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Research

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The synthesis of medium-long-medium structured lipid (MLM-SL) by lipase-catalyzed transesterification using palm olein and tricaprylin in packed-bed reactor (PBR)

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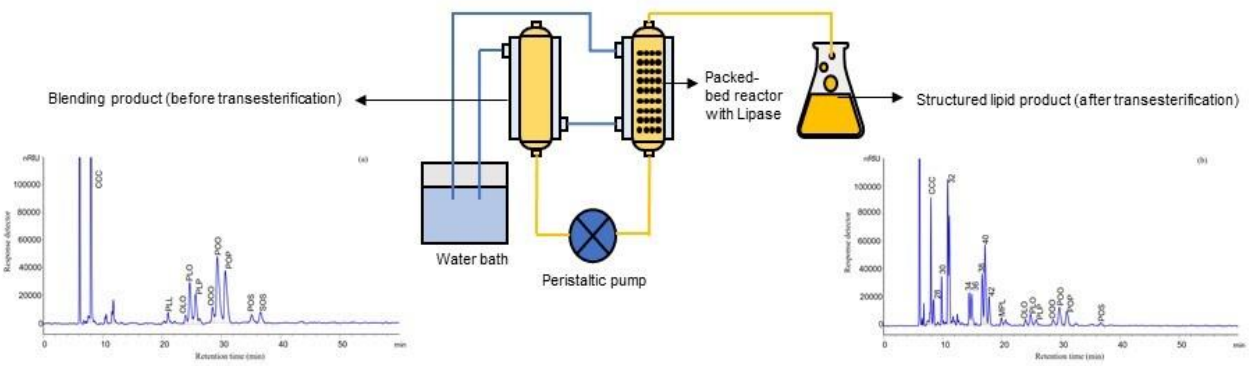
Abstract

Lipase-catalyzed transesterification between refined bleached deodorized palm olein (RBDO) and tricaprylin to produce medium-long-medium structured lipid (MLM-SL) in a packed bed reactor has been investigated. A specific *sn*-1,3 commercial Lipozyme TL IM was used as biocatalyst. Within this study, the progress of transesterification was monitored especially for triacylglycerol (TAG) formation with equivalent carbon number (ECN) of 32, presumably 1,3-dicapryoyl-2-oleoyl-*sn*-glycerol (COC). Transesterification conditions investigated were residence times (*i.e.*, 15, 30, and 60 min) and enzyme loadings (2 and 4.5 g). The highest yield of ECN 32 (13%) and transesterification degree (71%) were obtained at residence time of 15 mins for both enzyme loadings. Longer residence time seemed to facilitate lipid hydrolysis over transesterification. This was

indicated by the number of peaks appearing in the high-performance liquid chromatography (HPLC) chromatograms and the reduction of of fat slip melting point (SMP). Additionally, at 4.5 enzyme loading the highest productivity was obtained for one-cycle reaction. Conclusively, this study has demonstrated the potential use of packed-bed reactor with immobilized Lipozyme TL IM for continuous synthesis of MLM-SLs especially TAG ECN32.

Keywords, Lipase, 1,3-dicapryoyl-2-oleoyl-*sn*-glycerol, palm olein, packed bed reactor, structured lipids

Graphical Abstract



Introduction

Medium-long-medium structured lipid (MLM-SL) is a typical structured lipid that contains medium chain fatty acids (MCFAs, C6-C12) at *sn*-1,3 positions and long chain fatty acid (LCFA, C14-C24) at *sn*-2 position. The presences of MCFA and LCFA on a TAG molecule poses benefits especially for clinical nutrition purposes such as to improve fat malabsorption and managing obesity. MCFAs at *sn*-1,3 positions are directly

49 transported to liver as quick energy sources. In addition to this, structured lipid where
50 LCFA is at *sn*-2 position is also directly absorbed. MLM-SLs are not commonly found
51 from natural resources. Both chemical and enzymatic synthesis are used in an attempt to
52 produce it. The enzymatic interesterification was preferably to synthesize MLM-SL due
53 to its selectivity, less by-products, mild reaction conditions, and easy recovery of the
54 biocatalysts. Herein, enzyme-based MLM-SL synthesis gains popularity in recent years
55 (Utama et al., 2019)
56 The continuous synthesis of MLM-SL was of importance especially at industrial scale.
57 Continuous synthesis leads to the reduction of unproductive times (due to start-, and end-
58 procedures in repetitive batch cycles), and also minimization of batch-to-batch oscillation
59 in product quality (Sitanggang et al., 2014a, 2015, 2016). Generally, MLM-SL synthesis
60 in continuous system was conducted using packed bed reactor (PBR), micro-channels
61 (MC), enzymatic membrane reactor (EMR). PBR has several advantages such as ease of
62 operation, better product control, and high reaction rate and mass transfer (Itabaiana et
63 al., 2013; Silva et al., 2011). In PBR system, flow rate or residence time plays important
64 role for reaction kinetics and thus, volumetric productivity. The operation of PBR requires
65 the enzyme to be immobilized. Herein, another consideration for successful and efficient
66 MLM-SL synthesis in PBR is the cost of biocatalyst. Lipozyme TL IM (Novozymes A/S)
67 is an *sn*-1,3 specific lipase originating from *Thermomyces lanuginosus,* and immobilized
68 on a non-compressible silica gel carrier (Yang et al., 2014). It has been reported for its
69 economically low price and larger active side pockets possible for rapid catalysis of lipid
70 transesterification (Basri et al., 2013; Wang et al., 2008).
71 Based on this rationale, within this work we demonstrated the continuous synthesis of
72 MLM-SL using palm olein and tricaprylin under PBR. The synthesis was catalyzed by

Lipozyme TL IM. Refined bleached deodorized palm olein (RBDO) is always considered as a potent substrate to produce MLM-SL. It is due to high content of oleic acid at *sn*-2 position (May and Nesaretnam, 2014). Ong and Goh (2002) reported that consumption of oleic acid has shown positive effect for prevention of cardiovascular diseases. In addition to this, caprylic acid has been shown to be more effective to increase plasma ketone for rapid energy sources as compared to other MCFAs (Vandenberghe et al., 2017). The incorporation of caprylic acid into RBDO is expected to yield MLM-SL with equivalent carbon number (ECN) of 32, presumably1,3-dicapryoyl-2-oleoyl-*sn*-glycerol (COC).

Materials and Methods

Materials

RBDO with iodine value (IV) of 60 was obtained from PT. Salim Ivomas TBK, Indonesia. Caprylic acid, tricaprylin (TC), molecular sieve 4 Å and triglyceride standard mixture (tricaprin, tricaprylin, trilaurin, trimyristin, and tripalmitin) were purchased from Sigma-Aldrich, Singapore. Lipozyme TL IM was obtained from Novozyme A/S, Denmark. Hexane, chloroform, ethanol, octanol, sodium hydroxide, acetonitrile, and acetone were analytical grade and purchased from Merck, Germany.

Synthesis of MLM-SL in continuous system (packed bed reactor)

The schematic design of PBR system is shown in Figure 1. The reactor system was consisted of substrate reservoir, peristaltic pump (BT 100-IF longer Peristaltic Pump, Baoding longer Precision Pump Co., Ltd), column, water bath (Stephen Hacke, Germany), and product reservoir. Packed bed reactor column (ID =11 mm and H = 80 mm) with jacketed wall was made from glass material. The upper and lower ends of

column were equipped with filter which was impermeable for the biocatalyst resins. The column was packed with either 2.0 or 4.5 gram of biocatalysts. For 2.0 g of enzyme loading, molecular sieve 4 Å (Sigma-Aldrich) was used as "dummy enzyme" to avoid catalysts floating within the column. The mixture of substrates (RBDO and tricaprylin with molar ratio of 1:1) was pumped into the reactor from the upper end of the column. Three different residence times were realized (*i.e.*, 15, 30, and 60 min) within this study. The residence time was calculated according to Levenspiel (1999) and Sitanggang et al. (2014b) as follows (eq. 1).

$$\tau = \frac{V}{v_0} \qquad (1)$$

where τ is the residence time (min), V is the the working volume of the reactor (mL) and v_0 is the volumetric flow rate (mL/min). Temperature of reaction (50^{o}C) was maintained by circulating water continuously into substrates reservoir and jacketed column of PBR. Samples were taken from product reservoir after 3 h of reaction (without recycle procedure).

