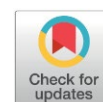


Synthesis of Long-Chain Alkyl Epoxides via Chemoenzymatic Epoxidation of Fatty Acids of *Terminalia catappa* L. Oil

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Received: 4th September 2026; Revised: 22th September 2026; Accepted: 23th September 2026
Available online: 26th September 2026; Published regularly: December 2026



Abstract

Long-chain alkyl epoxides (LCAEs) derived from renewable vegetable oils are promising bio-based chemical intermediates and functional materials. This study aimed to synthesize LCAEs from unsaturated fatty acids derived from *Terminalia catappa* L. seed oil (FA-TCO) via chemoenzymatic epoxidation. TCO was first hydrolyzed using Lipzyme® TL IM, an immobilized lipase from *Thermomyces lanuginosus*, to obtain FA-TCO, which was subsequently subjected to epoxidation using H₂O₂ as the oxygen donor and lipase as the biocatalyst. The effects of reaction parameters were systematically investigated to determine the optimum epoxidation conditions. The optimum conditions were achieved at 50 °C, with an FA-TCO-to-enzyme mass ratio of 10:1 (w/w), an FA-TCO-to-H₂O₂ mass ratio of 5:4 (w/w), and an epoxidation time of 5 h. Under these conditions, the relative conversion to oxirane (RCO) reached 67.67 %, demonstrating effective conversion of C=C bonds into oxirane groups. FTIR analysis confirmed oxirane formation through characteristic bands at 884 and 1247 cm⁻¹. The formation of oxirane groups was further supported by ¹H and ¹³C NMR spectroscopy, which showed resonances consistent with oxirane associated protons at 2.75–2.77 ppm and oxirane carbons at 62.2–62.5 ppm, respectively. Overall, the results demonstrate the feasibility of chemoenzymatic epoxidation for producing FA-TCO-based LCAEs from a renewable feedstock.

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Keywords: *Terminalia catappa* L. seed oil; unsaturated fatty acid; epoxidation; chemoenzymatic; long chain alkyl epoxides

How to Cite: Suhendra, D., Gunawan, E. R., Murniati, M., Liana, B. N. H., Kusmiati, E. (2026). Synthesis of Long-Chain Alkyl Epoxides via Chemoenzymatic Epoxidation of Fatty Acids of *Terminalia catappa* L. Oil. *Bulletin of Chemical Reaction Engineering & Catalysis*, 21 (4), 976-988. (DOI: 10.9767/bcrec.20836)

Permalink/DOI: <https://doi.org/10.9767/bcrec.20836>

1. Introduction

The key structural characteristic of epoxides is the presence of a three-membered oxirane ring consisting of one oxygen and two carbon atoms. This strained ring geometry contributes to its high chemical reactivity, making it widely used as an important intermediate in various chemical industrial processes. Furthermore, its high reactivity and broad spectrum of biological activity make compounds containing the epoxide group an interesting object of study in the fields of pharmaceuticals and drug development [1-3]. The high reactivity of epoxides makes them important

precursors in the development of a wide range of value-added chemical products and bioactive compounds, including polymers, agrochemicals, pharmaceuticals, surfactants, alkaloids, and terpenoids [4,5].

Alkenes are one of the main raw materials in epoxide synthesis. Ethylene oxide (EO), the simplest carbon-chain epoxide, is produced through the epoxidation of ethylene and is the epoxide produced on the largest industrial scale, with a production volume reaching 30.7 million tons in 2021 [6]. Meanwhile, long-chain epoxides can be synthesized from long-chain alkenes through the Prile-schajew reaction, an epoxidation process using meta-chloroperoxybenzoic acid as an oxidizing agent [7].

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However, the alkenes used as raw materials are generally based on petroleum which is non-renewable. As an alternative, vegetable oils can be utilized as a source of alkenes, particularly to produce long-chain epoxides. The use of vegetable oils as a renewable feedstock for bio-based epoxide production can support the transition toward more sustainable chemical synthesis by reducing carbon footprints and reliance on fossil resources. This potential is closely related to the composition of vegetable oil, which is dominated by triglycerides, which are esters composed of fatty acids and glycerol. The presence of C=C bonds in the fatty acid chains of vegetable oils provides reactive sites that allow for oxidation and the formation of epoxide groups. Therefore, the utilization of vegetable oils as a renewable feedstock source offers a promising opportunity for the development of more sustainable bio-based epoxide synthesis routes.

LCAEs, particularly those derived from vegetable oils, possess unique physicochemical characteristics, including amphiphilic properties that allow them to interact with both hydrophilic and hydrophobic phases. These characteristics make LCAEs have the potential to increase added value and expand the functions of various materials [8]. LCAEs have been utilized in various applications, including as more environmentally friendly biolubricants [9], diluents in the coating industry, viscosity regulators, and additives in biolubricant formulations [10]. Furthermore, LCAEs are also used as plasticizers [11] and as raw materials and precursors in the polymer industry, including for the synthesis of polyurethane and polyvinyl chloride foams [12,13].

Conventionally, the synthesis of LCAEs is carried out via the epoxidation of C=C bonds in unsaturated fatty acids derived from vegetable oils. This process generally employs a peroxy-acid as the oxidizing agent to convert the C=C bonds into oxirane rings. In that conventional route, the formation of peroxyacids generally proceeds *in situ* through the reaction of H₂O₂ with an organic acid, such as formic acid, catalyzed by a strong acid, including H₂SO₄. In this case, H₂O₂ acts as a source or donor of oxygen, while organic acids act as oxygen acceptors [14]. However, the use of strong mineral acids as catalysts, especially under epoxidation conditions with relatively high temperatures, can trigger side reactions in the form of opening of the oxirane ring that has been formed. These side reactions can reduce the epoxide conversion efficiency and negatively impact the characteristics and quality of the resulting LCAEs [15,16].

In the epoxidation process, peroxyacids can also be produced *in situ* through a biocatalytic approach by utilizing lipase as a biocatalyst. This process is more accurately categorized as

chemoenzymatic epoxidation, as lipase replaces conventional acid catalysts in the formation of peroxyacids, which subsequently oxidize double bonds in unsaturated fatty acids. A number of previous studies have documented the catalytic activity of various types of lipases in catalyzing epoxidation reactions. Some biocatalysts that have been used include Novozym® 435 [17,18], and lipase isolated from *Cladosporium cladosporioides* 01 [19]. The catalytic ability of lipase remains effective in both aqueous and non-aqueous reaction systems, making it a widely used biocatalyst in various industrial applications. In addition, lipases generally have good stability against various organic solvents and temperature conditions, and show broad tolerance to various types of substrates. The advantages of lipases are also reflected in their ability to catalyze a wide range of chemical transformations with high selectivity, including chemoselectivity, enantioselectivity, and regioselectivity, thereby offering the potential to support the development of more selective, sustainable, and efficient synthetic routes [20].

