

Isolation on Unsaturated Fatty Acids from *Terminalia Catappa* L. Seed Oil Using Urea Crystallization Method as A Basic Material for Dihydroxy Octadecanoic Acid

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Abstract

Dihydroxyoctadecanoic acid (DHOA) is a fatty acid-based compound with industrial applications along with cosmetic applications. This compound is synthesized by chemical transformation of unsaturated fatty acids in vegetable oil, one of which is *Terminalia catappa* L. (TC) seed oil. In this study, we have tried to synthesize and characterize DHOA by taking the unsaturated fatty acid (UFA) fraction of TC seed oil, enriched by urea crystallization and then used as a precursor for DHOA synthesis with a sequence of epoxidation and hydroxylation reactions to convert double bonds into dihydroxy groups with an epoxide intermediate. The unsaturated fatty acid fraction was a viscous yellow liquid with a maximum yield of 27.14% at a urea to fatty acid ratio of 8.57:1 (w/w). The fraction contained an iodine value of 74.23 mg I₂ g⁻¹ and a hydroxyl value of 33.66 mg KOH g⁻¹ indicating unsaturation and hydroxyl groups (–OH) in the product. GC-MS showed that the urea crystallization product had 73.28% unsaturated fatty acids. The resulting DHOA was a pale yellow semi-solid with an oxirane oxygen content (OOC) of 0.051% and a hydroxyl value of 186.96 mg KOH g⁻¹. The product was characterized by low residual epoxide groups and hydroxyl groups in the product. The spectroscopic characterization of the product showed that an O–H absorption band in the FT-IR spectrum at 3432.84 cm⁻¹ was present and that the epoxide C–O–C band was lost. The ¹³C NMR spectrum showed a resonance at approximately δ 74 ppm indicating hydroxylated carbon and the ¹H NMR spectrum at approximately δ 3.3 ppm indicated a methine proton in a –CH–O– environment. Overall, these spectroscopic results provide structural evidence for the formation of DHOA.

Keywords

Dihydroxy Octadecanoic Acid, Unsaturated Fatty Acids, Urea Crystallization, *Terminalia catappa* L. Seed Oil

Received: 4 May 2026, Accepted: 14 August 2026

<https://doi.org/10.26554/sti.2026.11.4.1549-1565>

1. INTRODUCTION

Dihydroxystearic acid (DHSA), also known as 9,10- dihydroxy-octadecanoic acid (DHOA), is a hydroxylated fatty acid which is of high functional and commercial value. The hydroxyl groups present in the hydrocarbon chain (DHOA) and carboxyl group at the end of the chain provide an interesting chemical structure and a strong intermolecular interaction (Hadi et al., 2023). The hydroxyl compounds can be considered to enhance the functional properties of DHOA which can be used in various oleochemical applications. In the cosmetics field, the emulsifying agent is used as a stabilizing agent for oil or wax and water phases (Mahadi et al., 2022; Ismail et al., 2024). DHOA can be produced from unsaturated fatty acids (UPAs) in epoxidation and hydroxylation processes, and in situ using formic acid and peroxide as oxidative reagents (Jalil et al., 2021). Various vegetable oils have been shown to be sources of UPAs used for DHOA synthesis: coconut oil (Boateng et al., 2016), soybean oil (Lee et al., 2016), palm oil (Mancini et al., 2015; Ng et al.,

2025; Silvianti et al., 2018), peanut oil (Akhtar et al., 2014), olive oil (Revelou et al., 2021), and avocado oil (Flores et al., 2019). While these sources are commercially available, they have economic value and play a significant role in the food supply. Utilizing them as oleochemical feedstocks can compete with food requirements. It is therefore important to explore non-commercial and underutilized oil sources in order to find more sustainable feedstocks for DHOA production.

Non-commercial vegetable oils represent a potential source of raw material for the production of bio-based compounds, as their use does not directly compete with food supplies and allows for the utilization of underused lipid resources (Atabani et al., 2013). One interesting source to explore is TC, known as tropical almond and widely grown and cultivated as a shade plant in tropical regions, including Southeast Asia (Silalahi, 2022; Ramanan S et al., 2025). The relatively abundant availability of its seeds positions the plant as a potential source of alternative vegetable oil. However, the utilization of TC seed

oil is still relatively limited, so its development as a value-added oleochemical raw material still has broad opportunities (Gunawan et al., 2023).

The oil content of TC seeds is reported to be 55–65%, although the yield can be determined differently from the different studies. Orhevba et al. (2016) reported an oil yield of 50.33%, whereas Suhendra et al. (2018). and Menkiti et al. (2015). reported yields of 59.75% and 60.45%, respectively. These yield variations suggest that the oil content in TC seeds could be influenced by genetic factors, agroecological and farming processes. Despite these variations, a number of studies have shown that TC seed oil has a fatty acid profile dominated by unsaturated elements (Halaldeen et al., 2024). (Suhendra et al., 2018) report that oleic acid (C18:1) is the dominant UFA, accounting for 38–39%, linoleic acid (C18:2) for 20–21% and palmitoleic acid for 0.3–0.5%, while saturated fatty acid is made up of palmitic (35–36%) and stearic (4–5%). This indicates that TC seed oil is highly unsaturated fatty acid rich and thus is a good source for development of vegetable oil-based derivatives as a raw material for vegetable oil-based derivative production (Gunawan et al., 2023). The unsaturated fatty acids are highly abundant in TC seed oil so the unsaturated fatty acid content is a strategic concern since it provides a large number of carbon–carbon double bonds and can be used for chemical modification with the presence of the double bonds. In the epoxidation process, the double bond becomes the reaction center and epoxide can be used as the intermediates that can be converted into diol compounds (such as DHOA) and therefore, the high unsaturation in TC seed oil is significant for the production of DHOA as it indicates that it can be a renewable feedstock for DHOA synthesis, via the selective double-bond functionalization (Cogliano et al., 2024; Habri et al., 2024).

TC seed oil is promising as a potential feedstock for DHOA synthesis, compared to other non-commercial vegetable oil sources, such as *Calophyllum inophyllum* oil (CIO) and *Jatropha curcas* oil (JCO). Although CIO is reported to have a high oil yield (~75%), its unsaturated fatty acid content may limit its use as a substrate for epoxidation reactions. Low unsaturation has consequences for the low number of carbon–carbon double bonds that can be used as the active groups for epoxide reactions (Crane et al., 2005; Jain et al., 2018; Kusumaningtyas et al., 2022). JCO has a relatively high oleic acid content and good reactivity in many chemical transformation processes. However, the use of this oil as a raw material for DHOA synthesis is still a challenge due to the presence of anti-nutritional and toxic compounds, especially phorbol esters, that need to be detoxified or purified before use. By doing so, such pre-processing steps can increase energy use, operational costs, and complexity of the whole production process (Cao et al., 2025; Silitonga et al., 2013). With the fatty acid profile, reactivity, and processing factors of TC seed oil, we are able to develop it as an alternative material for DHOA synthesis. Therefore, further study on the properties, reactivity, and potential applications can be done on TC seed oil in order to develop sustainable, renewable resource-based, value-added chemicals.