TAG composition analysis

The TAG composition was analyzed using a Hewlett Packed Series 1100 HPLC system equipped with a refractive index detector (RID), Agilent Technologies, USA. The TAG peaks were identified using TAG mixture standard peaks and equivalent carbon numbers (ECNs). ECN can be obtained as CN-2(DB), where CN shows the total amount of carbon in the TAG molecule without glycerol, and DB is number of double bonds on the TAG molecule (Holčapek et al., 2005). The change of tricaprylin concentration before and after interesterification was used to determine transesterification degree and as follows (eq. 2):

120 $$TD = \frac{(P_E - P_O)}{P_O} \quad (2)$$

121 where P_O and P_E were percentage area of tricapylin prior to and after reaction,
122 respectively.

123

124 **Determination of acylglycerol fractions**

125 The acylglycerol fractions were determined by AOCS Official Method Cd 11b-91
126 (AOCS, 1997) with modification. The acylglycerol fractions were analyzed using a
127 Hewlett Packed Series 6890 autoinjector gas chromatography system equipped with a
128 flame ionization detector (FID) and DB-5HT column (L = 15 m, ID = 320 nm, and
129 thickness = 0.1 µm). The sample (0.0250-0.0255 g) was added with 10 µL of
130 tetrahydrofuran and 50 µL of N-methyl-N-trimethylsilyl-trifluoroacetamide and vortexed
131 at 2400 rpm for 90 s. The test tube was placed in the dark for 10 min. Thereafter, a 2 mL
132 of heptane was added and thoroughly vortexed at 2000 rpm for 30 s. Sample was left for
133 30 min at room temperature (27°C) and ready for analysis.

134

135 **Differential scanning calorimetry**

136 Melting and crystallization point of blending and the produced structured lipids were
137 determined using differential scanning calorimetry (DSC) (model TA-60, TA instrument,
138 New Castle) according to Saberi et al. (2011). Samples (6-10 mg) were sealed
139 hermetically using aluminium pan. The exothermic curves were obtained by holding
140 samples at 80°C for 10 min followed by cooling down to -50°C at a rate of 5°C/min. The
141 samples were held at -50°C for 10 min to obtain endothermic curves and followed by

heating to 80°C at 5°C/min. The crystallization was indicated by peaks in cooling curves, whereas melting point was indicated by heating curves.

Slip melting point (SMP)

Slip melting point (SMP) was determined according AOCS Official method Cc 3-25 (AOCS, 2017). Sample was tempered around 10 mm in a capillary tube at 4-10°C for 16 h. The tube was slowly heated in a beaker glass filled with water as heating medium. The temperature when samples started to rise was reported as SMP. The measurements were run in triplicate and reported as a mean ± standard deviation.

Results and Discussion

TAG compositions of structured lipids

The formation of MLM-SL was determined by comparing peaks (*i.e.*, TAG composition) between TAG mixture standard and transesterification products. In the blended mixture (*i.e.*, RBDO:tricaprylin (1:1)), the dominant TAGs were mainly those with ECN > 42. TAGs of blended mixture were dominated by tricaprylin (CCC), palmitic-oleic-oleic (POO), palmitic-oleic-palmitic (POP), and palmitic-linoleic-oleic (PLO). After transesterification reaction, these TAGs were depleted, leading to the emergences of several new TAG species especially with ECN 32, 38, and 40 (see Figure 2b-d). This change was presumed as the results of caprylic acid incorporation (mono- or di-substitution) within TAG molecules found in RBDO. During batch transesterification using the same substrates and biocatalyst, several new TAG species were also produced including ECN 30, 32, 34, 36, 38, 40, and 42 (Utama et al., 2020). In our previous work (Utama et al., 2020), such a high concentration of caprylic-oleic-caprylic (ECN 32) was

obtained batch-wise. Within this work, the incorporation of caprylic acid into RBDO catalyzed by Lipozyme TL IM also showed higher possibility to produce caprylic-oleic-caprylic (COC). From Figure 2 (a-d), it is indicated by higher chromatogram areas of ECN 32 compared to that of blended mixture's peak area. Herein, COC was selected as TAG of interest and representative of MLM-SL in this study.

In continuous reaction, residence time plays a key role to determine the rate of disappearance or formation of interest chemical species. In this study, 15 min of residence time was considered as the best residence time for both enzyme loadings due to high concentrations of ECN 32 (Figure 3). The increasing of residence time was found to have no influence on the concentration of TAG dominant. Yang et al. (2014) reported that 30-40 min of residence time was optimum to produce MLM-SL using soybean oil medium chain triacylglycerol (MCT) catalyzed by Lipozyme TL IM in PBR system. In addition, Xu et al. (2002) also reported lipase catalyzed interesterification between fish oil and MCT in PBR system with Lipozyme TL IM as catalyst. The results showed that degree of reaction reached equilibrium at 30-40 min residence time.

In general, increasing amount of enzyme in reaction will affect the reaction rate. Zhang et al., (2001) reported that interesterification degree was positively influenced by enzyme loading and reached equilibrium at 6 % of enzyme loading. Based on this, we realized two enzyme loadings in this study (*i.e.*, 2.0 and 4.5 g) had no effect on the product concentration obtained. The results also showed similar patterns for the reduction of initial dominant TAGs and increase of new TAGs (Figure 3). We considered that two enzyme loadings employed within this study might be excessive. This could be indicated by relatively short time to reach concentration plateau for both loadings. Additionally, the

189 reaction times needed to reach this equilibrium were also similar, approximately within
190 15 min. (Figure 4).

191 **Productivity rate and productivity of structured lipid**

192 Productivity and productivity rate of MLM synthesis in continuous system were
193 determined based on the kinetics of enzyme inactivation during batch production.
194 Productivity rate (PR) was determined as rate of MLM concentration per gram enzyme
195 used and per hour of reaction. For optimum residence time (*i.e.*, 15 min), 2.0 g of enzyme
196 loading (7.70 ml/ g_{enzyme}. h) showed higher PR compared to 4.5 g of enzyme loading (5.19
197 ml/ g_{enzyme}. h). This condition was assumed as initial PR when residual activity of enzyme
198 was 100%. As mentioned above, for this calculation, we assumed the kinetics of
199 Lipozyme TL IM inactivation in batch system (Utama et al., 2020) was the same with
200 continuous system. Herein, the integration of residual activity from batch-wise
201 transesterification was used to predict enzyme productivity in one cycle reaction in
202 continuous system (Equation 3). One cycle reaction was defined as the operation time
203 performed to reach 50% of enzyme's residual activity.

204 $$\text{Productivity (mL/gram enzyme)} = \int_0^t (-0.1486x + PR)dt \qquad (3)$$

205 Based on this, enzyme loading of 4.5 g showed higher productivity (6846.04 mL/gram
206 enzyme) than that of 2.0 g (6220.56 mL/ gram enzyme).

207

208 **Acylglycerol fraction after transesterification**

209 Despite of its small amount is required (*i.e.*, microaqueous system), water still has
210 important role during lipase-catalyzed interesterification. In lipase-catalyzed
211 interesterification reaction, water was included in the enzyme materials or substrates.
212 High content of water in system will shift the progress of reaction towards hydrolysis.

213 Herein, the formations of new TAGs in transesterification are accompanied by the
214 formations of by-products such as diacylglycerol (DAG), monoacylglycerol (MAG) and
215 the fatty acid (FFA) in reaction system. Kadhum and Shamma (2017) determined the
216 formation acyl glycerol complexes as results of lipase-catalyzed interesterification.
217 However, Hermansyah et al. (2010) reported that hydrolysis of triacylglycerol by the
218 enzyme was stepwise process to produce DAG, MAG and glycerol, while FFA is released
219 at each reaction step. The enzyme-substrate complexes are formed at the respective steps.
220 For blending product, acyl glycerol fraction only consisted of TAGs and DAGs. After
221 transesterification reaction, changes on acylglycerol fractions were observed (Figure 5).
222 Within 15 min of residence time, TAG concentration was slightly increased while DAG
223 concentration was decreased. In addition to this, MAG, glycerol, and FFA were also
224 detected. Increased residence time (30 and 60 min) could increase DAG, MAG, glycerol,
225 and FFA concentration. This might be due to facilitation of a longer contact time between
226 the initial and produced TAGs with enzyme molecules that favored hydrolysis reaction.
227 Higher amounts of side products from the transesterification might be detrimental
228 especially for the separation of produced structured lipids. In addition to this, formation
229 of FFA could lead to pH shift that has influence on the stability of the enzyme.
230 Different enzyme loadings relatively showed similar concentrations of acylglycerol
231 fractions. In contrast, Zhang et al., (2001) reported that the increase of enzyme loading
232 had positive impact on the increased formation of FFAs and DAGs. This was due to a
233 higher amount of water from the enzyme materials involved during the reaction.
234 Moreover, in higher enzyme loading, such higher active pockets were also available to
235 perform hydrolysis on the TAGs. For our study, we focused on the production of

structured lipid, especially higher concentration of ECN 32. Therefore, the optimum residence time for continuous production of ECN 32 of 15 min was selected.