This study explores the synthesis of FA-TCO-based LCAEs via a chemoenzymatic epoxidation approach. Several previous studies have demonstrated the potential of various vegetable oils as renewable feedstock sources for LCAEs synthesis, including tall oil [21], soybean oil [22], palm oil [23], and sunflower oil [24]. Most of these raw materials are commercial vegetable oils that also have the potential to be used as food ingredients. Based on these considerations, this study explores the potential of FA-TCO as a sustainable alternative feedstock for the synthesis of LCAEs. TCO was chosen because it is a non-commercial and non-edible vegetable oil source that has the potential to provide added value to biological resources that have not been optimally utilized. In addition, lipase is used as an alternative biocatalyst to replace strong mineral acids in the epoxidation process. In this study, lipase acts as a biocatalyst in the hydrolysis stage of TCO to produce FA-TCO. In addition, lipase plays a role in catalyzing the formation of peroxyacids *in situ* through the reaction between FA-TCO and H₂O₂. The resulting peroxyacids then function as oxidizing agents by transferring oxygen atoms to the unsaturated bonds in FA-TCO, resulting in LCAEs. The use of lipase as a biocatalyst in this process offers several advantages over conventional acid-catalyst-based approaches. Biocatalytic processes generally operate under milder operating conditions, including relatively low reaction temperatures, and allow for enzyme reuse when using immobilized lipase systems. Furthermore, this approach has the potential to reduce byproduct formation and result in a more environmentally friendly process. These advantages are related to

the biocatalyst's high activity, specificity, and selectivity toward both the substrate and the catalyzed reaction, thereby increasing process efficiency while minimizing byproduct formation [25].

2. Materials and Methods

2.1 Materials

Lipozyme® TL IM (Novozymes, Denmark) was used as the biocatalyst in this study, while n-hexane served as the solvent for the extraction of TCO from *Terminalia catappa* L. seeds. The chemicals used included glacial acetic acid (99.7%, ACS grade), potassium iodide, methanol, high-purity silica gel, hydrogen peroxide (30%), hydrogen bromide in glacial acetic acid (0.1 N), anhydrous sodium sulfate, sodium hydroxide, n-hexane, Wijs solution, and crystal violet indicator (ACS grade). All chemicals used in this study, unless otherwise stated, were pro-analysis (p.a.) grade reagents supplied by Sigma-Aldrich (Merck, Germany).

2.2. Methods

2.2.1. Hydrolysis of TCO

Enzymatic hydrolysis of TCO was conducted following the procedure reported by [26], with modifications to accommodate the experimental conditions of the present study. Briefly, 10 g of TCO was transferred into a 100 mL Erlenmeyer flask, followed by the addition of 5 mL of distilled water and Lipozyme® TL IM at 2.5% (w/w) relative to the initial TCO mass. The reaction mixture was incubated at 40 °C for 18 h in a shaking water bath. Upon completion of the hydrolysis, the immobilized enzyme was removed by filtration. Subsequently, 20 mL of n-hexane was added to the hydrolyzed mixture, and the mixture was transferred to a separatory funnel to facilitate phase separation and recovery of the FA-TCO fraction. The solvent was then removed under reduced pressure using a rotary evaporator, and the resulting FA-TCO was stored prior to further characterization and epoxidation. The FA-TCO yield was determined from the mass ratio of the recovered FA-TCO to the initial mass of TCO.

2.2.2. Chemoenzymatic epoxidation

The chemoenzymatic epoxidation method was adapted from [27] with some modifications. The reaction system was prepared by mixing 2.5 g of FA-TCO, 0.20–0.40 g of Lipozyme® TL IM, and 25 mL of n-hexane in a 250 mL three-necked flask (equipped with a mechanical stirrer, a reflux condenser, and a thermometer). The reaction mixture was then heated at temperatures of 20–60 °C with continuous stirring at 300 rpm. During stirring, a 30% aqueous H₂O₂ solution was added

dropwise at varying amounts of 1.0, 1.5, 2.0, 2.5, and 3.0 g, corresponding to 8.82, 13.24, 17.65, 22.06, and 26.47 mmol of H₂O₂, respectively. Evaluation of the effect of epoxidation time was carried out at reaction time variations of 3–7 hours. At the end of the reaction, Lipozyme® TL IM was separated from the reaction mixture using a filtration method to recover the biocatalyst. Residual H₂O₂ in the organic phase was removed by repeated washing with excess distilled water. Residual water in the organic phase is removed by drying the phase over anhydrous sodium sulfate. The purified product was comprehensively characterized by determining oxirane oxygen content (OOC), RCO, iodine value, and viscosity, as well as by identifying functional groups and molecular structures using FTIR and NMR spectroscopy.

The selected optimum conditions and the corresponding neighboring conditions were independently repeated three times (n = 3) to assess experimental reproducibility. The results are expressed as mean ± standard deviation (SD). To assess the contribution of Lipozyme® TL IM to the epoxidation process, an enzyme-free control experiment was conducted under the optimum reaction conditions, using the same amounts of FA-TCO, H₂O₂, and n-hexane and the same reaction temperature, stirring rate, and epoxidation time, but without the addition of Lipozyme® TL IM.

2.3. Product Characterization

2.3.1. Oxirane Oxygen Content (OOC)

OOC, representing the oxirane ring content in a compound, is an important parameter for evaluating epoxide group formation during the epoxidation process. In this study, OOC was determined titrimetrically using an HBr solution in glacial acetic acid and calculated based on Equation (1) [14]:

$$OOC (\%) = \frac{N \times V \times 1.6}{W} \quad (1)$$

where *W* represents the sample mass (g), *V* represents the volume of the HBr solution used in the titration (mL), and *N* represents the normality of the HBr solution.