Many approaches have been developed to obtain and separate unsaturated fatty acids from vegetable oils, such as column chromatography, methanol crystallization, and urea crystallization (AOAC International, 1996; Gunawan et al., 2023; Jumaah et al., 2019; Yong et al., 2021). While column chromatography can produce fatty acid fractions of very high purity, its applications are still limited due to the long separation time and the high level of chemicals needed for preparation and modification of silica-based stationary phases. Conversely, methanol crystallization can use the difference in solubility between fatty acids as a basis for separation in a relatively simple way, but the selectivity for fatty acids is not very high. On the other hand, urea crystallization is easier to separate and has a higher selectivity, and therefore the separation efficiency can be better than those. The reason for the separation principle is that fatty acids do not form inclusion complexes with urea in the molecular structure. Saturated fatty acids and some monounsaturated fatty acids can be incorporated into the crystalline structure of urea, whereas fatty acids with higher degrees of unsaturation are more likely to remain in the liquid phase. This process of urea inclusion complex formation allows the enrichment of unsaturated fatty acids efficiently. According to several previous studies, urea crystallization can recover above 80% with purity up to 98%, while column chromatography generally recovers lower from 50–60% (González-Fernández et al., 2020). Besides the high separation efficiency, this method provides a simple procedure, relatively low costs, and a promising growth potential for larger-scale applications (Liu and Tao, 2022; Rastegari et al., 2022). For this reason, urea crystallization was selected as the separation method for this study as it allows enriching UPAs in a chemical way without affecting the chemistry of the UPAs and requires less organic solvent, making it more efficient and sustainable for the separation process.

Urea crystallization was used to increase the percentage of unsaturated fatty acids in the fraction obtained from TC seed oil. The separation process is based on urea forming inclusion complexes with straight-chain fatty acids, particularly saturated fatty acids. However, double bonds in unsaturated fatty acids distort the carbon chain structure and thus block the formation of inclusion complexes with urea and keep these components in the liquid phase. After the crystallization process, the liquid fraction and solid fraction are separated by filtration or centrifugation (Japir et al., 2018; Setyawardhani et al., 2018). The effectiveness of this method is related to the urea-to-fatty acid ratio. While an excessively low urea ratio can lead to the formation of inclusion complexes, excess urea can lead to the inefficient separation process. Therefore, the urea-to-fatty acid ratio is a key parameter to obtain unsaturated fatty acid fractions with the desired composition and enrichment. The enriched fraction is used as a precursor in the synthesis of DHOA. There are no studies on the optimization of the urea-to-fatty acid ratio as a parameter to achieve high enrichment and recovery of UPAs. This work aims at elucidating the ideal fractionation process conditions for high recovery of UPAs in a sustainable and efficient manner.

2. EXPERIMENTALL SECTION

2.1 Materials

The equipment used consisted of standard laboratory glassware and several supporting instruments: a pH meter, a Soxhlet extraction apparatus equipped with a condenser, a rotary evaporator (B-ONE RE-1000VN), a shaking water bath (Biosan WB-4MS), a vacuum filtration system, and an analytical balance. The functional group characterization and semi-quantitative analysis of the composition were performed using a PerkinElmer Spectrum Two FTIR spectrometer. GC-MS analysis was performed to detect components and to determine the relative molecular mass of the product using a Stabilwax-DA column (30 m × 0.25 mm × 0.25 μm; polyethylene glycol) with a temperature ramp of 5 °C min⁻¹ from 40–250 °C. The molecular structure was determined with an Agilent DD2 NMR spectrometer with 500 MHz of frequency for ¹H and 125 MHz for ¹³C. The spectra were taken at 298 K using CDCl₃ as the solvent, and the residual solvent signal is used as the basis of the chemical shift. The thermal properties of DHOA were analyzed through Differential Scanning Calorimetry (DSC-60 Plus, Shimadzu, Japan) to determine the thermal transition parameters of the product.

Unless otherwise stated, all chemicals used were of pro-analysis (p.a.) grade. The materials used included laboratory-extracted TC seed oil, acetone, *n*-hexane, silica gel 60, silica gel F254-coated TLC plates, anhydrous Na₂SO₄, diethyl ether, Lipozyme[®] TL IM (immobilized lipase from *Thermomyces lanuginosus*), urea, 96% ethanol, Wijs' reagent, 98% (v/v) formic acid, 50% hydrogen peroxide, sulfuric acid, and 0.1 N KOH, 0.1 N Na₂S₂O₃, and 0.1 N HBr solutions. All materials were used without further purification, unless specifically stated in the experimental procedures.

2.2 Methods

2.2.1 Hydrolysis of TC Seed Oil

The hydrolysis of TC seed oil was done by following Karouw et al. (2016) by modifying the procedure. TC seed oil was extracted from *n*-hexane first and purified by column chromatography as reported by Gunawan et al. (2024). The purified oil was mixed with distilled water at a 2:1 (w/w) oil-water ratio in a 3-necked flask and Lipozyme[®] TL IM was added at 2.5% (w/w) relative to the oil mass. The reaction mixture remained in a 40 °C water bath for 18 hours with stirring. When the reaction was complete, the mixture was separated in a separatory funnel to obtain the organic phase where the hydrolysis products were. Next, the organic layer was passed through a Büchner funnel to separate the enzyme from the resulting fatty acids. The fatty acid product is then characterized based on the acid value (S Method Ca 5a-40), iodine value (AOCS Method Cd 1d-92), thin layer chromatography (TLC), and FTIR spectroscopy. The percentage of free fatty acids (FFA) was determined using Equation 1.

$$\text{FFA (\%)} = \frac{V_{\text{KOH}} \times N_{\text{KOH}} \times M_{\text{FA}}}{m_{\text{sample}} \times 1000} \times 100 \quad (1)$$

The reusability of Lipozyme[®] TL IM was evaluated over five consecutive reaction cycles. After each cycle, the enzyme was recovered by filtration, washed with *n*-hexane, dried, and reused under the same reaction conditions. The residual enzyme activity was determined based on the relative product yield in each cycle.

2.2.2 Urea Crystallization

The separation of saturated and unsaturated fatty acids using the urea crystallization method is carried out through the following process steps.

2.2.2.1 Urea Crystallization Stage

The urea crystallization stage was carried out by dissolving fatty acids in a 96% ethanol-urea solution; the mixture was then heated to 60 °C and stirred magnetically until a homogeneous solution was obtained (González-Fernández et al., 2020; Setyawardhani et al., 2024). The ethanol-urea solution was maintained at 60 °C for 30 minutes, after which the fatty acids were added at predetermined urea-to-fatty acid ratios. Following homogenization, the mixture was cooled for 30 hours to facilitate the formation and maturation of urea inclusion crystals. The variations in urea-to-ethanol and urea-to-fatty acid ratios used in the crystallization process are presented in Table 1.