Thermal profile of structured lipid product

The concentrations of MAGs and DAGs may influence melting point, crystal formation, and the hardness of lipids (Basso et al., 2010; Saberi et al., 2011). After transesterification, SMP was increased because of changes on the acylglyerol fractions. Generally, the increasing of DAGs concentration will reduce slip melting point of lipid (Figure 6). SMP of blending product was 4.33^{o}C. At 15 min of residence time, SMP was higher than at 30 and 60 min. At 15 min of residence time, the formations of TAGs (also MLM TAG) were favored whereas for longer residence times the hydrolysis was pronounced. Thus, the concentrations of DAGs were higher than TAGs for these longer residence times (30 and 60 min). The effects of DAGs and MAGs concentration on SMP were also depend on types of fatty acid (*i.e.*, length of carbon chain, saturated or unsaturated) and isomeric positions of fatty acids. Siew, (2002) reported that 1, 2 isomers of DAG was found to be more effective to reduce melting point as compared to that of 1,3 isomers of DAG. Moreover, Subroto et al., (2019) mentioned that higher concentrations of saturated fatty acids in DAG and MAG structures could increase melting point of lipids.

The information about melting and crystallization temperature of fats is important for designing their food applications. Within this study, transesterification was also found to reduce melting and crystallization point of blended product (Figure 7 and Table 1). Furthermore, the obtained structured lipid product showed more narrow range in melting temperature and wider range in crystallization temperature as compare to that of blending product. Moreover, for 4.5 g of enzyme loading showed lower melting and crystallization

temperature as compared to that of 2.0 g. Different of melting and crystallization temperatures might be influenced by the changes in TAG and acylglycerol fractions.

Conclusions

Lipase-catalyzed transesterification reactions can potentially be used to synthesize 1,3-dicapryoyl-2-oleoyl-*sn*-glycerol (COC, ECN32), potential lipid of MLM type-structured lipid. Within this study, the enzyme loadings utilized might be excessive as indicated by little or no effect on especially TAG concentrations was observed. However, increasing residence time higher than 15 min (30 and 60 min) showed decreasing concentrations of TAGs which further had influence on the slip melting point of structured lipid. Conclusively, in continuous transesterification, residence time of 15 min and 4.5 g of enzyme loading were selected as the optimum conditions to produce highest productivity of COC one-cycle reaction.

Abbreviations

COC : 1,3-dicapryoyl-2-oleoyl-*sn*-glycerol / caprylic-oleic-caprylic

DAG : Diacylglycerol

DSC : Differential scanning calorimetry

ECN : Equivalent carbon number

FFA : Fatty acid

HPLC : High-performance liquid chromatography

IM : Immobilized

LCFA : Long chain fatty acid

MAG : Monoacylglycerol

MCFA : Medium chain fatty acid

MLM-SL : Medium-long-medium structured lipid

MPL : Myristic-palmitic-linoleic

OLO : Oleic-linoleic-oleic

OOO : Oleic-oleic-oleic;

PBR : Packed-bed reactor

PLO : Palmitic-linoleic-oleic

PLP : Palmitic-linoleic-palmitic

POO : Palmitic-oleic-oleic

POP : Palmitic-oleic-palmitic

PR : Productivity rate

RBDO : Refined bleached deodorized palm olein

SL : Structured lipid

SMP : Slip melting point

sn- : Stereospecific number

TAG : Triacylglycerol

TC/CCC : Tricaprylin

TD : Transesterification degree

TL : *Thermomyces lanuginosus*

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interest

The authors declare that they have no competing interest.

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Authors' contributions

QDU conducted the research, analyzed the data, and drafted the manuscript. ABS, DRA, and PHA supervised the research, reviewed the manuscript, and provided comments to enhance the quality of manuscript. All authors read and approved the final manuscript.

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List of Figures

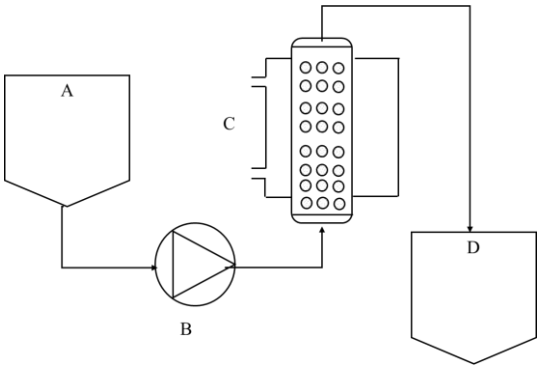


Figure 1. Schematic design of reactor system: (A) sample reservoir, (B) peristaltic pump, (C) packed bed reactor, and (D) product reservoir

