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Synthesis of structured lipids mimicking human milk fat from palm stearin and oleic acid via enzymatic interesterification using a packed bed reactor

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Abstract

Human milk fat substitute (HMFS) is a structured lipid containing 1,3-dioleoyl-2-palmitoyl-glycerol (OPO) as the primary triacylglycerol. However, conventional infant formulas fail to replicate the regiospecific triacylglycerol structure of human milk, leaving palmitic acid at the outer sn-1,3 positions and reducing fat and calcium absorption in infants. This study utilized a palm fraction to synthesize structured lipids via an interesterification reaction between hard palm stearin and oleic acid at a molar ratio of 1:6, conducted at 60°C for 1 hour and catalyzed by the *sn*-1,3-specific lipase Lipozyme TL IM. The reaction was carried out using a packed bed reactor under two flow configurations: a recirculating (recycle) loop system and a single-pass (direct continuous) flow system.The results indicated that the recirculating system provided optimal conditions for producing structured TAG, yielding 50.78% palmitic acid at the *sn*-2 position and 19.23% oleic acid at the *sn*-1,3positions. Meanwhile, the single-pass system resulted in 46.06% palmitic acid at the sn-2 position and 21.11% oleic acid at the *sn*-1,3 positions. Although the palmitic acid level in the raw material was not fully maximized, the application of a packed bed reactor demonstrated a clear ability to retain palmitic acid at the internal *sn*-2 position during enzymatic structured lipid synthesis.

[Keywords: hard palm stearin, human milk fat substitute, interesterification, lipozyme TL IM, oleic acid, packed bed reactor, structured lipids].

Introduction

Human milk (Human Milk Fat / HMF) is widely recognized as the optimal source of nutrition and energy for infants, covering almost all nutritional requirements during the period when they cannot digest other dietary intakes (Nurcholis, 2013). Fat in breast milk contributes up to 50% of the total energy required for infant nutrition, despite accounting for only 3-5% of the milk composition (H. Lee et al., 2018). The government and the Ministry of Health strongly emphasize the importance of exclusive breastfeeding during the first 6 months; however, national health data indicate that approximately 17.9% of infants experience nutritional deficiencies (Septiani et al., 2025).

This issue arises because many infants cannot receive human milk directly due to various constraints, such as insufficient milk production, maternal health issues, or specific medical conditions. Under these circumstances, infant formula becomes an indispensable alternative to fill the nutritional gaps (Niar et al., 2021; Afrilia et al., 2018) Lipid components are among the most critical elements in infant formula, playing a pivotal role in providing energy and supporting infant growth and development (Sinaga & Siahaan, 2018).

Although commercial infant formulas have advanced significantly, most products on the market still fail to fully replicate the regiospecific lipid structure of human milk, particularly regarding the triacylglycerol (TAG) fatty acid distribution. This specific layout profoundly dictates the efficiency of digestion and absorption in infants; pancreatic lipases

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selectively hydrolyze fatty acids at the external *sn*-1 and *sn*-3 positions, leaving the fatty acid at the central *sn*-2 position as a 2-monoacylglycerol, which is highly bioavailable and easily absorbed (Hageman et al., 2019). Conversely, conventional infant formulas formulated with standard vegetable oils place palmitic acid predominantly at the external *sn*-1,3 positions; upon hydrolysis, these free palmitic acids react with dietary minerals to form insoluble calcium soaps in the intestinal lumen, causing constipation and impeding overall nutrient absorption (Koletzko, 2017).

1,3-dioleoyl-2-palmitoylglycerol (OPO) is a symmetrically structured triacylglycerol that mimics human milk fat and is widely used as a premium ingredient in infant formula. The architecture of OPO features a unique distribution where two oleic acid chains are esterified at the *sn*-1 and *sn*-3 positions of the glycerol backbone. In contrast, one palmitic acid resides at the central *sn*-2 position (Ghide & Yan, 2021). The synthesis of OPO typically involves pure tripalmitin (PPP) or lard as the source of the palmitic core, which is reacted with oleic acid or ethyl oleate using an *sn*-1,3-specific lipase (Tecelão et al., 2019). However, large-scale production of pure tripalmitin is cost-prohibitive, and the use of animal fats, such as lard, is subject to severe restrictions due to cultural and religious constraints (Karouw, 2014).