2.2.2.2 Unsaturated Fatty Acid Separation Stage

The urea crystals formed as the urea-complexed fraction (UCF) were separated from the filtrate with the non-urea-complexed fraction (NUCF) by filtration. The filtrate was then purified by adding distilled water in the same volume as that of the filtrate itself. The filtrate was then acidified with 6 N HCl to a pH of 4–5 to separate fatty acids from other dissolved components of the mixture. From the filtrate, *n*-hexane was then added until the total volume of the mixture was equal to the initial filtrate volume and then stirred for 1 hour for a more efficient extraction process. Once two phases of the mixture were formed, the organic phase of *n*-hexane and fatty acids was separated from the aqueous phase by the separation funnel. The organic phase was then dried with anhydrous Na₂SO₄ to remove the remaining water. After drying, the *n*-hexane was removed using a rotary evaporator for the unsaturated fatty acid (UFA) fraction concentrate.

2.2.2.3 Epoxidation and Hydroxylation

The UFA was oxidized using performic acid that was generated *in situ* during the reaction. The UFA-to-reagent molar ratio is 1:1:6 and produced from the process reported by Jalil (2019). The UFA and formic acid are charged into a three-necked flask with a thermometer and condenser. After 5 minutes of constant temperature, 17.65 mmol of H₂O₂ solution is added slowly and 3–5 drops of concentrated H₂SO₄ are added over 5 minutes as a catalyst and the mixture is stirred magnetically. The epoxidation reaction was done at 65 °C

Table 1. Urea and Fatty Acid Ratio Variation

Formulation	Fatty Acid (g)	Urea (g)	Ethanol (mL)	Urea:Ethanol (w/v)	Urea:Fatty Acid (w/w)	Crystallization Time (h)
F1	7	20	100	1:5	2.86:1	30
F2	7	40	200	1:5	5.71:1	30
F3	7	60	300	1:5	8.57:1	30

for 75 minutes and the reaction time was measured after the addition of H_2SO_4 . The colour change of the mixture from a white to a pale whitish yellow was visible to be the first sign that the reaction was happening. After the reaction was over, the mixture was transferred to a separatory funnel for phase separation. The epoxide product was separated from the other phase by organic phase separation. The hydroxylation stage was carried out by mixing the epoxide with hot water in a three-necked flask in an oil bath. The epoxide to water ratio was set at 300:2000 mL and the mixture temperature was taken to be 78–86 °C, as it was done by [Mohamed et al. \(2014\)](#). After hydroxylation was completed, the product was washed to remove residual reagents and impurities and the solvent was removed from the mixture using a rotary evaporator to yield DHOA.

2.2.2.4 Analysis and Characterization

The thermal properties of DHOA were analyzed with Differential Scanning Calorimetry (DSC-60 Plus, Shimadzu, Japan) in a nitrogen atmosphere at a gas flow rate of 10 mL min⁻¹. The sample was heated from 30 to 300 °C at 10 °C min⁻¹. DSC thermograms were used for the onset temperature, peak temperature, final temperature, and enthalpy of fusion (ΔH) transition parameters to be determined using TA-60 software. These were then used to evaluate the thermal properties and purity level of the DHOA. In order to ensure the correctness and reliability of physicochemical analysis, all titrant solutions are first standardized with validated analytical procedures. The sodium thiosulfate solution is used for determination of iodine value using potassium dichromate as the primary standard. The HBr solution in glacial acetic acid for OOC is standardized using the AOCS Cd 9-57 method. On the other hand, the KOH solution for hydroxyl value is standardized with potassium hydrogen phthalate (PHP) as the primary standard. Each measurement was performed in triplicate to ensure the accuracy of the analytical results. Product characterization was performed by determining the iodine value ([AOCS, 1996](#)), acid value [AOCS, 2009a](#); [AOCS, 2009b](#), and OOC ([AOCS, 1997](#)). The degree of epoxidation was subsequently evaluated based on the OOC value calculated using Equation 2.

$$\text{OOC}_{\text{exp}} = \frac{V_{\text{HBr}} \times 1.6 \times N_{\text{HBr}}}{w} \quad (2)$$

The experimental OOC (OOC_{ex}) was calculated using Equation 3.

$$\text{OOC}_{\text{ex}} = \frac{V_{\text{HBr}} \times 1.6 \times N_{\text{HBr}}}{w} \quad (3)$$

where:

V_{HBr} HBr titration volume (mL)

N_{HBr} HBr solution concentration (N)

w Weight of sample (g)

The theoretical OOC value (OOC_{th}) is determined from the iodine value of the starting material and subsequently used to calculate the theoretical oxirane content based on Equation 3.

$$\text{OOC}_{\text{th}} = \frac{A_{\text{O}} \times IV_0}{2A_{\text{I}} + A_{\text{O}}} \quad (4)$$

where:

OOC_{th} Theoretical OOC (%)

A_{I} 126.9045 (relative atomic mass of iodine)

A_{O} 16 (relative atomic mass of oxygen)

IV_0 Sample iodine value (mg Iod/g)

The experimental OOC (OOC_{ex}) and theoretical OOC (OOC_{th}) values were used as the basis for calculating the percentage of relative conversion to oxirane (RCO) according to Equation 5.

$$\text{RCO} = \frac{\text{OOC}_{\text{ex}}}{\text{OOC}_{\text{th}}} \times 100\% \quad (5)$$

The experimental and theoretical DHOA values, as well as the percentage efficiency of DHOA formation, were calculated based on Equations 5 6 7.

$$\text{DHOA}_{\text{theoretical}} = \frac{m_{\text{UFA}}}{M_{\text{UFA}}} \times M_{\text{DHOA}} \quad (6)$$

$$\text{DHOA}_{\text{Efficiency}} = \frac{\text{DHOA}_{\text{experimental}}}{\text{DHOA}_{\text{theoretical}}} \times 100\% \quad (7)$$

3. RESULT AND DISCUSSION

3.1 Hydrolysis of TC Seed Oil

Lipzyme[®] TL IM is used as a biocatalyst in the hydrolysis of TC seed oil. Such lipase can efficiently catalyze the reaction in mild circumstances and is suitable for processing suboptimal materials ([Baena et al., 2022](#)). Triglyceride hydrolysis also results in free fatty acids and glycerol, which are broken down into separate phases given their polarity. Based on the reaction, the fatty acid-rich phase is separated into separate parts by a



Figure 1. Comparison of R_f Values of TC Seed Fatty Acids and Standards Using *N*-Hexane:diethyl Ether (87:13 v/v) as Eluent. A = Linoleic Acid Standard, B = TC Seed Oil, C = Oleic Acid Standard.

separatory funnel, and the enzyme residue is disposed of via filtration with a Büchner funnel. The FFA content in the hydrolysis process is $75.56 \pm 1.71\%$ with a RSD of 2.26%, which is excellent for the process repeatability. Since hydrolysis is primarily the cleavage of ester bonds in triglycerides to produce FFAs, the FFA percentage is a direct indication of the extent of hydrolysis conversion. Therefore, the FFA value shows that Lipozyme® TL IM can catalyze the hydrolysis of TC seed oil.