2.3.2. Iodine Value (IV)

Changes in the level of unsaturation during the epoxidation process were evaluated based on the iodine value (IV). The IVs of TCO and the epoxidized product were determined in accordance with ASTM D 5554-95 and calculated using Equation (2) [28].

$$IV = \frac{12.69 \times N \times (V_1 - V_2)}{W} \quad (2)$$

where W represents the sample mass (g), V_1 and V_2 represent the volumes of sodium thiosulfate solution used for the blank and sample titrations (mL), respectively, and N represents the normality of the sodium thiosulfate solution. The value of 12.69 is a conversion factor that represents the amount of iodine equivalent to sodium thiosulfate used during the titration process.

2.3.3. Relative Conversion to Oxirane (RCO)

The epoxidation success rate is determined based on the RCO (%) value, which reflects the amount of C=C bonds successfully converted into oxirane rings and is calculated using Equation (3) [14]:

$$RCO (\%) = \frac{OOC_e}{OOC_n} \times 100 \quad (3)$$

OOC_e , which represents the experimental OOC value, is determined based on Equation (1). Meanwhile, OOC_p , which indicates the maximum theoretical OOC value, is calculated using the following equation:

$$OOC_p = \left[\frac{\left(\frac{IV_0}{2A_i} \right)}{100 + \left(\frac{IV_0}{2A_i} \right)(A_0)} \right] \times A_0 \times 100 \quad (4)$$

where A_i is the atomic weight of iodine (126.9 g.mol⁻¹), A_0 is the atomic weight of oxygen (16.0 g.mol⁻¹), and IV_0 is the iodine value of FA-TCO, the substrate used for the epoxidation reaction.

2.3.4. Viscosity

The dynamic viscosity of the solvent-free epoxidized product was determined using an Ostwald viscometer at 28–29 °C, with distilled water as the reference liquid. The density of the distilled water used as the reference liquid was 0.995–0.996 g.mL⁻¹ at the measurement temperature. The density of each epoxidized product was determined at the same temperature. The flow times of the reference liquid and the sample were measured under identical experimental conditions. The dynamic viscosity value is then calculated using Equation (5) [14]:

$$\eta_L = \frac{\eta_r \times \rho_L t_L}{\rho_r \times t_r} \quad (5)$$

where η_r = viscosity absolute of reference and η_L = viscosity absolute of product; ρ_r = density of reference and ρ_L = density of product; t_r = time of flow of reference and t_L = time of flow of product.

2.3.5. Product characterization

The chemical structure of the synthesized product was confirmed based on its spectral

characteristics obtained through FTIR and NMR analysis. The presence of the oxirane group in the epoxidation product was verified through characterization using ^{13}C and ^1H NMR spectroscopy, focusing on signals related to the chemical environment of carbon atoms and protons in the typical C–O–C structure of the oxirane ring. NMR spectra were obtained using a JEOL-ECP 500 DD2 spectrometer (Agilent Technologies, USA) with resonance frequencies of 125 MHz for ^{13}C NMR and 500 MHz for ^1H NMR, respectively. The NMR spectrum data were then processed and analyzed using Delta 5.0.4 software. The FTIR spectral profiles of TCO, FA-TCO, and LCAEs were analyzed to identify changes in functional groups associated with chemical transformations during the hydrolysis and epoxidation stages. Characterization using FTIR spectroscopy was performed with a PerkinElmer Frontier Spectrophotometer (PerkinElmer, USA), and spectra were recorded in the 4000–650 cm^{-1} wavenumber range in transmission mode (%T).

3. Results and Discussion

Table 1 presents the physicochemical characteristics of TCO produced through the *Terminalia catappa* L. seed extraction process. Enzymatic hydrolysis of TCO (Figure 1), followed by a purification step, yielded 59–60% of FA-TCO. The transformation of triglycerides into free fatty acids was further evaluated by comparing the FTIR spectra of TCO (depicted in next Figure 7a) and FA-TCO (depicted in next Figure 7b). The FTIR spectrum of FA-TCO shows a strong absorption band at 1710–1700 cm^{-1} and a broad band at 4000–3000 cm^{-1} , which can be attributed to the stretching vibrations of carbonyl (C=O) and hydroxyl (O–H) groups, respectively, which are characteristic of carboxylic acid groups. In contrast, the TCO spectrum does not show a clear O–H absorption band in the 4000–3000 cm^{-1} region, while the band at 1710–1715 cm^{-1} is

Table 1. Physicochemical characteristics of TCO.

Parameter	Value
Color	Pale yellow
Iodine value (g I ₂ 100 g ⁻¹)	71.07
Acid value (mg NaOH g ⁻¹)	0.90

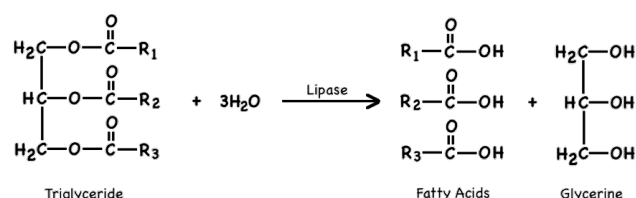


Figure 1. TCO hydrolysis reaction.

related to the carbonyl (C=O) stretching vibration of the ester group. The difference in spectral profiles between TCO and FA-TCO indicates a change in the functional group environment consistent with the cleavage of ester bonds in the triglyceride structure during enzymatic hydrolysis. These results provide spectroscopic evidence supporting the formation of a free fatty acid fraction (FA-TCO) as a hydrolysis product, which is then used as a substrate in the epoxidation stage.

3.1. Chemoenzymatic Epoxidation

In general, epoxides characterized by the presence of an oxirane ring can be synthesized through the oxidation of unsaturated bonds in fatty acids using peroxyacids. The formation of peroxyacid species occurs *in situ* through the transfer of oxygen from H_2O_2 to organic acids as oxygen acceptors under catalyzed conditions. Catalysts used for the in-situ formation of peroxyacids include homogeneous catalysts, such as sulfuric acid [29], heterogeneous catalysts, such as TiO_2 [30] and Amberlite IR-120H [31], as well as enzyme-based catalysts, biocatalysts. The epoxidation reaction was enzymatically catalyzed using Lipozyme® TL IM as a biocatalyst. The use of biocatalysts has several advantages, including allowing the reaction to take place under milder operating conditions, especially at relatively low temperatures, and demonstrating high catalytic activity, selectivity, and specificity towards substrates. Furthermore, the use of biocatalysts is considered a more environmentally friendly approach compared to conventional chemical catalysis systems. Therefore, biocatalytic strategies hold promising prospects for developing more sustainable epoxide compound synthesis.