Enzymatic interesterification offers substantial advantages over chemical methods: exceptional positional selectivity, no formation of trans fatty acids, improved product quality, reduced environmental impact, and operational simplicity (Amir et al., 2015). Continuous-flow systems, particularly packed-bed reactors (PBRs), offer superior productivity and conversion efficiency compared to batch processes (Patel & Niranjan, 1994; Hamam & Budge, 2010). A packed-bed reactor is a tubular column with an immobilized enzyme catalyst, maintained at a constant temperature by an external water jacket. Substrates flow continuously through the enzyme-packed column for catalytic conversion (Denčić et al., 2013; Utama et al., 2022). Continuous and repeated-pass continuous systems offer higher production capacity, improved time efficiency, and greater opportunities for enzyme–substrate contact (Zou et al., 2016).

Previous studies have explored diverse synthetic approaches. Hasibuan (2021) achieved 41% OPO via acidolysis using Lipozyme TL IM with 75.8% sn-2 palmitate and 58.7% sn-1,3 distribution. Xu et al. (2024) synthesized 71.57% sn-2 palmitate via interesterification using lipase UM1 (Xu et al., 2024). Lee et al. (2010) produced 31.43% OPO via transesterification catalyzed by Lipozyme TL IM with 80.6% sn-2 palmitate (Lee et al., 2010). However, comparative investigations using packed bed reactor systems for continuous synthesis remain limited. This study implements a continuous Packed Bed Reactor (PBR) operated under two flow configurations: single-pass direct continuous flow and recirculating (recycle loop) continuous flow. This research aims to evaluate the effectiveness of hard palm stearin (HPS) as a palmitic donor substrate and the performance of the PBR system in synthesizing a high-quality human milk fat substitute that conforms to regiospecific standards.

Materials and Methods

Materials

The primary raw material, refined, bleached, and deodorized (RBD) palm stearin, was generously provided by the Indonesian Oil Palm Research Institute (IOPRI) Medan. The acyl donor substrate, analytical-grade oleic acid, was procured from Tokyo Chemical Industry (Japan). The biocatalyst employed was the immobilized sn-1,3 regiospecific lipase Lipozyme TL IM (50,000 U/g), sourced from Novozymes A/S (Denmark). Analytical chemicals and reagents, including WS 90 thinner, n-hexane, diethyl ether, chloroform, methanol, boron trifluoride (BF3) in methanol, tricaprin, MSTFA, and porcine pancreatic lipase were purchased from Sigma-Aldrich, Merck, and Supelco as pro-analysis grade.

Fractionation of hard palm stearin (HPS)

Solvent fractionation was carried out to isolate and purify HPS from non-triacylglycerol components and other impurities. Briefly, 100 g of RBD palm stearin was blended with 700 mL of WS 90 thinner. The mixture was heated to 50°C under constant magnetic stirring for 3 hours to ensure complete dissolution of crystalline triacylglycerols. The solution was subsequently cooled slowly to room temperature to induce selective crystallization. The precipitated solid fraction (HPS) was separated via filtration and air-dried at ambient temperature until entirely free of residual solvent (Hasibuan, 2021).

Synthesis of structured lipids equivalent to HMFS

The enzymatic interesterification reaction was conducted inside a temperature-controlled packed bed reactor (PBR) unit maintained at 60°C. A 100 g substrate mixture consisting of HPS solid fraction and free oleic acid at a fixed molar ratio of 1:6 was prepared. Lipozyme TL IM enzyme at a concentration of 2.7% (w/w of total substrate) was carefully packed into a water-jacketed cylindrical glass column reactor to ensure isothermal conditions.

The liquefied substrate mixture was driven through the enzyme bed using a calibrated peristaltic pump for a total reaction residence time of 1 hour. The reactor was operated in two comparative configurations: (a) Single-Pass Continuous System, where the effluent was collected immediately after a single transit through the column; and (b) Recirculating Continuous System, where the output was continuously recycled back into the substrate reservoir and passed through the enzyme bed repeatedly.

Analysis of acylglycerol composition and total fatty acids

The acylglycerol distribution was profiled using a Gas Chromatograph (GC) equipped with a Flame Ionization Detector (FID). Samples were prepared by adding tricaprin as an internal standard, derivatized using MSTFA, and diluted with THF and n-hexane prior to injection (Tecelão et al., 2019). For total fatty acid profiling, pure triacylglycerols were isolated via Preparative Thin-Layer Chromatography (PTLC) using an n-hexane:diethyl ether:acetic acid solvent system (80:20:1, v/v/v). The triacylglycerol band was scraped and extracted, saponified with methanolic NaOH, and converted into fatty acid methyl esters (FAME) using boron trifluoride (BF3) reagent at 100°C. The FAME phase was extracted with n-hexane prior to GC injection.