The reusability of Lipozyme® TL IM was evaluated with 5 consecutive reaction cycles. After each cycle, the biocatalyst was separated by filtration, washed with *n*-hexane and dried and reused under the same reaction conditions. The hydrolysis yield after five cycles was $67.51 \pm 2.07\%$ with RSD = 3.06%, which means Lipozyme® TL IM is stable during use. These results are in line with [Marín-Suárez et al. \(2019\)](#) who showed that the activity of Lipozyme, especially the Lipozyme® TL IM and Lipozyme® RM IM, generally decreased about 10–20% after 5 cycles but still maintained 80–90% of the initial activity after 5 cycles. The enzyme's activity decreases during repeated use due to partial desorption from the support matrix, conformational changes in the protein after repeated exposure to the reaction environment, and the accumulation of substrates or products on the biocatalyst surface. Such factors decrease substrate accessibility to the active site and decrease the enzyme's catalytic activity ([Silva et al., 2022](#)). However, the activity retention after five cycles indicates Lipozyme® TL IM is stable enough to maintain activity and contribute to the efficient utilization of biocatalyst and lower the operating cost of the process.

TLC profile shows the presence of UPAs in the hydrolysis fraction of TC seed oil. Separation using *n*-hexane/diethyl ether (87:13, v/v) yielded spots with R_f values that were compared against oleic acid and linoleic acid standards. The matching R_f values between the sample and the standards indicate the presence of these two UPAs in the hydrolysis fraction (Figure 1).

TLC analysis revealed three spots in the TC seed oil sample with R_f of 0.157 ± 0.02 (spot 1), 0.485 ± 0.01 (spot 2) and 0.610 ± 0.02 (spot 3). For comparison, the oleic and linoleic acid standards had R_f values of 0.585 ± 0.01 and 0.500 ± 0.01 , respectively. The close R_f values for the two spots (spot 2 and spot 3) are consistent with the presence of these two unsaturated fatty acids in the TC seed oil fraction. The lowest R_f of spot 1 is thought to be associated with a more polar lipid, probably due to intermediate compounds or lipid residues not completely hydrolyzed into free fatty acids. As the latter was more strongly interacting with the polar silica stationary phase, this component might have been less likely to migrate in the relatively nonpolar *n*-hexane/diethyl ether mobile phase ([Christie, 2003](#)). The results from the FTIR analysis of TLC were also confirmed (Figure 2) and provided additional information regarding the presence of the functional groups of unsaturated fatty acids in the TC seed oil.

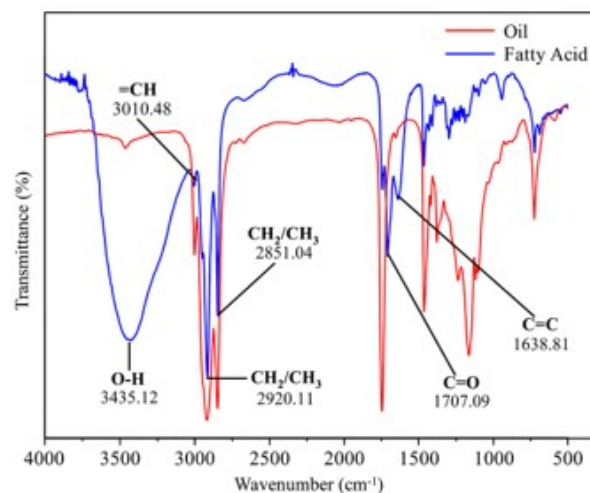


Figure 2. FTIR Spectrum of Oil and Fatty Acids.

The FTIR spectrum of TC seed oil after hydrolysis shows the characteristic change of free fatty acids (Figure 2 and Table 2). The broad and intense absorption band at 3435.12 cm^{-1} corresponding to the O–H stretching vibration of the carboxyl group was detected in the hydrolyzed sample whereas this band was not present in the triglyceride spectrum. A strong absorption band at 1707.09 cm^{-1} corresponding to the C=O stretching vibration of the carbonyl group was also observed. This large and strong band suggests the presence of the carboxyl group which is a key characteristic of free fatty acids. The unsaturation of the fatty acid structure is also shown by an absorption band at 1638.81 cm^{-1} (C=C stretching vibration) and a weak band at 3010.48 cm^{-1} (=C–H stretching vibration of alkene). Collectively, these bands represent spectroscopic evidence of carbon–carbon double bonds in the hydrocarbon chains of the fatty acids produced through hydrolysis. These FTIR results are consistent with the TLC analysis (Figure 1) that revealed that several spots in the sample were within similar

Table 2. Comparative FT-IR Spectra of Oil and Fatty Acids

Bond	TC Seed Oil	TC Seed Oil Reference*	Fatty Acid	Fatty Acid Reference**
O–H	–	–	3435.12	3420
=C–H	3007.52	3007.71	3010.48	3009
CH ₂ and CH ₃	2924.67 and 2854.09	2925.85 and 2854.91	2920.11 and 2851.04	2930 and 2850
C=O	1746.68	1741.73	1707.09	1740
C=C	1655.00	1641.00	1638.81	1670

R_f to oleic acid and linoleic acid standards. The compatibility of these two analytical methods further confirmed the presence of unsaturated fatty acids in the hydrolysis products of TC seed oil.

The FTIR absorption characteristics reported in this study are in general in agreement with [Suhendra et al. \(2018\)](#) and [Hassabo et al. \(2018\)](#) in the bands of O–H and =C–H vibrations as well as CH₂/CH₃. However, the absorption bands of C=O and C=C bonds are slightly shifted in contrast to the results of [Hassabo et al. \(2018\)](#). Those differences can be attributed to the fatty acid composition, which depends on carbon chain length, unsaturation, and double bond position and can affect the chemical environment and vibration qualities of the functional groups. Overall, the FTIR results show the hydrolysis of TC seed oil triglycerides to free fatty acids and unsaturated fatty acid structures in the hydrolysis products. The TLC analysis results and the hydrolysis results together show that there are oleic acid and linoleic acid in the hydrolysis fraction as well. The data also confirm the potential of TC seed oil as renewable feedstock for a variety of oleochemical applications such as a precursor of fatty acid-based compounds.

3.2 Urea Crystallization

UFAs are separated through urea crystallization, based on the formation of inclusion complexes between urea (host molecule) and fatty acids (the guest molecules) (Figure 3) ([Ohlan et al., 2008](#)). Through this process, the urea molecules form a crystalline lattice in which cavities are able to contain fatty acid molecules. Urea–fatty acid complexes are formed by noncovalent interactions, such as hydrogen bonds, and do not change the chemical structure of fatty acids ([Engkvist et al., 2000](#)). Fatty acids that are saturated and have relatively linear chain structures are easier to include into the urea lattice and these fatty acids are separated by filtration. The UPAs, which have distorted chain structures due to double bonds, are not easily incorporated in the urea lattice and are kept in the liquid phase, and the UFAs fraction is enriched.

