

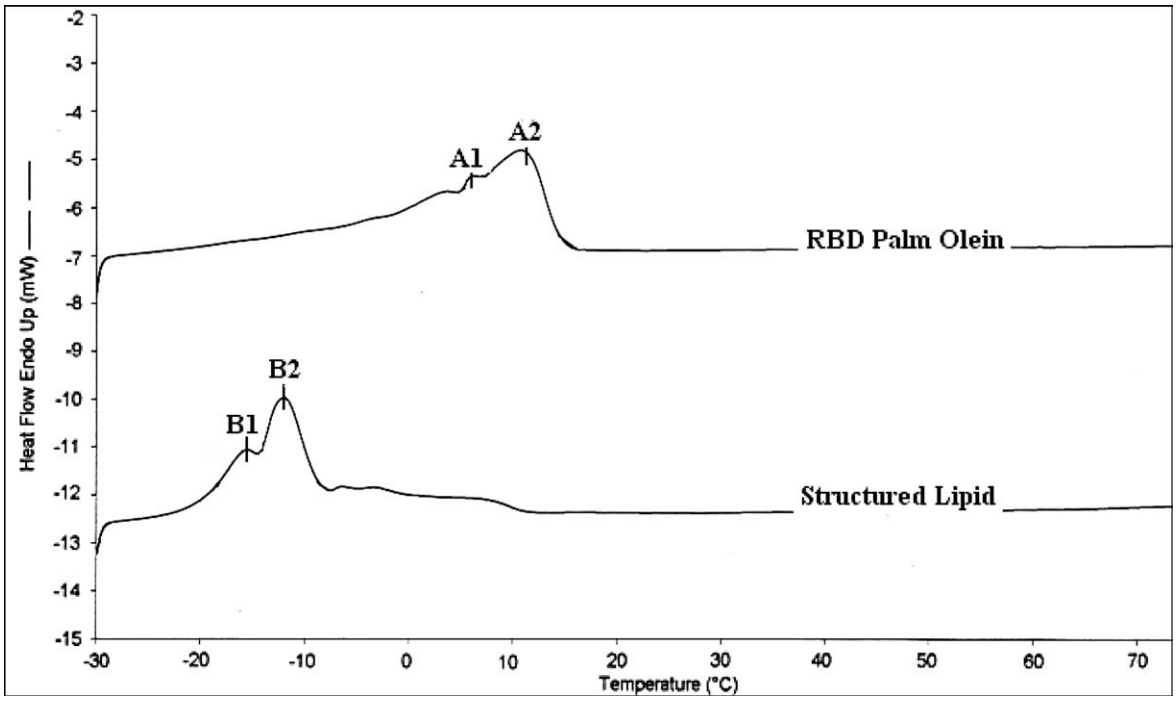

Fig. 3. Melting profile of structured lipid synthesized through lipase-catalyzed acidolysis compared to refined, bleached and deodorized (RBD) palm olein. See DSC conditions in text. Peak A1, onset: 5.08 °C, melting point: 5.88 °C; peak A2, onset: 7.25 °C, melting point: 11.26 °C; peak B1, onset: −18.43 °C, melting point: −15.97 °C; peak B2, onset: −14.03 °C, melting point: −11.78 °C.

the presence of C8:0 at the 2-position demonstrates some occurrence of acyl migration even though a 1,3 specific lipase was used in the reaction. A prolonged reaction time might cause the phenomena to happen, together with other factors, such as lipase load, water content, lipase type, temperature and acyl donor type used for the reaction (Xu et al., 2000a, 2000b). However, this was not investigated in this study.

3.4. Iodine value

Lipase-catalyzed acidolysis reduced the IV of RBD palm olein from 60.4 to 48.2. The reduction in IV can be explained by the successful incorporation of caprylic acid (C8:0) into palm olein to formed SL. Caprylic acid is a saturated fatty acid; therefore its incorporation resulted a decrease in IV as the degree of saturation for SL increased. Akoh and Moussata (2000) reported the same trend in their work with fish and canola oils.

3.5. Slip melting point and thermal profile

SMP is one of the essential physical characteristics of fatty compounds that is useful in many technological aspect of fatty materials. It is proportional to the increase in chain length and degree of saturation. SMP fats usually contain 5% solid fat; thus SMP generally happens at a lower temperature than the melting point of the fat itself. In this experiment, SMP for RBD palm olein drastically decreased from 13 to 4.2 °C after the incorporation of caprylic acid. An 8.8 °C drop in SMP is caused by increase in the amount of shorter fatty acids chain length which was contributed by caprylic acid.

Similarly, the results of the heating profile, using DSC, showed a shift of the peaks for RBD palm olein from high to lower melting ranges after acidolysis, due to the incorporation of caprylic acid. Fig. 3 shows that two onset melting temperatures were detected for the SL at −18.43 °C (B1) and −14.03 °C (B2) with melting peaks of −15.97 and −11.79 °C, respectively. As for RBD palm olein, the onset melting temperatures were recorded at 5.08 °C (A1) and 7.25 °C (A2) with melting peaks of 5.88 and 11.26 °C. This suggests that the SL has potential to be used in salad dressing, mayonnaise, liquid and pourable margarine products, as liquid margarine fats have been reported to have low melting ranges between −26 and −17 °C (Fomuso & Akoh, 2002).

In summary, 30.5% caprylic acid was successfully incorporated into RBD palm olein and produced significant amounts of SL, in which cluster C32 recorded the highest content with 35.3% of the total TAG composition. The TAG of interest, caprylic–oleic–caprylic, was predicted to be in this cluster. Also, the SL produced will have metabolic benefit as the medium chain fatty acids were strategically located at positions *sn*-1,3 while retaining the benefits of oleic acid at the *sn*-2 position.

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