Chemoenzymatic epoxidation catalyzed by lipases has been widely studied using various substrates, including fatty acids [17,32], fatty acid esters [33], vegetable oils [34], and olefins [5,19]. In this reaction system, epoxidation proceeds within a liquid–solid–liquid triphasic system consisting of immobilized Lipozyme® TL IM lipase as the solid phase, fatty acids as the organic phase component, and H_2O_2 as the oxidizing agent in the liquid phase. One of the main characteristics of the chemoenzymatic approach is that it does not require the addition of external organic acids as oxygen acceptors in the formation of peroxycarboxylic acids, because the carboxyl groups present in the fatty acids themselves can play a role in the formation of these peroxy species. Mechanistically, the reaction is initiated by the perhydrolysis of fatty acids and H_2O_2 catalyzed by immobilized lipase, producing peroxycarboxylic acids *in situ* as active oxidants for the subsequent epoxidation step. Once formed *in situ*, the peroxycarboxylic acid acts as an

oxidizing species that non-enzymatically converts the C=C bonds into epoxide groups. The proposed chemoenzymatic epoxidation reaction pathway for FA-TCO is presented in Figure 2. Reaction conditions, including enzyme loading, epoxidation temperature and time, and H_2O_2 concentration, play a crucial role in determining the efficiency of the epoxidation reaction. Based on these considerations, the influence of each reaction parameter on the epoxidation performance of FA-TCO was systematically evaluated to determine the optimal conditions for LCAEs formation.

3.2. Effect of Enzyme Loading

As an initial step in optimization, enzyme loading was systematically evaluated to identify the most effective amount of lipase in promoting oxirane ring formation from the C=C bond in FA-TCO. The OOC was used as a quantitative indicator of oxirane ring formation or the extent of epoxidation. As shown in Figure 3, the OOC was highly dependent on the amount of enzyme used. The maximum OOC value was achieved at a Lipozyme® TL IM loading of 0.25 g, which corresponds to a fatty acid to enzyme mass ratio of 10, indicating that this enzyme loading provided the most favorable catalytic conditions for FA-TCO epoxidation.

As shown in Figure 3, the OOC value tends to decrease when the mass ratio of FA-TCO to Lipozyme® TL IM is below 10:1. In this chemoenzymatic epoxidation reaction, lipase acts as a biocatalyst in the formation of peroxycarboxylic acid *in situ*, while the species acts as an oxidizer that transfers oxygen atoms to the C=C bond in the FA-TCO chain to form an epoxide group. The decrease in OOC at higher enzyme loadings is thought to be related to the FA-TCO composition, which consists of saturated and unsaturated fatty acids with varying levels of reactivity toward epoxidation. This condition has the potential to increase the occurrence of

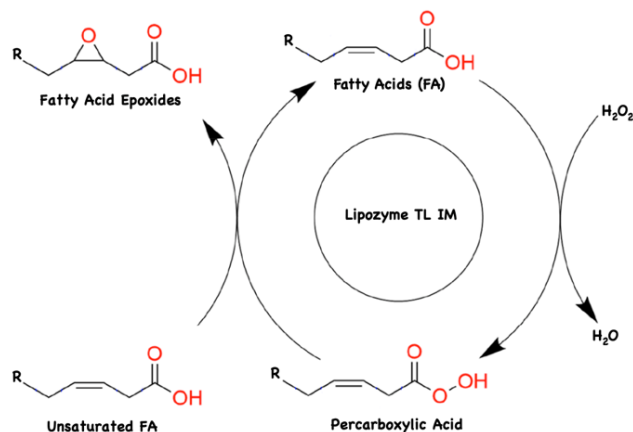


Figure 2. Chemoenzymatic epoxidation of FA-TCO.

undesirable side reactions, both involving peroxy species and epoxide products formed in situ. The decline in epoxidation performance at higher enzyme loadings is likely associated with increased reaction medium viscosity and interphase mass transfer resistance, which can hinder mixing and effective contact between reacting species. This condition can limit effective contact between the enzyme, substrate, and oxidant, thus negatively impacting the performance of the epoxidation process [27,35]. Based on these results, the mass ratio of fatty acids to Lipozyme® TL IM of 10:1 was determined as the optimum enzyme loading condition and was subsequently used in the subsequent epoxidation parameter optimization stage.

3.3. Effect of H₂O₂ Concentration

In the epoxidation reaction, H₂O₂ acts as an oxygen donor involved in the formation of a peroxyacid species, which subsequently transfers oxygen to the C=C bond to form an oxirane ring. Therefore, optimization of the amount of H₂O₂ is necessary to achieve optimal epoxidation efficiency while minimizing excess oxidant remaining in the reaction system. The results showed that the highest OOC was obtained with 17.65 mmol of H₂O₂, corresponding to 2.0 g of the 30% aqueous H₂O₂ solution. Thus, the optimum FA-TCO-to-H₂O₂ solution mass ratio was 5:4 (w/w), based on 2.5 g of FA-TCO and 2.0 g of the H₂O₂ solution (Figure 4).

A decrease in OOC values was observed at high hydrogen peroxide concentrations or at low fatty acid to hydrogen peroxide ratios. This phenomenon is thought to be caused by several factors. First, excessive H₂O₂ concentration can increase the possibility of side reactions, especially oxirane ring opening, thus limiting the increase in epoxidation conversion. The regeneration of carboxylate groups under these conditions can increase the acidity of the system and, in conjunction with the presence of water,

facilitate oxirane ring opening, subsequently yielding dihydroxy compounds [14]. Second, the presence of H₂O₂ in excessive concentrations can negatively affect the catalytic activity of lipase, thereby reducing the enzyme's effectiveness in catalyzing the epoxidation process [27,36].

3.4. Effect of Epoxidation Temperature on OOC

Enzymes are protein-based biocatalysts that facilitate chemical transformations by lowering activation energy through the formation of an enzyme-substrate complex, without undergoing permanent change at the end of the reaction. Structurally, enzymes have a complex three-dimensional configuration with active sites that specifically bind substrates and facilitate their transformation into products. The three-dimensional conformation of enzyme proteins is maintained through a network of molecular interactions, primarily hydrophobic interactions, ionic interactions, and hydrogen bonds, which collectively contribute to their structural stability. The stability of these interactions is strongly influenced by the temperature conditions during the reaction. Therefore, changes in reaction temperature have the potential to affect the structural stability and conformation of the enzyme, which ultimately determines its level of catalytic activity [37,38].