The fatty acid separation by urea crystallization is highly influenced by the structure of the fatty acid molecules and their unsaturation. In saturated fatty acid molecules, the carbon chains are relatively linear and flexible (only C–C bonds) which makes them easier to fit into the urea crystal channels. But since unsaturated fatty acid chains have double bonds, the chain geometry is not so flexible and the molecules are less

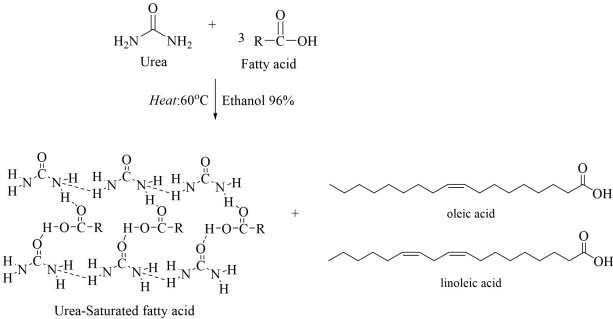


Figure 3. Urea Inclusion Reaction.

likely to form inclusion complexes with urea. As double bonds increase, the geometric mismatch between fatty acid molecules and urea crystal channels decreases the chance of inclusion ([Setyawardhani et al., 2015](#)). The mass balance and recovery efficiency for urea crystallization are illustrated in Table 3.

We can see from the mass balance calculation that the urea-to-fatty acid ratio plays an important role in the recovery of the UFA fraction during urea crystallization. Formulations 1 and 2 give relatively low UFA recoveries of 5.75% and 6.28%, respectively. Formulation 3 gives a much higher recovery of 27.145, the average UFA mass of 1.9 g out of 7 g fatty acids. This increase shows that the urea-to-ethanol ratio influences the formation of inclusion complexes and the distribution of fatty acids between the crystalline and non-complexed phases. Under Formulation 3, complex formation to include components that are easier to include in the urea lattice seems to be more successful. Thus the uncomplexed UFA fraction is still in the liquid phase and can be recovered through the separation process. Also, whereas Formulation 3 had the highest UFA recovery, Formulation 3 lost less mass than all the other formulations, indicating better separation efficiency.

The ratio of recovered UFA fraction to starting material was different from the formulations. Formulations 1 and 2 presented a ratio of 0.061 and 0.067, respectively, while Formulation 3 showed the highest value of 0.373 (Table 4). The higher value for Formulation 3 suggests that the crystallization conditions were more effective in retaining and recovering the UFA fraction in the non-complexed phase. Formulation 3 provides more favorable separation conditions for enriching UFAs and enhances fractionation selectivity against saturated

Table 3. The mass balance and recovery efficiency in the urea crystallization process

Formulation	Fatty acid (g)	Urea (g)	Ethanol (mL)	UFA Mass (g)	Recovery UFA (%)	Mass of other fractions (g)	Loss (%)
F1	7	20	100	0.403 ± 0.187	5.75	6.597	94.25
F2	7	40	200	0.440 ± 0.140	6.28	6.560	93.72
F3	7	60	300	1.900 ± 0.216	27.14	5.100	72.86

fatty acid (SFA) components.

Table 4. Selectivity Factor (SF) Separation of SFA and UFA

Formulation	SFA Mass	UFA Mass	SF
1	6.597 ± 0.188	0.403 ± 0.187	0.062 ± 0.031
2	6.560 ± 0.140	0.440 ± 0.140	0.067 ± 0.023
3	5.103 ± 0.220	1.900 ± 0.210	0.373 ± 0.058

Increasing the urea-to-fatty acid ratio tended to enhance the enrichment of the UFA fraction, a trend attributable to the increased capacity for urea inclusion complex formation with fatty acid components that are more readily included. These findings align with those of [Setyawardhani et al. \(2018\)](#), who reported that increasing the urea ratio could raise the concentration of polyunsaturated fatty acids (PUFA) in the concentrate fraction. In contrast, the use of urea at lower ratios resulted in more limited inclusion levels and, under the conditions of this study, resulted in lower UFA ratios of 2.86:1 and 5.71:1, respectively. Based on these results, a urea-to-fatty acid ratio of 8.57:1 (w/w) was selected as the optimal condition for GC-MS analysis, as it produced the highest level of UFA enrichment.

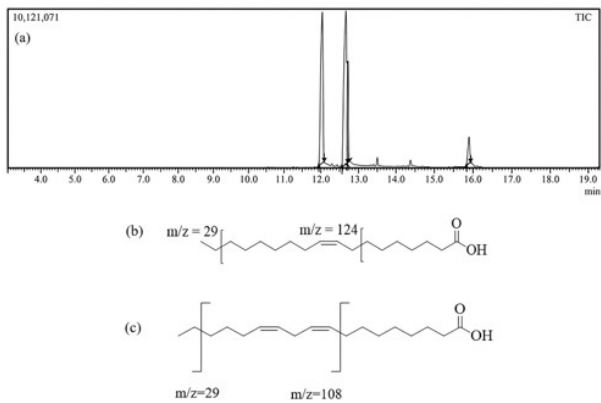


Figure 4. (a) Chromatogram of GC-MS of Fatty Acids in TC Seed Oil; (b) Fragmentation of Oleic Acid; (c) Fragmentation of Linoleic.

GC-MS data in Tables 5 and 6 show a significant change in the composition of fatty acids after urea crystallization. The saturated fatty acid (SFA) fraction (free of carbon-carbon double bonds) decreased from 49.85% to 9.21% in terms of relative peak area. On the other hand, the proportion of UFA (with one or more double bonds) increased from 50.15% to 73.28%

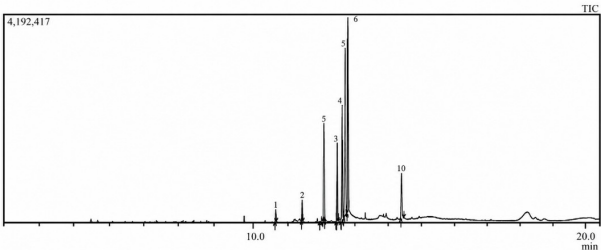


Figure 5. Chromatogram of GC-MS of Unsaturated Fatty Acids with an 8.57:1 Ratio.

with oleic acid and linoleic acid making up 46.26% and 16.56% of peak area respectively. These results indicate that urea crystallization can reduce the SFA fraction and enrich the UFA fraction at once. These results are consistent with [Chamidah and Wicaksono \(2021\)](#) who obtained UFA separation yield of 73.06% using urea crystallization. In this respect, we expect that urea crystallization is a promising and versatile method for UFA enrichment across various lipid sources. However, comparing the performance of different studies is challenging because raw material compositions and crystallization processes are different. The separated fraction has an iodine value of 74.23 mg I₂ g⁻¹ and hydroxyl of 33.66 mg KOH g⁻¹. The increase of the iodine value is related to the higher number of unsaturated fatty acids in the crystallized fraction, as the sample is unsaturated. The iodine value is within the 45–94 mg I₂ g⁻¹ range set in [AOCS \(1996\)](#) 993.20 for commercial oils. This implies that the unsaturation level is within an acceptable range.