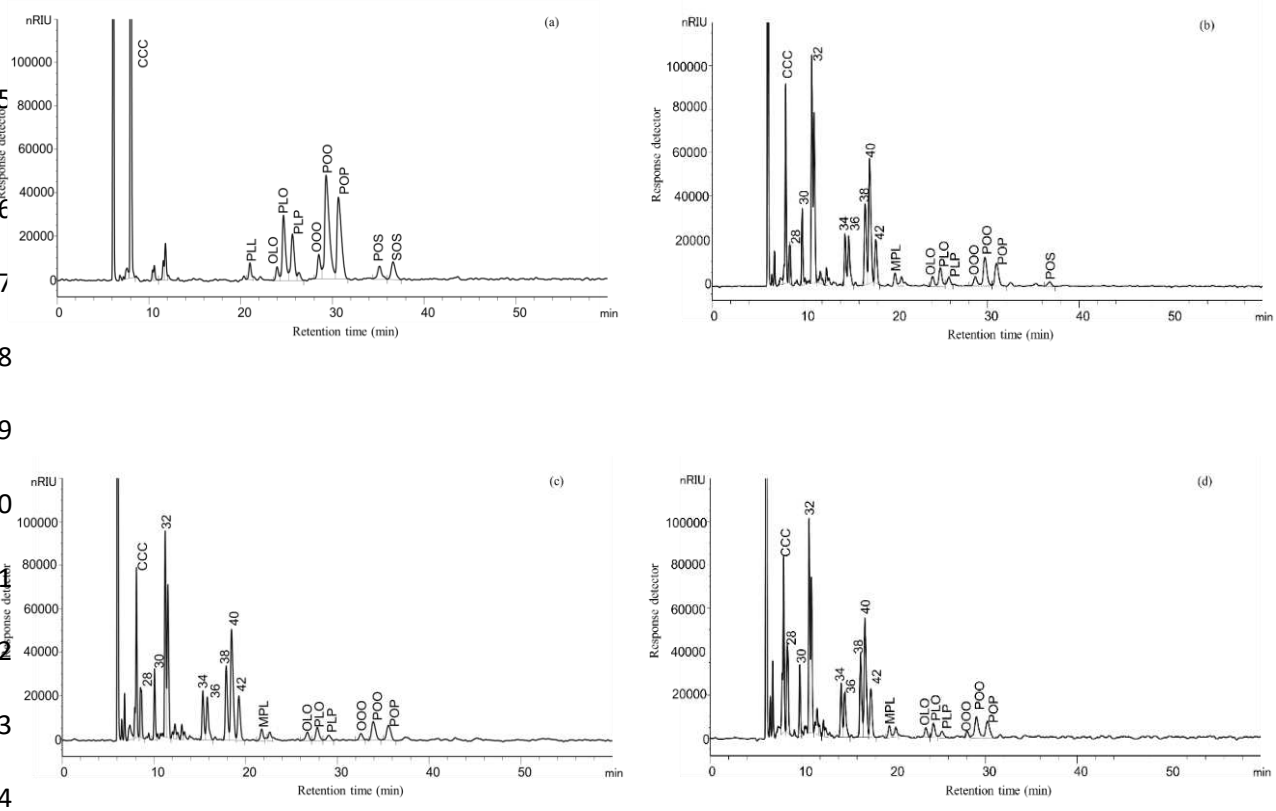
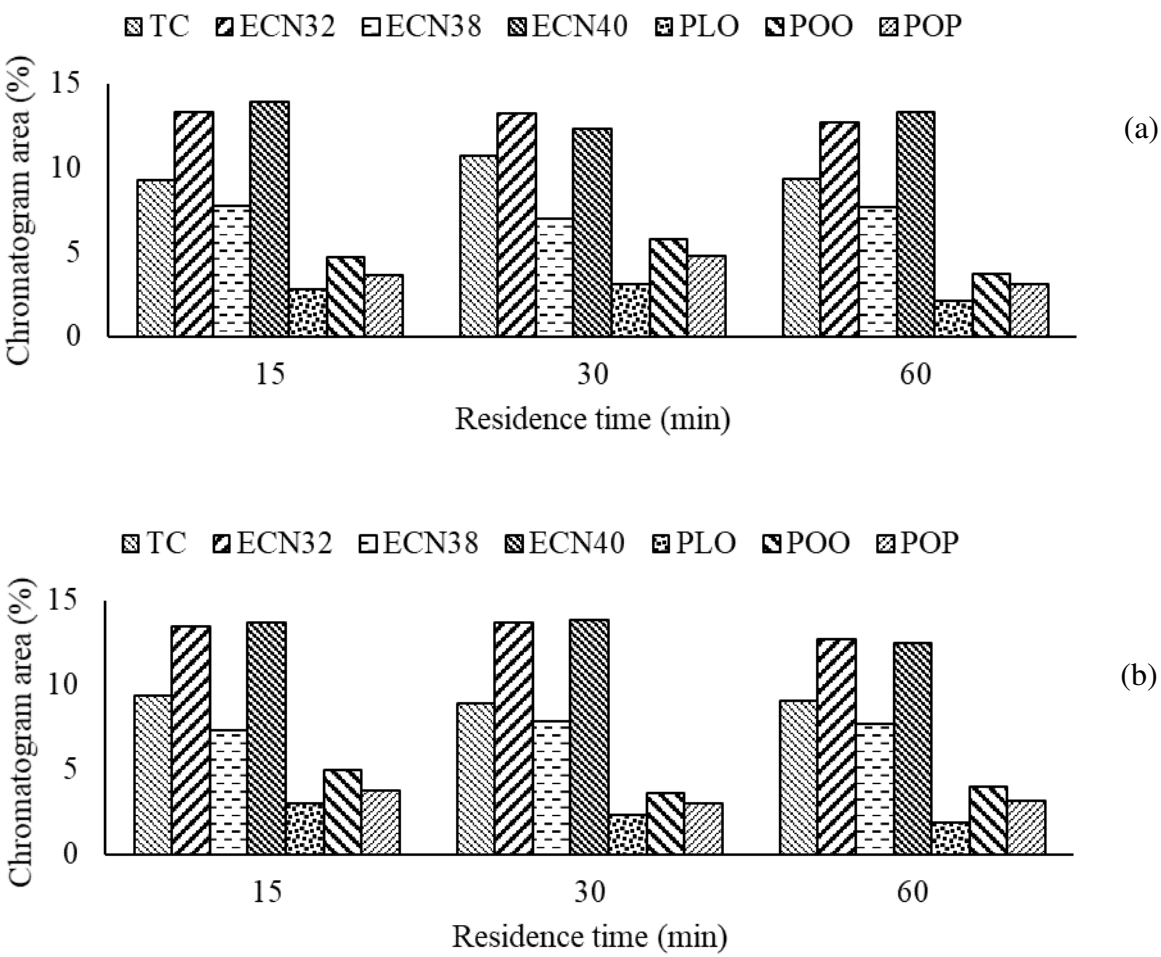


Figure 2. Chromatograms of (a) blending RBDO:tricaprylin (1:1), (b) RT 15 min, (c) RT 30 min, and (d) RT 60 min. Other reaction condition: enzyme loading 4.5 g, T= 50^{o}C. TC/CCC = tricaprylin; MPL=myristic-palmitic-linoleic; OLO=oleic-linoleic-oleic; PLO=palmitic-linoleic-oleic; PLP=palmitic-linoleic-palmitic; OOO=oleic-oleic-oleic; POO=palmitic-oleic-oleic; POP=palmitic-oleic-palmitic.



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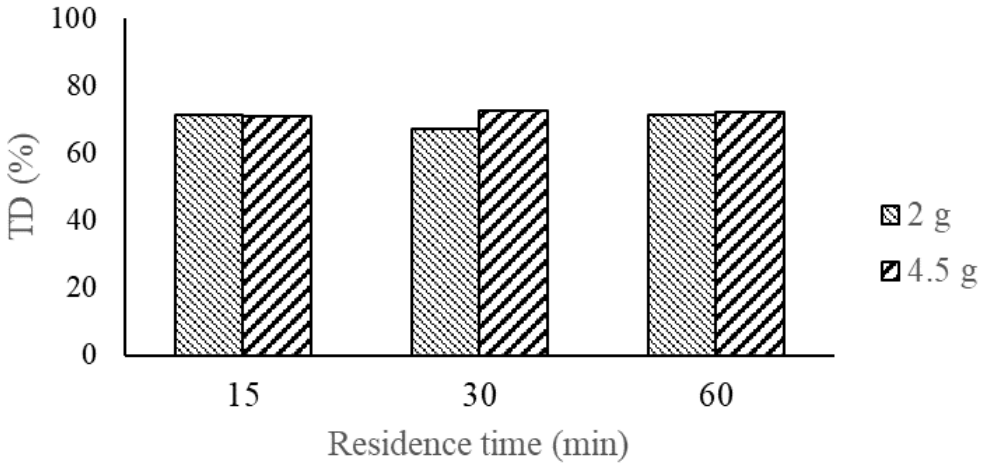

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461 Figure 4. Effect of residence time on transesterification degree (TD)

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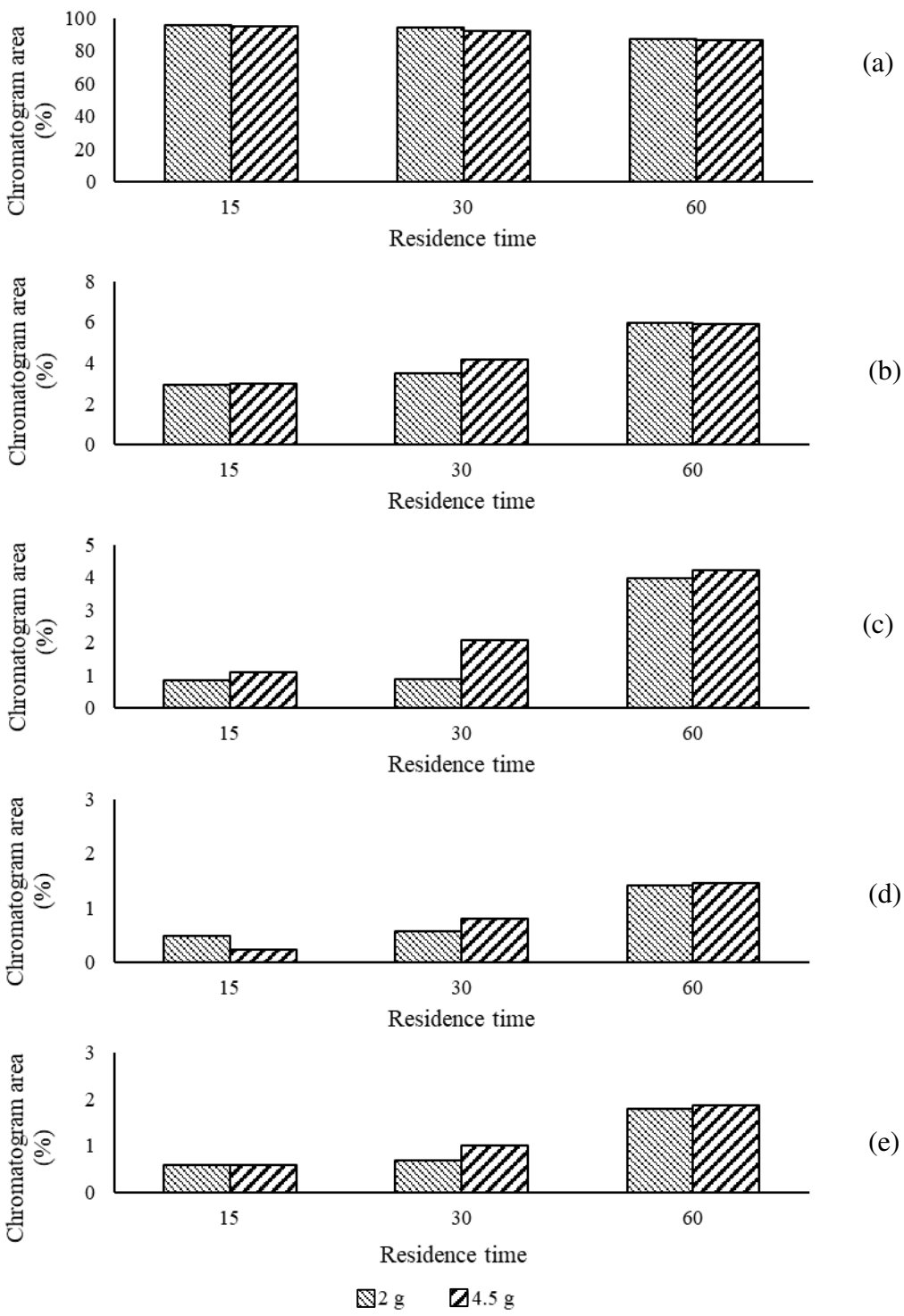