Analysis of regiospecific sn-2 and sn-1,3 positions

The positional fatty acid distribution at the central position was determined via pancreatic lipase hydrolysis, which selectively cleaves the external ester bonds at the *sn*-1 and *sn*-3 positions, isolating the 2-monoacylglycerol (2-MAG) core. The partial hydrolysis products were separated on PTLC plates. The band representing 2-MAG was recovered, methylated into FAME, and quantified by GC. The percentage of fatty acids located at the external positions was calculated using the standard mathematical formula:

$$sn-1,3\ FA = \frac{(3\times total\ FA)-(sn-2\ FA)}{2} \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

Notes:
sn-1,3 FA = Fatty acid on sn-1,3 position (%)
Total FA = Total fatty acid (%)
sn-2 FA = Fatty acid on sn-2 position (%)

Statistical analysis

Fractionation of RBD palm stearin was performed once, and the resulting HPS batch was used as the common substrate for all syntheses. Each interesterification configuration (single-pass and recirculating) was carried out as a single run. Analytical determinations (acylglycerol composition, total fatty acids, and sn-2 regiospecific analysis) were performed in duplicate (n = 2) on samples from that run. Data are presented as mean ± standard deviation of these analytical duplicates, calculated using Microsoft Excel. These values reflect analytical precision only and do not capture batch-to-batch or run-to-run variability of the fractionation or enzymatic process. Consequently, no statistical test of differences between configurations was performed, and comparisons are interpreted as trends.

Results and Discussion

Characterization of raw hard palm stearin (HPS)

Hard palm stearin (HPS), derived via solvent fractionation of RBD palm stearin, serves as the fundamental backbone, providing the palmitic core at the central *sn*-2 position of the target structured lipids. The initial purification using solvent fractionation aimed to remove non-glyceride impurities and minor components, thereby enriching the palmitic fraction. This extraction adheres to the like-dissolves-like principle, in which organic solvents dissolve unsaturated fatty acids while leaving highly saturated solid fats to form pure, crystalline precipitates.

This fractionation yielded substantial shifts in the physicochemical traits of the fat; the purified HPS exhibited a rigid, solid consistency, a higher melting point, and greater enrichment in saturated fatty acids than the starting RBD palm stearin, which was semi-solid. To ensure chemical uniformity of the specific triacylglycerol pool prior to downstream interesterification, an additional separation by Preparative Thin-Layer Chromatography (PTLC) was performed. This high-resolution separation step is vital because thermal dry fractionation has limited selectivity due to strong molecular co-crystallization and the inherently complex mix of natural palm TAGs. The overall fatty acid composition and the regiospecific layout at the *sn*-2 position of the isolated HPS were quantitatively monitored via Gas Chromatography-Flame Ionization Detection (GC-FID). The gathered profile is consolidated in Table 1.

Table 1. Fatty acid composition and regiospecific distribution of raw hard palm stearin (HPS)

Fatty acid type	Total composition (%)	Distribution at sn-2 position (%)
Palmitic acid (C16:0)	80.98 ± 0.19	46.06 ± 11.79
Stearic acid (C18:0)	4.66 ± 0.06	4.36 ± 0.06
Oleic acid (C18:1)	11.20 ± 0.08	45.55 ± 4.97

As detailed in Table 1, palmitic acid (C16:0) was confirmed as the dominant saturated fatty acid component, reaching a high total concentration of 80.98% ± 0.19. Nonetheless, its regiospecific attachment at the central *sn*-2 locus of the glycerol backbone was significantly lower, standing at 46.06% ± 11.79. This lower distribution clearly indicates that a large portion of the native palmitic acid in the HPS matrix remains bound to the outer terminal (*sn*-1,3). Conversely, oleic acid (C18:1), which possessed a minor initial total fraction of 11.20% ± 0.08, was intensely packed at the internal *sn*-2 position with a value of 45.55% ± 4.97.