GC-MS analysis of the separated fraction identified UFA components with different molecular masses and fragmentation patterns. Oleic acid and linoleic acid were identified by the same fragmentation patterns of their standards (Figure 4). The GC-MS chromatogram of the UFA mixture is shown in Figure 5 that shows good separation with distinct peaks at a composition ratio of 8.57:1. By using retention times and mass spectra, the major components in the separated fraction were well identified. These results are consistent with [Lee et al. \(2004\)](#) showing that urea complexation can enrich the non-complexed fraction (which is rich in UPAs) by selectively removing saturated fatty acids, because saturated fatty acids are the ones that form inclusion complexes with urea, whereas the majority of UPAs remain in the non-complexed fraction. In this scenario, the GC-MS profile obtained in the present

Table 5. Results of GC-MS Analysis of Fatty Acids in TC Seed Oil

Fatty Acid	Name	Molecular Weight (g/mol)	Retention Time	Area (%)	Library Match (%)
C16:0	Palmitic Acid	256	12.101	41.67	92
C18:1	Oleic Acid	282	12.709	45.54	90
C18:0	Stearic Acid	284	12.756	8.17	93
C18:2	Linoleic Acid	296	15.868	4.61	88
SFA				49.85	
UFA				50.15	

Table 6. GC-MS Analysis of UFAs with an 8.57:1 Ratio

Fatty Acid	Name	Molecular Weight (g/mol)	Retention Time	Area (%)	Library Match (%)
C16:1	Palmitoleic Acid	254	11.973	2.19	87.2
C16:0	Palmitic Acid	256	12.050	9.21	91.4
C18:2	Linoleic Acid	280	12.465	4.61	90.1
C18:1	Oleic Acid	282	12.700	46.26	93.5
C18:2	Linoleic Acid	280	12.768	16.56	91.8
C18:2	Linoleic Acid	280	14.398	3.66	88.6
SFA				9.21	
UFA				73.28	

study provides additional evidence for the contribution of urea crystallization in enriching the unsaturated fatty acid fraction.

Fatty acid fractionation is done by urea crystallization by mixing fatty acids with a urea-ethanol solution and heating the mixture for 30 minutes at 60 °C. The mixture is then cooled at low temperature for 30 hours in order to form urea-fatty acid inclusion complexes. Saturated fatty acids (which have relatively linear carbon chains) are more easily incorporated into the urea crystal channels and form long needle-like crystals. Unsaturated fatty acids, by contrast, are often left in the non-complexed fraction. The unsaturated fatty acids change the molecular structure and steric barriers to the formation of inclusion complexes (Maruyama et al., 2022; Setyawardhani et al., 2024). The resulting crystals are separated by filtration and filtration leads to a filtrate with unsaturated fatty acids and ethanol. The water was then distilled to remove components soluble in polar phases, such as the residual urea (Setyawardhani et al., 2018), while acidifying with 6 N HCl to convert fatty acid components into nonionic acids and the fatty acid components were converted to organic state to increase the separation of the organic phase. The fatty acid components were extracted again with n-hexane, which is a solvent with high solubility for fatty acids and low solubility for the aqueous phase (Gunawan and Suhendra, 2012). After about 60 minutes of extraction, the mixture remained in the water and two parts were left until a mixture of two different phases was formed. The organic part of the mixture was produced in the higher layer due to the lower density of n-hexane (0.6548 g mL⁻¹ vs. 1.00 g mL⁻¹ in the aqueous phase) and the unsaturated fatty acid fraction was recovered with n-hexane-rich organic phase.

The ability of fatty acids to be included in the urea crystal lattice depends on unsaturation and the structure of their

carbon chains. The presence of double bonds restricts chain conformational flexibility compared to SFA and thus makes it difficult for fatty acids to fit into urea crystal channels. As the number of double bonds and the effective size of the molecule increase, the fatty acid is less likely to form inclusion complexes with urea. Therefore, this paper tests the impact of the urea-to-fatty acid molar ratio on the efficiency of fractionation even if the crystallization temperature of urea is not changed. We did not test the crystallization temperature at all in this study, but it is a key parameter to be investigated in order to improve the selectivity and efficiency of the urea crystallization process.

3.3 Epoxidation of Unsaturated Fatty Acids

The UFAs obtained after separation and purification through urea crystallization were subsequently subjected to epoxidation using sulfuric acid as a catalyst, with formic acid and hydrogen peroxide serving as the oxidizing system (Figure 6). In this reaction, the carbon-carbon double bonds of unsaturated fatty acids are converted into epoxide groups (oxirane rings) along the fatty acid chains. The reaction mixture was subsequently separated by liquid-liquid extraction using a separatory funnel to separate the organic and aqueous phases. The organic phase, which was rich in epoxidized fatty acid products, appeared pale yellow and relatively viscous, whereas the aqueous phase while the aqueous part of the mixture was clear and settled on the bottom layer.

The iodine value of the UFA fraction and epoxidized product was determined in triplicate to evaluate the repeatability of the analytical method and to monitor the reduction in unsaturation during epoxidation. The epoxidized product showed a decrease in iodine value of 64.71 mg I₂ g⁻¹ compared with the initial UFA fraction. This decrease indicates that the number

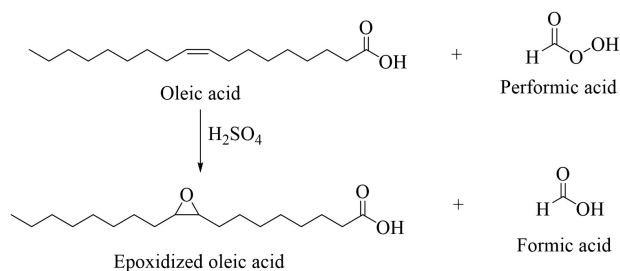


Figure 6. Epoxidation Reaction.

of carbon–carbon double bonds decreased as the double bonds were converted into oxirane groups during the epoxidation reaction. The repeatability of the iodine value determination was satisfactory, with an RSD of less than 5%.

The experimental oxirane oxygen content (OOC_{ex}) of the epoxidized product was 3.35%, whereas the theoretical oxirane oxygen content (OOC_{th}) was 4.47%. Based on these values, the relative conversion to oxirane (RCO) was calculated to be 75%. This result indicates that approximately 75% of the theoretically available unsaturated sites were converted into oxirane groups during the epoxidation process. The RCO obtained in this study was higher than the 50% reported by Roslan et al. (2025) for epoxidation of oleic acid using a peroxide-based system. The difference may be attributed to variations in reaction conditions, catalyst type, oxidizing system, reaction time, temperature, and the characteristics of the starting material.