In this study, the OOC value increased with increasing epoxidation temperature and reached a maximum value at 50 °C (Figure 5). Increasing temperature increases the kinetic energy of the substrate and enzyme, thereby increasing the frequency and effectiveness of collisions between the substrate and the enzyme's active site, ultimately promoting the formation of oxirane groups. However, excessively high temperatures can cause enzyme denaturation and disrupt the integrity of the active site. Furthermore, in chemoenzymatic epoxidation, temperatures above 30–50 °C can trigger oxirane ring opening, thereby decreasing the OOC value [39,40].

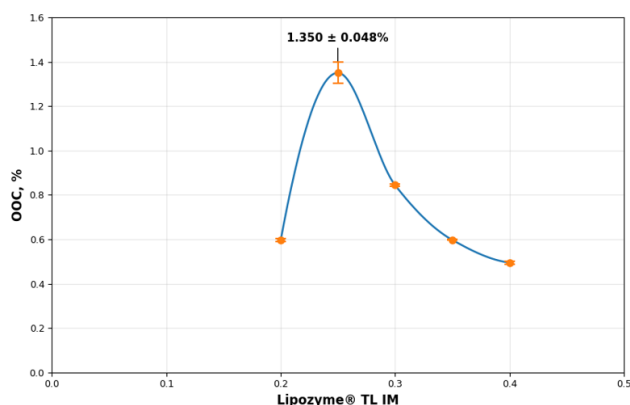


Figure 3. Effect of enzyme loading (H₂O₂ of 22.06 mmol; temperature of 50 °C; epoxidation time of 4 h).

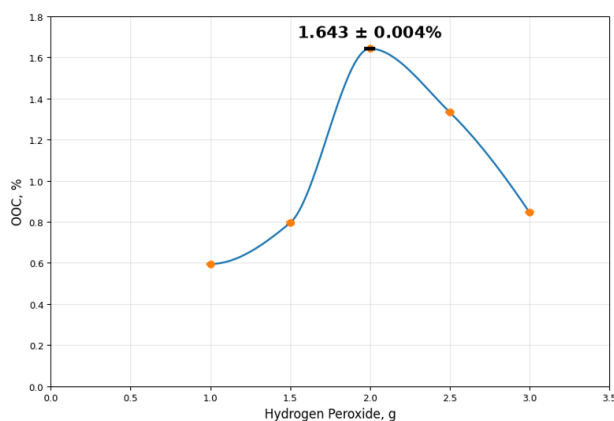


Figure 4. Effect of H₂O₂ concentration (temperature of 50 °C, amount of enzyme of 0.25 g, epoxidation time of 4 h).

3.5. Effect of Epoxidation Time on OOC

Reaction time and temperature are important parameters in chemoenzymatic epoxidation because they affect enzyme stability and catalytic activity. As shown in Figure 6, the OOC value increases with increasing epoxidation time. An increase in reaction time provides a greater opportunity for interaction between the substrate and the enzyme during peroxy-acid formation, as well as between the peroxy-acid and the unsaturated bond, subsequently leading to the formation of an oxirane ring. Thus, increasing the epoxidation duration has the potential to increase the degree of conversion of C=C bonds into epoxide groups.

After reaching the optimum reaction time, the OOC gradually decreased, suggesting that extending the reaction beyond 5 h did not promote further oxirane formation. This decline may be associated with a decrease in the catalytic activity of Lipozyme® TL IM during prolonged exposure to the reaction conditions. As the enzyme activity decreases, the formation of peroxycarboxylic acid may also be limited, thereby reducing the extent of further epoxidation of the remaining C=C bonds [37]. At the same time, the oxirane groups formed during the reaction may undergo secondary reactions, including ring opening, which could reduce the amount of epoxide groups retained in the final product [41]. These processes have different effects on the reaction outcome: reduced enzyme activity primarily limits the formation of new oxirane groups, whereas ring opening may consume oxirane groups that have already been formed. Thus, the decrease in OOC after 5 h may reflect a reduction in net oxirane formation as the reaction progresses, resulting from limited further epoxidation and the possible occurrence of secondary reactions involving the formed oxirane groups. This epoxidation time is shorter than the chemoenzymatic epoxidation of soybean oil (*Glycine max* L.) for 11 hours [42] and Keranja oil

(*Pongamia pinnata*) for 8–9 hours [36]. Variations in optimum epoxidation time can be attributed to differences in substrate characteristics, including their composition and reactivity, as well as the type and catalytic characteristics of the lipase used.

The FTIR spectra further support the formation of oxirane groups, as indicated by the appearance of characteristic bands at 1247 and 884 cm^{-1} and the disappearance of the C=C-related bands at 3008 and 1644 cm^{-1} . However, the O–H absorption observed at 3429 cm^{-1} in the epoxidized product should be interpreted with caution because FA-TCO itself exhibits an O–H band at 3433 cm^{-1} (Table 3). Therefore, the presence of the O–H band in the product cannot, by itself, be considered conclusive evidence of oxirane ring opening. Rather, it indicates the presence of hydroxyl-containing species, which may originate from the starting FA-TCO and/or from secondary reactions occurring during epoxidation. Accordingly, oxirane ring opening may have occurred as a secondary reaction, but its occurrence cannot be confirmed solely from the FTIR O–H absorption.

3.6. Product Characterization

Characterization was performed on the epoxide products obtained under optimum chemoenzymatic epoxidation conditions, as summarized in Table 2. Under the optimum epoxidation conditions, the experimental OOC_e was 1.9421%, while the OOC_p calculated from the iodine value of FA-TCO (46.96 g $\text{I}_2/100$ g) was 2.987%. Accordingly, the RCO was calculated to be 67.67% using Equation (3). This value is higher than the RCO of 48.94% reported by previous researcher [43] on the epoxidation reaction of tung oil (*Reutealis trisperma*) with H_2SO_4 as a catalyst. These differences indicate that the chemoenzymatic process has the potential to be a more efficient alternative

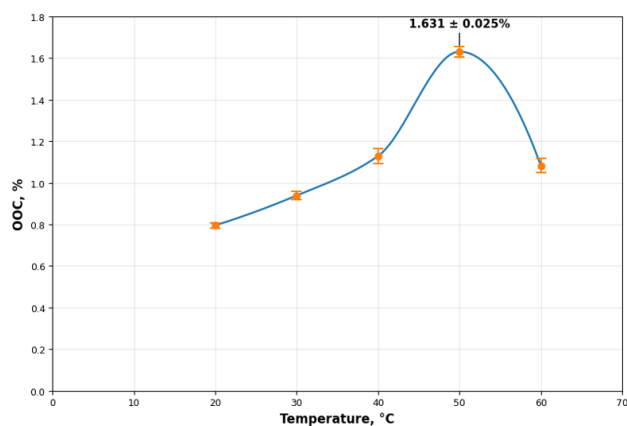


Figure 5. Effect of epoxidation temperature (amount of enzyme of 0.25 g, concentration of H_2O_2 of 17.65 mmol, epoxidation time of 4 h).