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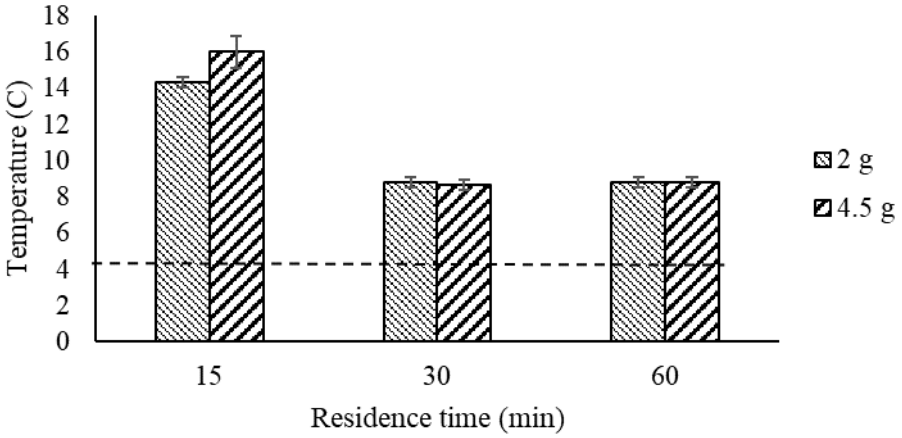

Figure 6. Slip melting point of blending product (dashed line) and structured lipid product

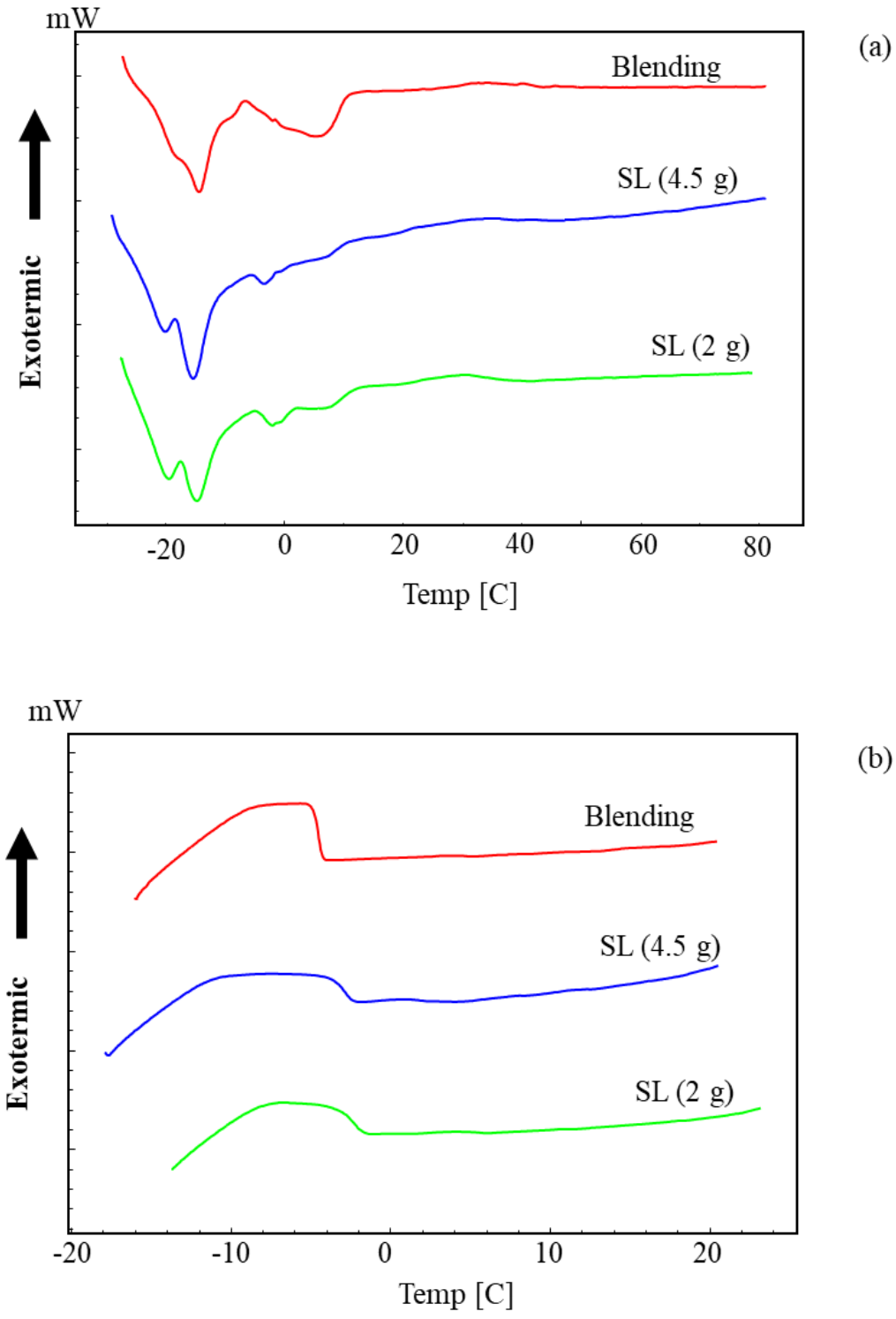

Figure 7. Differential scanning calorimetry (DSC) melting (a) and crystallization (b) curves of blending and structured lipid product. SL = structured lipid.