The iodine value further confirmed the occurrence of the epoxidation reaction. The epoxidized product had an iodine value of $9.491 \text{ mg I}_2 \text{ g}^{-1}$, which was substantially lower than that of the UFA fraction before epoxidation. This decrease can be attributed to the consumption of carbon–carbon double bonds during the formation of oxirane rings. Therefore, the reduction in iodine value, together with the OOC_{ex} and RCO values, provides quantitative evidence that the epoxidation reaction successfully occurred. The formation of oxirane groups is important because these epoxide groups serve as reactive intermediates that can subsequently undergo ring-opening reactions during the hydroxylation stage to produce hydroxylated fatty acids such as DHOA.

3.4 Epoxide Hydroxylation

The fatty acid epoxides obtained from epoxidation are then reacted with hot water to produce DHOA via a hydroxylation reaction. When distilled water is treated with high temperatures, this process not only removes residues or contaminants from the epoxidation product but also allows for oxirane ring opening. During this process, water molecules attack the epoxide ring and form dihydroxyl groups on the fatty acid chain. Hot water in the range of $80.4\text{--}86.5^\circ\text{C}$ is able to open the ring in this manner and lead to the formation of DHOA (Figure ??). This temperature range is the melting point of DHOA as defined by Maisaroh et al. (2016), and thus these thermal conditions enable the hydroxylation process to proceed effectively.

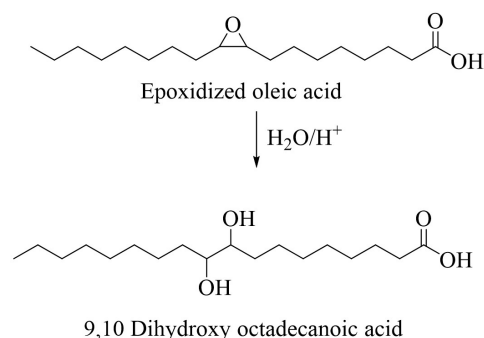


Figure 7. Reaction of DHOA Formation

The residual acid and catalyst that remained after the reaction were removed by washing the crude DHOA product with distilled water. Volatile components and residual solvent were then removed under reduced pressure, and the product was dried to a constant mass. For this study, since DHOA is semi-solid or liquid at room temperature, no conventional recrystallization was used for purification. Product purity may be improved via other purification methods, such as solvent-based fractionation or adsorption, which can provide better separation.

The theoretical yield of DHOA was based on the stoichiometric conversion of the oxidized substrate to the dihydroxylated product. Based on the ratio of the molecular mass of DHOA to the epoxide, e.g., the structure of epoxidized oleic acid, the theoretical mass of DHOA was estimated as 2.966 g from 2.800 g of oxidized substrate.

$$m_{\text{DHOA, theoretical}} = m_{\text{epoxide}} \left(\frac{M_{\text{DHOA}}}{M_{\text{epoxide}}} \right) = 2.966 \text{ g} \quad (8)$$

The experimentally obtained mass of DHOA was $1.513 \pm 0.012 \text{ g}$, with an RSD of 0.77%, which indicates a high degree of process repeatability. The average effective formation efficiency of $53.57 \pm 0.39\%$ shows that the hydroxylation process was carried out in a consistent manner with the reaction conditions.

$$\text{RSD} = \frac{s}{\bar{x}} \times 100\% \quad (9)$$

The formation efficiency can be calculated using the following equation:

$$\text{Formation Efficiency} = \frac{m_{\text{DHOA, experimental}}}{m_{\text{DHOA, theoretical}}} \times 100\% \quad (10)$$

The difference between the theoretical and experimental yields indicates that the conversion of substrate to DHOA did not take place quantitatively. The lower yield is explained

by incomplete epoxide conversion, side reactions in oxirane ring opening, and product loss during separation, purification, and recovery. Hence, optimal hydroxylation conditions and purification strategies will improve the formation efficiency and the recovery of DHOA.

After the hydroxylation process, the iodine value of DHOA dropped drastically from 9.52 to 0.58 mg I₂ g⁻¹ (RSD = 3.24%), indicating reduced unsaturation because most of the C=C double bonds were converted to hydroxyl groups. In theory, DHOA would have an iodine value close to zero (Rasib et al., 2025).

The iodine value can be expressed as:

$$IV = \frac{(V_{\text{blank}} - V_{\text{sample}})N_{\text{Na}_2\text{S}_2\text{O}_3}(12.69)}{m_{\text{sample}}} \quad (11)$$

The fact that the iodine value is still 0.58 mg I₂ g⁻¹ is probably due to a small amount of unreacted UPAs. However, the low iodine value clearly shows that the hydroxylation process was successful. The success of DHOA formation will be confirmed by parameters that directly reflect its formation. High-quality DHOA products are characterized by low residual oxirane and high hydroxyl content (Rasib et al., 2025).

Just the oxirane content is not enough to confirm that all the epoxide groups have been converted into hydroxyl groups, as oxirane ring consumption can also occur through side reactions. Therefore, the hydroxyl value is used as an additional parameter to evaluate the formation of -OH groups in DHOA. DHOA, unsaturated fatty acids, and epoxide precursors are compared in Table 9.

In the characterization, DHOA shows a lower OOC value compared to the epoxide precursor, which indicates fewer oxirane rings after the hydroxylation step. The decrease indicates that most of the epoxide groups opened and converted to dihydroxyl groups (DHOA) during the preparation process.

Moreover, the higher hydroxyl value of DHOA compared to unsaturated fatty acids indicates more hydroxyl groups in the product structure, which indicates that the oxirane ring-opening process occurs. This is in agreement with previous research in which the hydroxyl value of DHOA produced by epoxidation-hydrolysis steps could be as high as 129.4 mg KOH g⁻¹ in terms of effective hydroxyl group formation.

In general, the hydroxyl values of DHOA range from 90 to 400 mg KOH g⁻¹ for various raw materials, reaction conditions, and product purity. As a result, higher hydroxyl values correspond to a higher degree of conversion from epoxide groups to dihydroxyl groups. Thus, the low OOC value and higher hydroxyl value are complementary to support the successful conversion of the epoxide precursor into DHOA (Rasib et al., 2025).

Although epoxidation proceeds effectively, a number of side reactions may occur during prolonged reaction times, including oxirane ring opening, further oxidation, and the formation of secondary hydroxylated products. A relatively small decrease

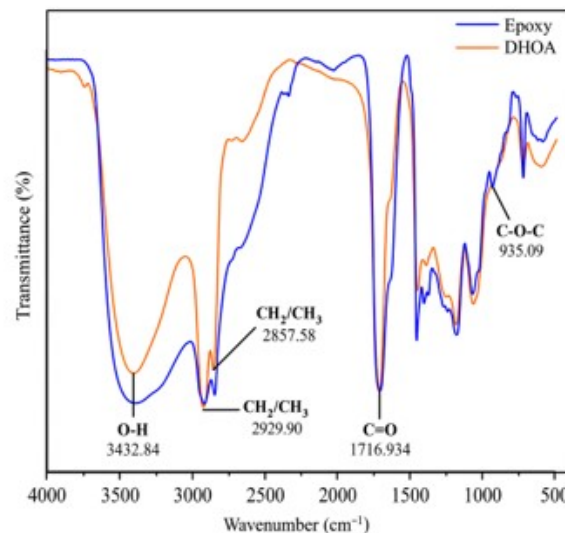


Figure 8. FTIR Spectra of Epoxide and DHOA

in the oxirane value at longer reaction times may indicate the partial consumption of epoxide groups via side reactions or oxirane ring opening.