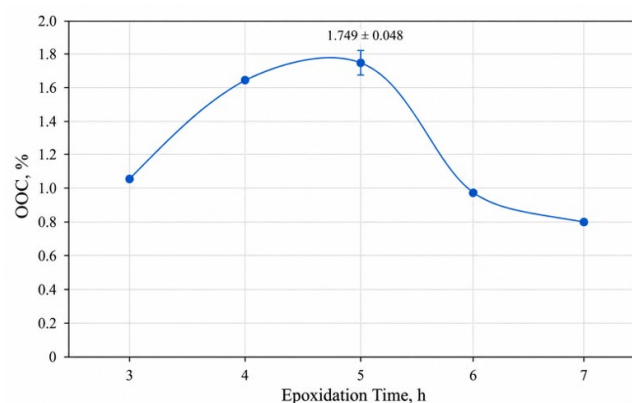


Figure 6. Effect of epoxidation time (amount of enzyme of 0.25 g, concentration of H_2O_2 of 17.65 mmol, temperature of 50 °C).

compared to the use of homogeneous catalysts, including HCl and H₂SO₄, which have corrosive properties in the epoxidation process [31]. Despite exhibiting good epoxidation performance, the RCO obtained in this study (67.67%) is still lower than the 77% reported by [42] using Novozym 435 in an ionic liquid medium for the epoxidation of soybean oil. The differences in RCO values are likely related to variations in substrate characteristics, lipase type, and the reaction media used.

3.6.1. FTIR, ¹H-NMR and ¹³C-NMR

The FTIR spectral profiles of TCO, FA-TCO, and epoxidized of FA-TCO are shown in Figure 7, and their characteristic absorption bands are listed in Table 3. The FTIR spectrum of the epoxidized product shows characteristic absorption bands at 884 and 1247 cm⁻¹, which can be assigned to C–C and C–O vibrations associated with the oxirane ring, respectively, supporting the formation of epoxide groups (Figure 7c). In the FA-TCO spectrum (Figure 7b), absorption bands at 3008 cm⁻¹ and 1644 cm⁻¹, corresponding to =C–H and C=C vibrations, respectively, were clearly observed but were no longer detected after epoxidation (Figure 7c). The disappearance of these bands indicates that the unsaturated C=C bonds were consumed during the reaction, consistent with their conversion into oxirane groups. The epoxidized product also exhibits a broad O–H absorption band at 3429 cm⁻¹.

Table 2. Optimum operating conditions of the chemoenzymatic epoxidation of FA-TCO.

Parameter	Optimum condition
Epoxidation time (h)	5
FA-TCO-to-30% H ₂ O ₂ solution mass ratio (w/w)	5:4
Reaction temperature (°C)	50
FA-TCO-to-enzyme mass ratio (w/w)	10:1

However, this feature should be interpreted cautiously because FA-TCO already shows an O–H band at 3433 cm⁻¹ (Table 3). Thus, the O–H absorption in the product cannot be attributed solely to oxirane ring opening. Instead, it indicates the presence of hydroxyl-containing species, which may originate from the starting FA-TCO and/or secondary reactions occurring during epoxidation. Therefore, while the FTIR results clearly support the formation of oxirane groups, the O–H absorption alone does not provide conclusive evidence for oxirane ring opening.

The observed differences in FTIR profiles between the substrate and the product indicate a change in chemical structure consistent with the formation of epoxide groups during the epoxidation reaction. The disappearance of the C=C stretching band in the FTIR spectra of TCO and FA-TCO, along with the appearance of vibration bands associated with C–O and C–C of epoxide groups, provides strong evidence for the formation of an epoxide structure during the epoxidation process.

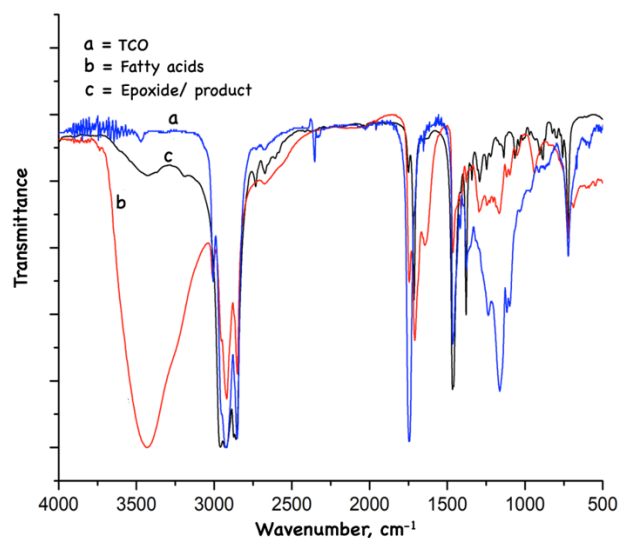


Figure 7. FTIR spectrum of TCO (a) , FA-TCO (b) and epoxide/ product (c).

Table 3. Characteristic absorption bands of TCO, FA-TCO, and the product.

Functional Group	Wavenumber (cm ⁻¹)			References
	TCO	FA-TCO	Epoxide	
=C-H	3005	3008	-	-
Alkyl (CH ₂ -/CH ₃ -)	2923 and 2852	2918 and 2850	2926 and 2859	2927 and 2855 ^[14]
C=O	1745	1708	1714	1744,5 ^[14]
C=C	1653	1644	-	-
C-O in epoxide	-	-	1247	1239 ^[14]
C-C in epoxide	-	-	884	840-956 ^[46]
C-C	721	720	724	723 ^[46]
-OH	-	3433	3429	3473 ^[47]

The ^1H NMR spectra of FA-TCO and the epoxidized product are compared in Figure 8 to elucidate the structural changes associated with epoxidation. FA-TCO exhibited resonances in the olefinic region at approximately 5.31–5.37 ppm, characteristic of protons in unsaturated fatty acid chains. Upon epoxidation, the changes in this region were accompanied by the emergence of a resonance at 2.75–2.77 ppm, which is consistent with protons associated with the oxirane moiety. The signals at 1.25–1.45 ppm remained prominent in the product spectrum, corresponding primarily

to methylene groups along the long hydrocarbon chains. The ^1H NMR spectral profile of the product exhibits a pattern of changes comparable to that reported by [44] on the epoxidation of kapok seed oil. These spectral changes are consistent with the conversion of C=C bonds during epoxidation. In conjunction with the FTIR results, which indicate the consumption of C=C bonds and the formation of oxirane-related functional groups, the ^1H NMR data provide complementary evidence for the formation of the epoxidized product.