Compared with historical literature, the HPS batch evaluated in this study showed slightly lower palmitic acid retention at the *sn*-2 position and higher oleic acid levels at the sn-2 position, compared with values reported by Hasibuan (2024): 88% and 3%, respectively. This structural variation affirms that the solid HPS fraction represents a complex, heterogeneous crystalline pool consisting of diverse TAG molecules, including tripalmitin (PPP), dipalmitostearin (PPS), palmitostearopalmitin (PSP), dioleopalmitin (OOP), stearate-palmitat-stearat (SPS), palmitat-oleat-palmitat (POP), triolein (OOO), and associated regioisomers.

Enzymatic interesterification reaction

The design of structured lipids equivalent to human breast milk fat aims to restructure the native acyl distribution of HPS by introducing oleic acid as an active acyl donor, thereby synthesizing the target structured molecule 1,3-dioleoyl-2-palmitoylglycerol (OPO). The transesterification was driven inside a jacketed Packed Bed Reactor (PBR) using the commercial continuous-flow biocatalyst Lipozyme TL IM (an *sn*-1,3 specific immobilized lipase) at an operating temperature of 60°C with an excess molar substrate ratio of 1:6. The throughput efficiency was tested under two distinctive configurations: a single-pass continuous scheme and a continuous recirculating loop.

Visually, the final interesterified products from both operational flow configurations exhibited a prominent structural shift, transitioning from a completely dry, crystalline powder (the raw HPS) to a soft, semi-solid blend at room temperature. This physical modification stems directly from the successful rearrangement of the acyl networks, where long-chain saturated structures at the outer endpoints (*sn*-1,3) were replaced by fluid, monounsaturated oleic chains. A pair of simultaneous reversible hydrolysis and re-esterification reactions govern this enzymatic bioprocess.

The pathway begins with a nucleophilic attack from the hydroxyl (–OH) group of the active serine residue within the catalytic triad (flanked by histidine and aspartate) inside the lipase cavity onto the outer carbonyl ester groups of the HPS at the *sn*-1 or *sn*-3. This attack severs the ester bridge, releasing a free palmitic chain and forming a stable covalent acyl-enzyme intermediate. Next, an external oleic acid molecule enters the active catalytic pocket and initiates a secondary nucleophilic attack on the carbon core of the acyl-enzyme complex. This sequence regenerates the free, functional enzyme while forming a new ester bond, yielding the target-structured molecule (OPO). Operating with an excess of oleic acid (1:6), it leverages Le Châtelier's principle, forcing the reversible chemical equilibrium to the right to optimize structured yields within a short timeframe.

Acylglycerol composition profile

High-resolution gas chromatography was performed to monitor the chemical shifts of the glyceride groups and map the distribution of Triacylglycerols (TAG), Diacylglycerols (DAG), and Monoacylglycerols (MAG) post-reaction. The quantitative distribution is outlined in Figure 1.

The metrics shown in Figure 1 indicate that the interesterification process does not convert the substrate entirely into pure TAG; rather, it yields partial hydrolysis products, including secondary MAG and DAG structures. The recirculating system maintained a higher TAG fraction (76.52%) than the single-pass continuous setup (74.50%). Preserving a dominant TAG level is vital, as it dictates the product's functional physical traits, lipid crystallization kinetics, and final texture profile.

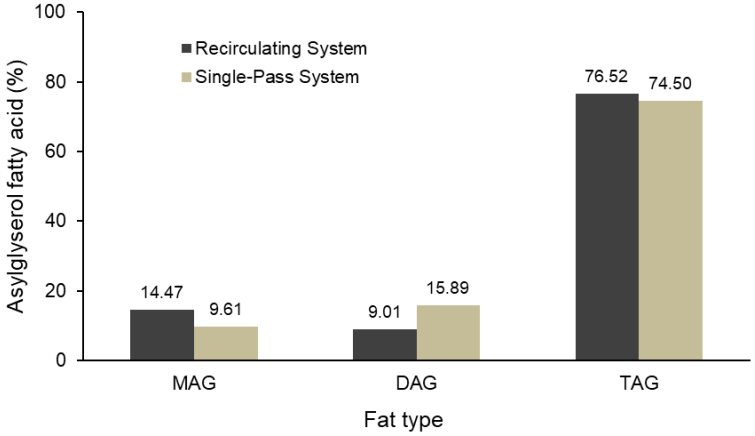


Figure 1. The acylglycerol composition of the interesterification product of hard palm stearin and oleic acid at a molar ratio of 1:6, at 60ºC for 1 hour. MAG= Monoacylglycerols, DAG= Diacylglycerols, TAG= Triacylglycerols.