520 List of Tables

521 Table 1. Thermal profile of blending and structured lipid product

Sample	Melting				Crystallization			
	Onset (°C)	Peak (°C)	Endset (°C)	Δh (J/g)	Onset (°C)	Peak (°C)	Endset (°C)	Δh (J/g)
Blending	-5.04	5.31	10.26	-10.50	-5.65	-4.18	-12.46	7.79
SL 2g	-4.23	-1.99	1.59	-1.25	-1.70	-6.68	-10.89	4.14
SL 4.5g	-4.97	-3.41	-1.37	-0.50	-2.35	-7.68	-13.66	4.76

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Figures

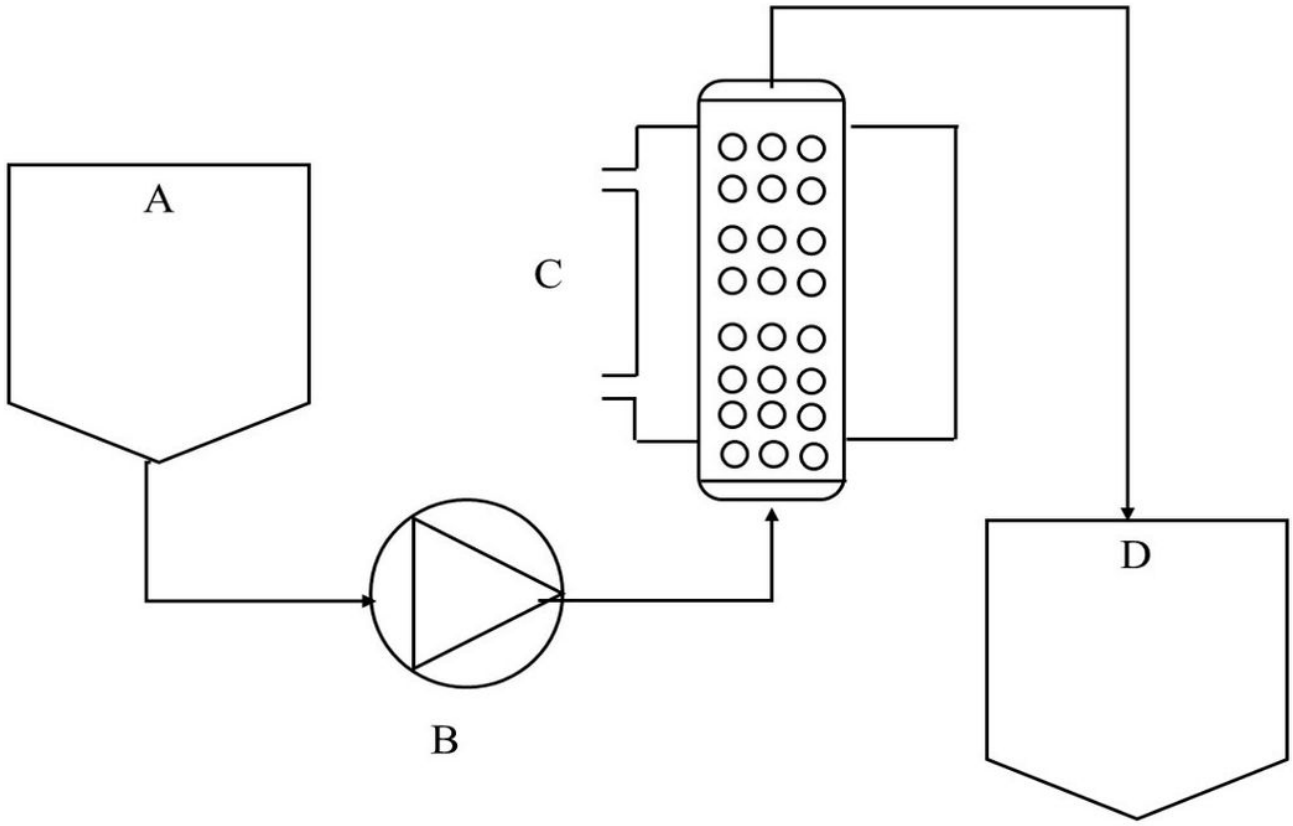
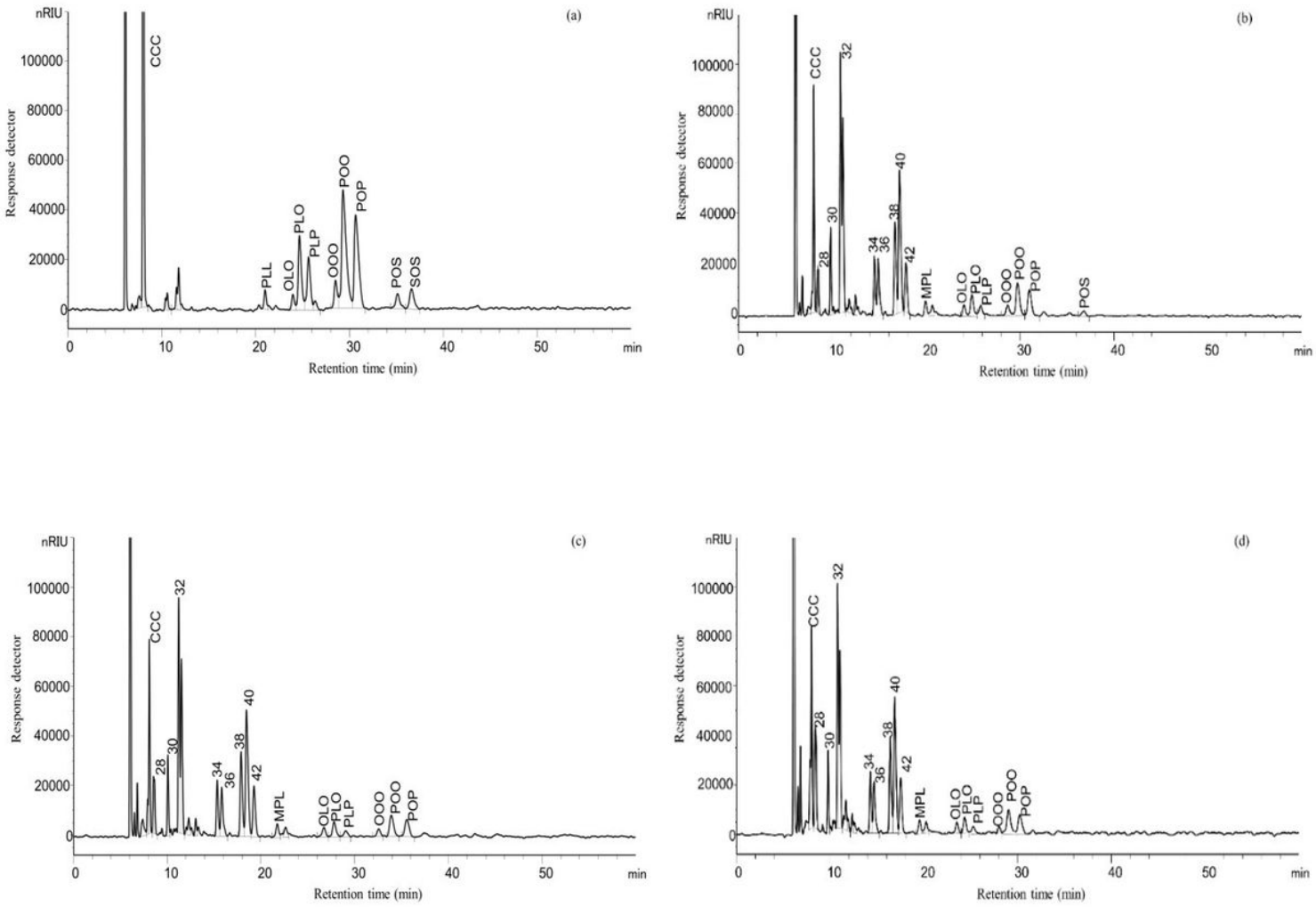


Figure 1

Schematic design of reactor system: (A) sample reservoir, (B) peristaltic pump, (C) packed bed reactor, and (D) product reservoir