The ^{13}C NMR spectra of FA-TCO and the epoxidized product are compared in Figure 9 to assess the structural changes resulting from epoxidation. The product exhibits distinct resonances at 62.2 and 62.5 ppm, which are consistent with oxirane carbons and fall within the reported chemical-shift range of 56–63 ppm for epoxidized fatty-acid derivatives [44,45]. The

Table 4. Physicochemical properties of FA-TCO and LCAEs.

Sample	IV (g I ₂ /100 g)	Viscosity(cP)
FA-TCO	46.96	2.94
LCAEs	11.67	27.32

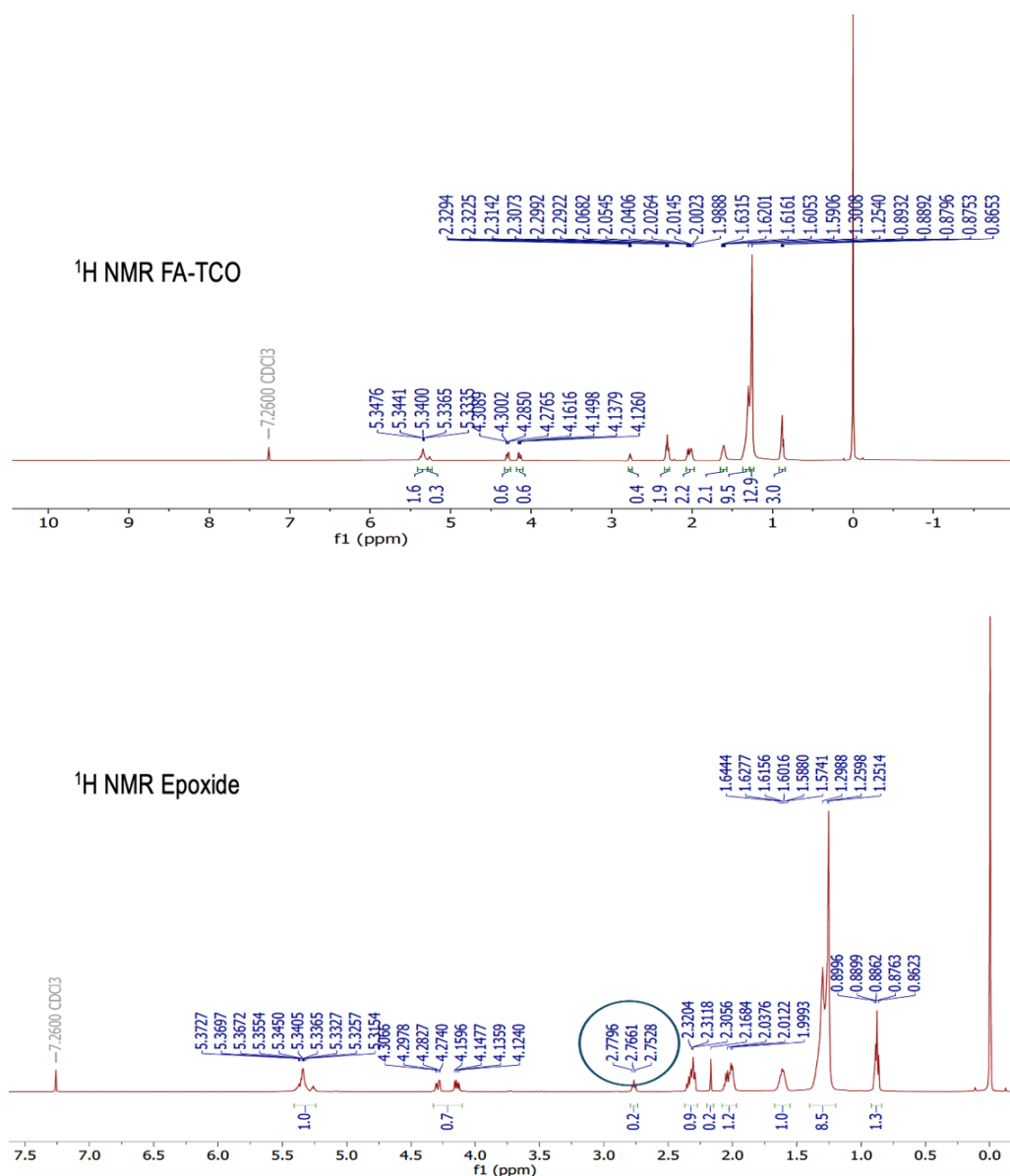


Figure 8. ^1H -NMR Spectra of FA-TCO and epoxide.

emergence of these resonances, together with the changes observed relative to FA-TCO, supports the formation of oxirane groups through the conversion of C=C bonds. These ^{13}C NMR findings, considered alongside the ^1H NMR and FTIR results, provide complementary evidence for the successful epoxidation of FA-TCO.

3.6.2. Iodine value (IV) and Viscosity

Iodine value (IV) and viscosity were also determined as complementary physicochemical parameters to evaluate the degree of epoxidation. Both parameters show a strong correlation with the unsaturation of a fatty acid. The iodine value is a parameter reflecting the degree of unsaturation of an oil or fat, with a higher value indicating a greater content of C=C bonds in the oil. Higher degrees of unsaturation generally correlate with lower viscosity, as a result of reduced intermolecular interactions and increased disorder of the fatty acid chain structure. Thus, changes in IV and viscosity

values can be used as indirect parameters to indicate the conversion of C=C bonds into epoxide groups in epoxidation reactions.