On the other hand, the DAG concentration in the single-pass reactor was notably higher (15.89%) than that of the recycle arrangement (9.01%). In comparison, the MAG value in the recirculating loop peaked higher (14.47%) than that the direct flow (9.61%). The accumulation of these partial compounds is a natural consequence of the reversible nature of enzymatic interesterification. This buildup aligns with findings by Hasibuan (2024), who reported that extended residence times can accelerate unwanted side hydrolysis. Restricting the reaction duration to 1 hour in this study effectively controlled runaway hydrolysis, achieving efficient conversion while minimizing energy consumption.

From a nutritional biochemistry standpoint, the regiospecific arrangement of the acyl chains is central to human infant lipid metabolism. Standard TAG molecules entering the small intestine undergo strict hydrolysis via gastric and pancreatic lipases, which target only the external *sn*-1 and *sn*-3 ester bonds. This action leaves the central core intact as a stable 2-monoacylglycerol (2-MAG). A 2-MAG molecule that holds a central palmitic core is immediately identified by enterocyte enzymes such as acyl-CoA: diacylglycerol acyltransferase (DGAT) and monoacylglycerol acyltransferase (MGAT) for direct resynthesis into new triacylglycerols. These resynthesized TAGs are efficiently packed into chylomicrons by microsomal triglyceride transfer protein (MTP) and released into the lymphatic network for systemic distribution, serving as essential energy fuels or building blocks for adipose tissue.

Total fatty acid composition analysis

The overall fatty acid distribution of the finalized fats was mapped by converting the fatty acid chains into volatile fatty acid methyl esters (FAME) and analyzing them by GC-FID. The comparative dataset is presented in Figure 2. The data in Figure 2 reveal that the recirculating continuous loop yielded a total palmitic concentration of 66.62% and an oleic incorporation of 24.94%. Conversely, the direct single-pass approach yielded 68.51% palmitic acid and 24.66% oleic acid. No extreme differences in total composition were noted between the two flow designs. The noticeable reduction of total palmitic acid from its initial baseline of 80.98% down to ~67%, paired with the enrichment of oleic acid to ~25%, confirms the successful exchange of acyl groups. The oleic acid incorporation level achieved here (~24-25%) is significantly higher than that reported by Hasibuan (2024), which ranged between 13% and 19%. However, this conversion efficiency remains slightly below the 30% threshold reported by Tecelão et al. (2019) using a *Candida parapsilosis* biocatalyst over an extended 4-hour runtime. Beyond the main palmitic and oleic pools, traces of minor fatty acids were recorded, including myristic (C14:0), stearic (C18:0), linoleic (C18:2), and arachidic (C20:0) acids, all staying well below the 5% mark.

Regiospecific distribution analysis at sn-2 and sn-1,3 positions

To map the enzyme's selectivity and verify the synthesis of structured OPO molecules, regioselective testing was performed to distinguish between the core internal position (*sn*-2) and the outer endpoints (*sn*-1,3). The gathered positional profile is structured in Table 2. As shown in Table 2, the recirculating continuous scheme achieved 50.78% palmitic retention at the central *sn*-2 position and 36.35% oleic migration to the internal position. Meanwhile, the direct continuous scheme yielded 46.06% palmitic acid at the *sn*-2 position and 31.75% oleic acid.