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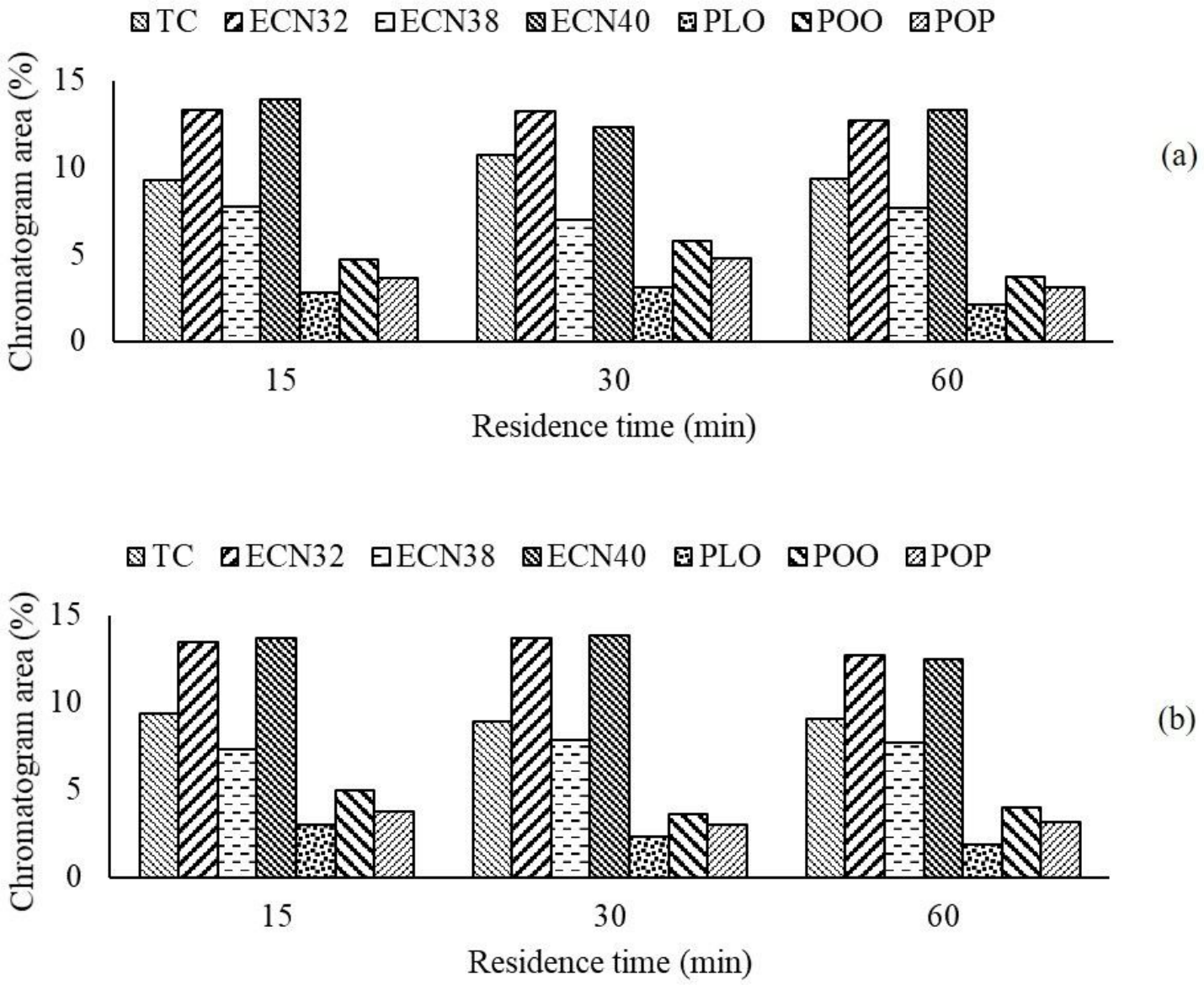

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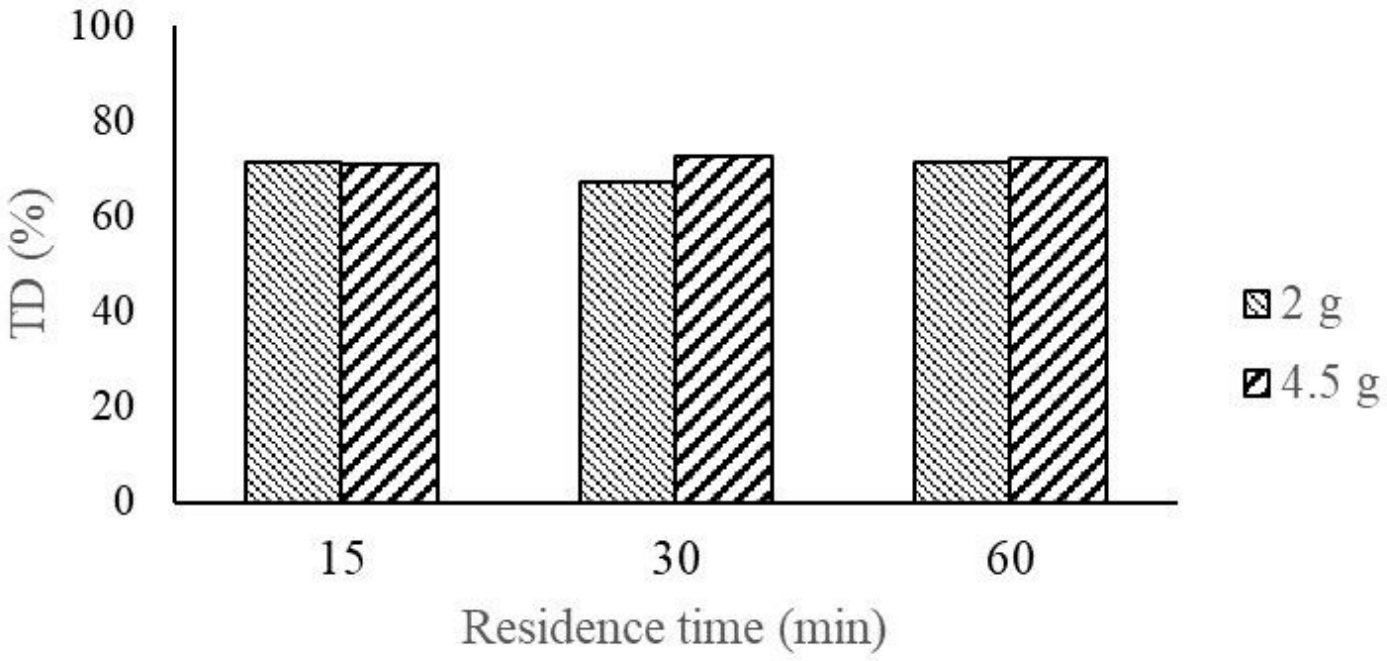

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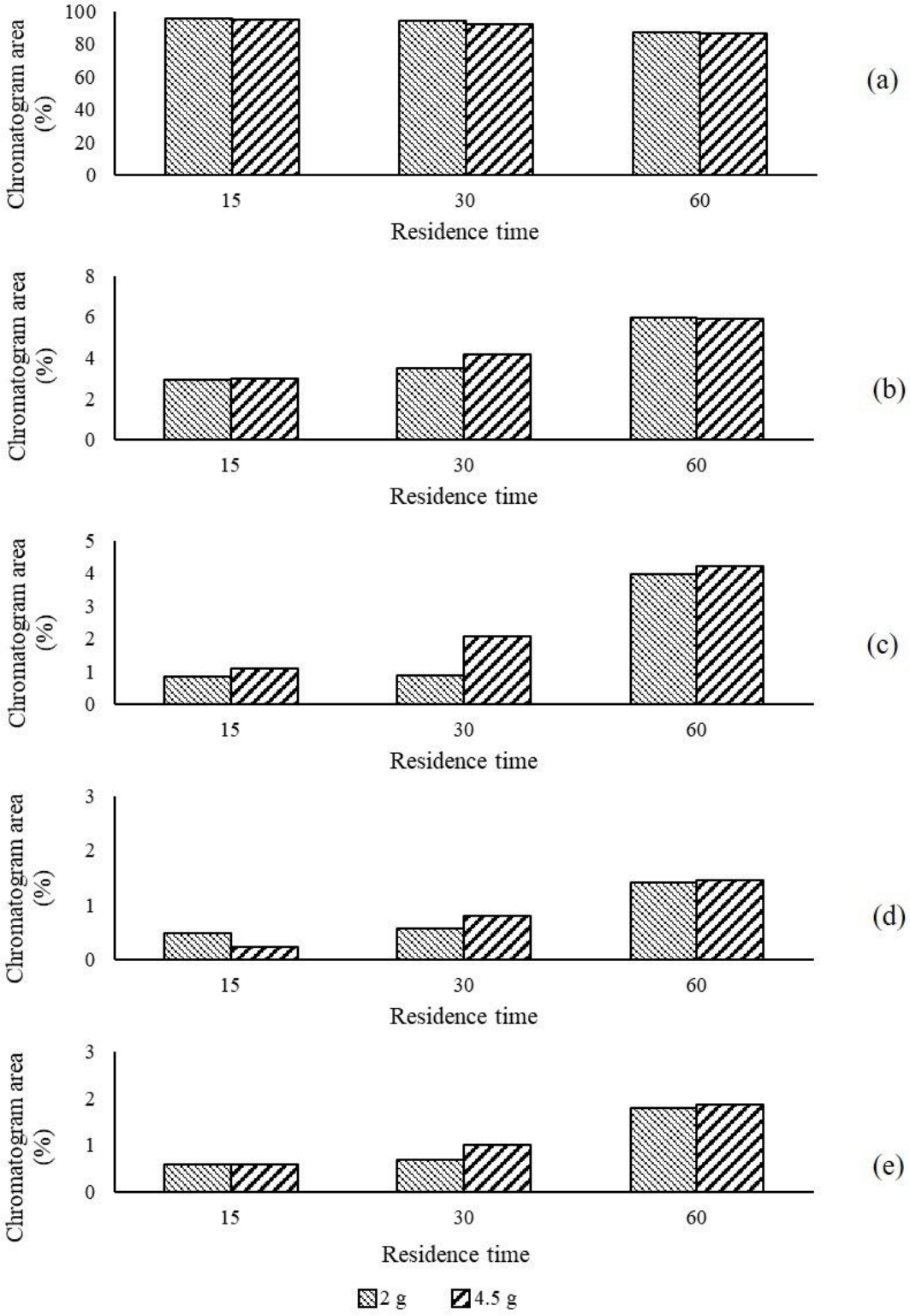