The iodine value (IV) of TCO was determined to be 71.07 g $\text{I}_2/100$ g, whereas the purified FA-TCO exhibited a lower IV of 46.96 g $\text{I}_2/100$ g. Because this decrease occurred during the hydrolysis and purification stages, prior to epoxidation, it cannot be directly attributed to the consumption of C=C bonds through oxirane formation. Instead, the observed difference may reflect changes in the composition and recovery of the lipid fraction following hydrolysis and purification. Since the distribution of residual glycerides and individual unsaturated fatty acids was not quantitatively determined before and after purification, the decrease in IV cannot be unequivocally attributed to a specific mechanism. Therefore, the measured IV of 46.96 g $\text{I}_2/100$ g was used as the experimental baseline for assessing the extent of unsaturation reduction during the subsequent epoxidation process.

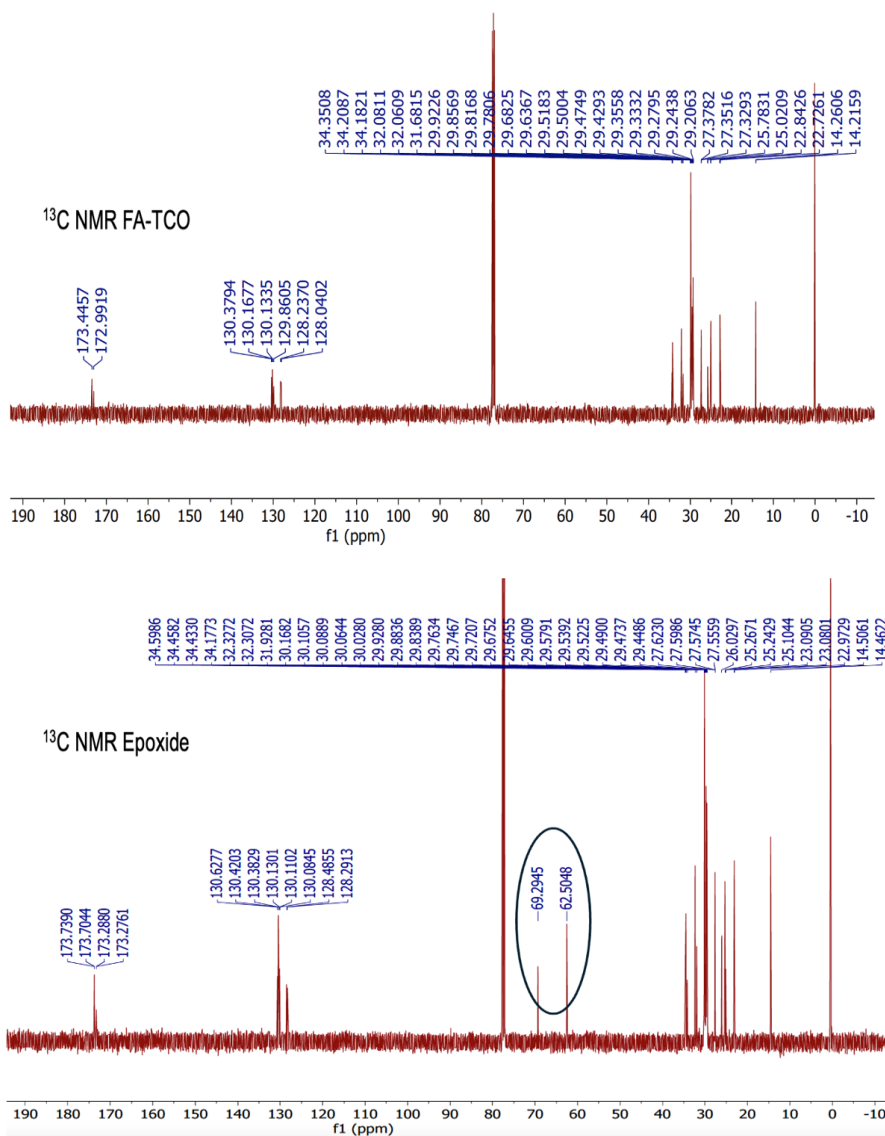


Figure 9. ^{13}C -NMR Spectra of FA-TCO and epoxide.

As shown in Table 4, the IV value decreased significantly from 46.96 g I₂/100 g in FA-TCO to 11.67 g I₂/100 g in LCAEs. The decrease in IV value from FA-TCO to epoxidation product indicates a decrease in the degree of unsaturation due to the consumption of C=C bonds and the formation of epoxide groups during the epoxidation reaction. Although the IV value decreased significantly, the value still measured in the product showed the presence of residual C=C bonds, which indicates that some of the unsaturation sites of FA-TCO have not been converted to oxirane rings during epoxidation. In line with the decrease in IV, the viscosity increased significantly from 2.94 to 27.32 cP after epoxidation. The increase in viscosity from FA-TCO to the epoxidized product reflects the change in molecular characteristics accompanying the conversion of C=C bonds into oxirane structures, thereby providing supporting evidence for the success of the epoxidation process.

4. Conclusion

Characterization results indicate a decrease in the degree of unsaturation of FA-TCO and the formation of an oxirane ring, collectively supporting the successful synthesis of LCAEs via chemoenzymatic epoxidation. Optimization results indicate that the optimum conditions for chemoenzymatic epoxidation were achieved at an FA-TCO-to-enzyme mass ratio of 10:1 (w/w), an FA-TCO-to-H₂O₂ mass ratio of 5:4 (w/w), a reaction temperature of 50 °C, and an epoxidation time of 5 hours, yielding an RCO of 67.67%. FTIR, ¹H NMR, and ¹³C NMR characterization collectively support the formation of an oxirane structure, indicating the successful epoxidation of FA-TCO. Physicochemical characterization results indicate that LCAEs has a viscosity of 27.32 cP and an IV of 11.67 g I₂/100 g. The decrease in IV and increase in viscosity following epoxidation provide supporting evidence for the reduction of FA-TCO unsaturation and the formation of epoxide structures, collectively confirming the successful synthesis of LCAEs.

Acknowledgment

The author expresses appreciation and gratitude to the Directorate of Research, Technology, and Community Service, Ministry of Education, Culture, Research, and Technology of the Republic of Indonesia, for the funding support provided through the 2024-2025 Basic Research Scheme.

CRedit Author Statement

Author Contributions: D. Suhendra: conceptualization, methodology, writing original draft, final review and editing; E.R. Gunawan: conceptualization, project administration,

funding acquisition & editing, revising manuscript; Murniati: data validation, review and editing, data curation; B.N.H. Liana: investigation, data curation; E. Kusmiati: investigation, data curation. All authors have read and agreed to the published version of the manuscript.

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