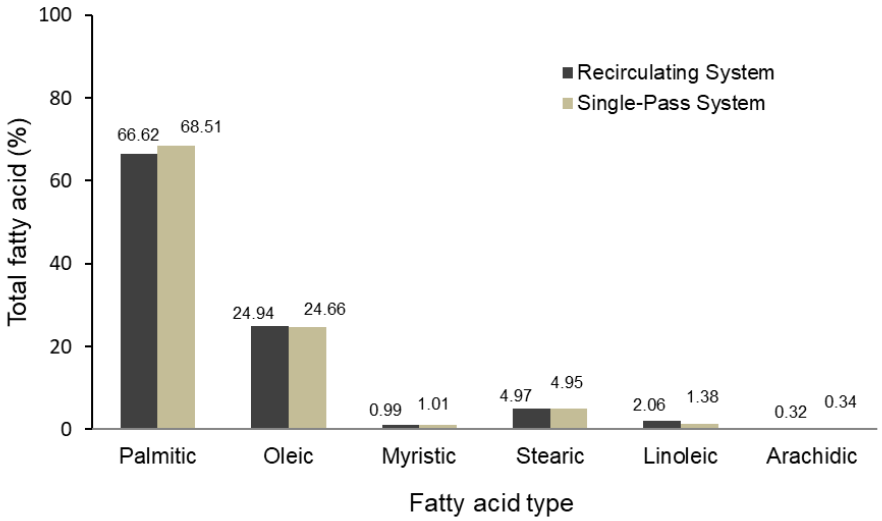


Figure 2. The total fatty acid composition of the interesterified product of hard palm stearin and oleic acid at a molar ratio of 1:6, 60ºC for 1 hour

Table 2. Regiospecific fatty acid distribution at the sn-2 and sn-1,3 positions (%)

System configuration	Palmitic sn-2 (%)	Oleic sn-2 (%)	Palmitic sn-1,3 (%)	Oleat sn-1,3 (%)
Recirculating system (recycle)	50.78	36.35	74.53	19.23
Single-pass system (direct)	46.06	31.75	79.74	21.11

The close preservation of the internal palmitic core relative to its initial raw baseline (46.06%) confirms the capacity of the packed bed configuration to preserve the desired internal fatty acids. The recycled design offered slightly better performance, likely due to enhanced mass transfer and repeated contact between the substrates and the immobilized enzyme bed throughout the continuous cycles. At the outer terminals (*sn*-1,3), oleic acid incorporation reached 19.23% in the recycle loop and 21.11% in the single-pass continuous system. This concentration at the endpoints confirms the synthesis of the target-structured OPO lipid, along with the symmetrical POP isomer. The outer oleic acid accumulation observed here is notably higher than that reported by Hasibuan (2024) which stood at 11%.

This spatial arrangement of fatty acids has critical clinical relevance for human infant digestion. Long-chain saturated palmitic acid (C16:0) located at the internal *sn*-2 tier is cleaved into highly polar 2-monopalmitin, which is readily emulsified and absorbed by the intestinal mucosa without binding crucial minerals like calcium (Ca^{2+}) or magnesium (Mg^{2+}). In contrast, when palmitic acid is located at the outer positions (*sn*-1,3), lipases release it as insoluble free palmitic acid. At typical intestinal pH levels, these free saturated chains react with free calcium ions to form an insoluble precipitate, calcium palmitate soap. The formation of these hard soaps triggers stool hardening, acute infant constipation, and drops the bioavailability of both lipids and calcium. Placing unsaturated oleic chains at the external *sn*-1,3 positions release free oleic acids that form soft, highly soluble calcium soaps, because their cis-double-bond geometry maintains a fluid state at body temperature. In summary, GC confirms the successful synthesis of OPO structured lipids. Nevertheless, to completely isolate the target OPO molecule from its regioisomers (such as POP) based on subtle polar differences, an additional high-resolution purification step using high-performance liquid chromatography (HPLC) is recommended.

Conclusion

The application of a packed bed reactor (PBR) via enzymatic interesterification demonstrates great potential for synthesizing structured lipids (specifically OPO) that mimic human milk fat (HMFS). The recirculating continuous configuration was shown to provide optimal conditions, yielding a high triacylglycerol (TAG) fraction of 76.52% while retaining 50.78% palmitic acid at the central sn-2 position and incorporating 19.23% oleic acid at the external sn-1,3 positions. Meanwhile, the single-pass system resulted in 46.06% palmitic acid at the sn-2 position and 21.11% oleic acid at the *sn*-1,3 positions. However, the ongoing formation of partial hydrolysis side-products (MAG and DAG) and the tendency of the highly saturated HPS raw material to solidify indicate that further optimization of reactor temperature profiles and fluid flow rates is required to maximize process efficiency.

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Declaration

Author contribution

Fauzi, A: Conceptualization, Methodology, Formal analysis, Writing - original draft, **Azzura, R. R**: Investigation, Data curation, Validation, **Amalia, R.**: Resources, Validation, Writing - review & editing, **Hasibuan, H.A**: Supervision, Project administration, Writing - review & editing

The used of Artificial Intelligence (AI) or IA assisted technology

During the preparation of this work, the author(s) used Jenni.ai and Grammarly for improving English language phrasing, grammar, and overall readability. After using this tool, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

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