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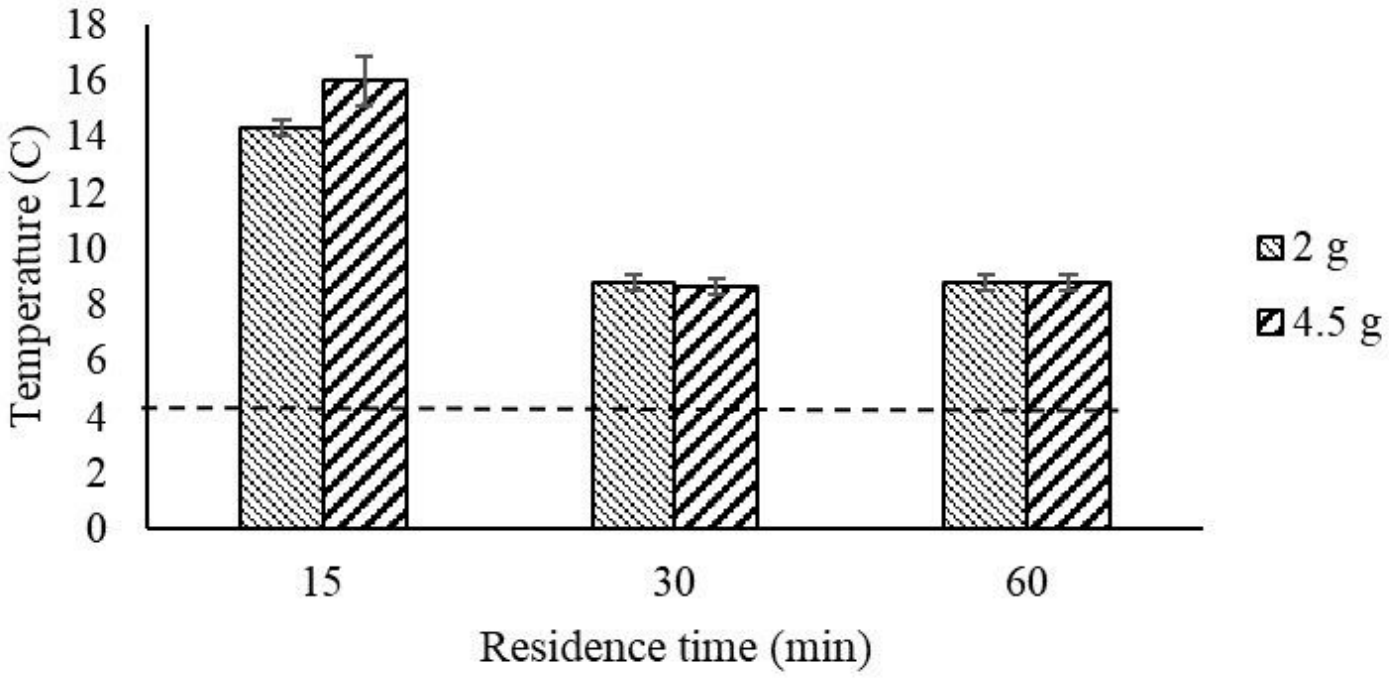

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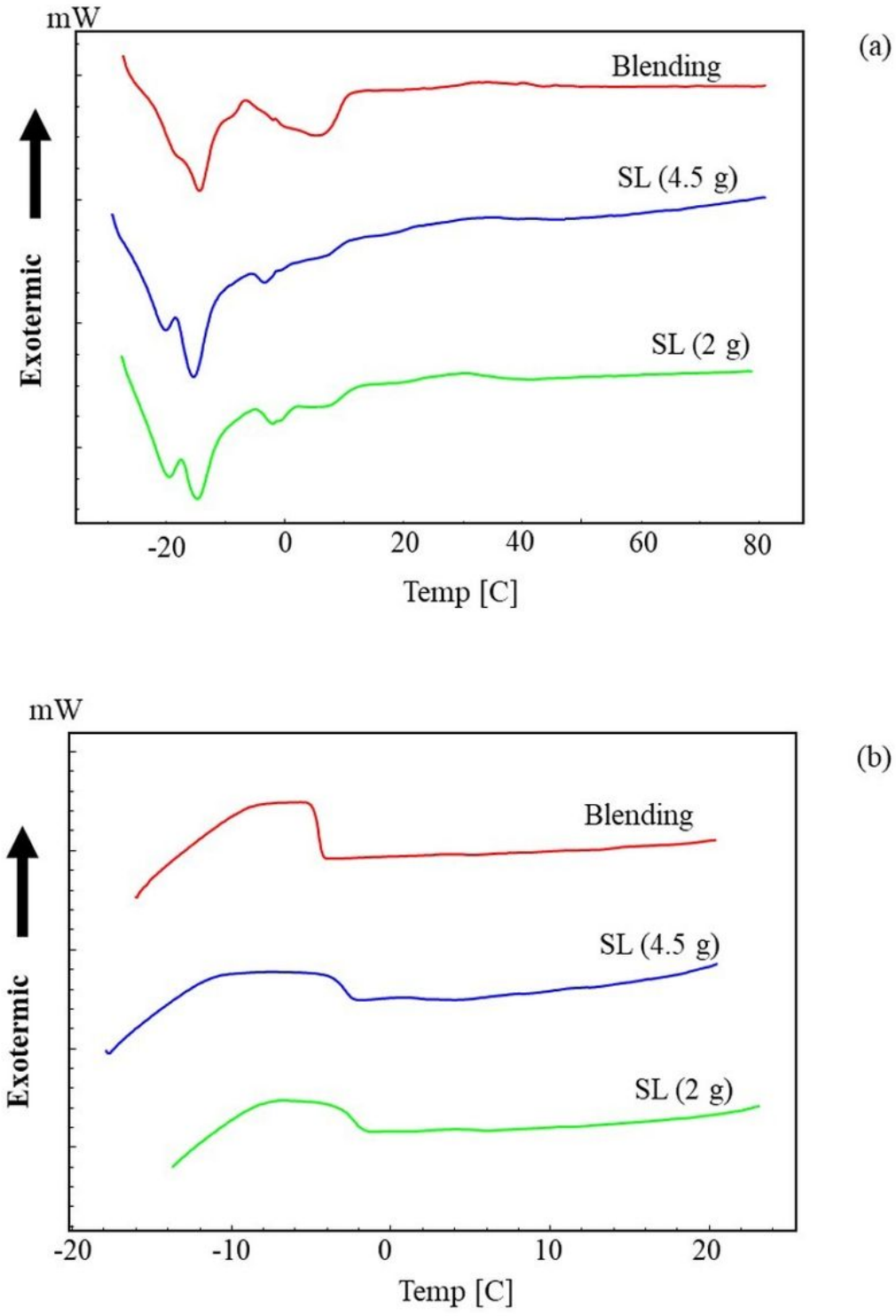

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Differential scanning calorimetry (DSC) melting (a) and crystallization (b) curves of blending and structured lipid product. SL = structured lipid.

Supplementary Files

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- GraphicalAbstractP3ed600.jpg