Furthermore, the presence of minor signals in the NMR spectrum suggests the possible formation of small amounts of by-products during the reaction process. Nevertheless, indications of oxidative degradation under the employed conditions are relatively limited, as evidenced by the absence of significant new FTIR absorption bands associated with the formation of degradation-related carbonyl groups. Further interpretation of chemical structural changes based on NMR and FTIR analyses is presented in the following section.

3.5 FTIR Spectrum Analysis of Epoxides and DHOA

FTIR spectrum was used to monitor the changes in chemical structure during the epoxidation and hydroxylation stages of UFAs from TC seed oil. The formation of epoxide groups during epoxidation and their subsequent conversion into hydroxyl (-OH) groups during hydroxylation could be identified through characteristic changes in absorption bands at specific wavenumbers.

These changes, whether in the form of the appearance of new bands, the disappearance of bands, or shifts in the position of absorption bands, provide information regarding the transformation of functional groups at the molecular level.

The FTIR spectra of the epoxide and DHOA (Table 9) reveal characteristic changes in absorption bands that support the occurrence of structural transformations during the epoxidation and hydroxylation stages.

In the epoxidation product, the absorption band at 935.09 cm⁻¹ is attributed to the C-O-C group vibration of the epoxide ring. This band falls within the characteristic 840–960 cm⁻¹ range reported for epoxide groups by Gunawan et al. (2023), thereby providing spectroscopic evidence of oxirane ring formation in

Table 7. Characteristics of Fatty acid, Epoxide, and DHOA

Parameter	UFA	Epoxide	DHOA
Iodine Value (mg I/g)	74.23 ± 2.54	9.52 ± 0.34	0.58 ± 0.15
RSD (%)	3.39	3.73	–
Oxirane Value (%)	–	3.35 ± 0.18	0.051 ± 0.003
RSD (%)	–	2.32	2.35
Oxirane Conversion (%)	–	75	–
Hydroxyl Value (mg KOH/g)	33.66 ± 1.12	–	186.96 ± 1.81
RSD (%)	3.37	–	2.10
Color	Yellow	Light yellow	Light yellow
Appearance	Viscous	Highly viscous	Semi-solid

Table 8. Comparison of FT-IR Spectra of Epoxide and DHOH

Functional Group	Wavenumber (cm ⁻¹)			
	Epoxide	DHOA	DHOA*	DHOA**
O-H	3418.67	3432.84	3200–3600	3326
=C-H	–	–	–	–
CH ₂ and CH ₃	2927.35 and 2855.81	2929.9 and 2857.58	2928.28 and 2854.05	2932.14
C=O	1714.52	1716.93	1708.82	1710.53
C=C	–	–	–	–
C-O-C	935.09	–	–	–

* Mohamed et al. (2014).
** Maisarah et al. (2016).

the epoxidation product. Conversely, the characteristic epoxy C–O–C band is no longer observed in the DHOA FTIR spectrum, indicating oxirane ring opening during the hydroxylation process. Under aqueous reaction conditions, water molecules act as nucleophiles, facilitating epoxide ring opening and the formation of hydroxyl groups on the fatty acid chain (Mohamed et al., 2014).

This transformation is further supported by the presence of a C=O absorption band at 1716.93 cm⁻¹ and a broad O–H band at 3432.84 cm⁻¹ in the DHOA spectrum. The O–H band lies within the characteristic 3200–3600 cm⁻¹ range and is consistent with values reported by Mohamed et al. (2014); Maisarah et al. (2016); Awang et al. (2001).

Overall, the disappearance of the epoxy C–O–C band accompanied by the appearance of the O–H band provides spectroscopic evidence supporting the conversion of epoxide groups into dihydroxyl groups during DHOA synthesis.

Semi-quantitative FTIR analysis was performed by calculating the absorbance ratio between the characteristic band and the CH₂ band in the wavenumber range of approximately 2920–2930 cm⁻¹, which served as an internal reference (Table ??). The CH₂ band at this wavenumber was selected because its intensity remained relatively stable throughout the reaction. Normalization against the CH₂ band enabled a semi-quantitative

assessment of changes in key functional groups while minimizing the influence of sample preparation and FTIR film thickness. Consequently, variations in the absorbance ratio between the characteristic band and the CH₂ band were used to monitor chemical transformations during the epoxidation and hydroxylation processes.

The absorbance ratio was calculated using:

$$R = \frac{A_{\text{characteristic}}}{A_{\text{CH}_2}}$$

(12)

where *R* is the absorbance ratio, *A*_{characteristic} is the absorbance of the characteristic functional group, and *A*_{CH₂} is the absorbance of the CH₂ reference band.

The FTIR spectrum of TC seed oil shows a relatively high absorbance ratio of the C=C band to the CH₂ band, which reflects the presence of double bonds in the UFA components. After the epoxidation process, an absorption band was identified at around 935 cm⁻¹, which is related to the vibration of the C–O–C group on the oxirane ring, thus supporting the formation of the epoxide group. In the DHOA product, a relative increase in O–H band absorbance compared to the starting material indicates the formation of hydroxyl groups during the hydroxylation stage. Concurrently, the disappearance of the characteristic epoxide band after hydroxylation indicates the

conversion of oxirane groups via a ring-opening reaction and the formation of dihydroxyl groups.

3.6 Analysis of DHOA Using GC-MS

Following FTIR spectral analysis, the DHOA product was analysed using GC-MS to identify its constituent components and calculate the relative molecular mass of each component. All samples were analysed under identical instrumental conditions to ensure consistency and reproducibility of measurement results. Due to the semi-quantitative nature of the analysis and comparison of component components, no standard was used. Thus, the mass spectral data were used to find the DHOA components and their relative molecular masses, not to measure the absolute concentration of each component.

GC-MS analysis revealed molecular ions at m/z 316–317 at a retention time of 10.635 minutes, consistent with the molecular mass of DHOA and supporting the formation of the target product (Figure 9). The ion at m/z 317 is likely to be due to the natural isotopic contribution of ^{13}C , which is consistent with Maisaroh et al. (2016), who reported that the molecular mass of DHOA in the m/z 316–317 range is obtained from oleic acid production. Other fragmentation patterns indicate the structure of the product. The fragment at m/z 74 is typical of fatty acid fragmentation, and the ion at m/z 299 is most probably produced due to the removal of a water molecule from the molecular ion, indicating a hydroxyl group in the DHOA structure.

The fragment at m/z 285 and the ions at m/z 216, 197, 177, 159, and 144 can be attributed to bond cleavage in the carbon chain region adjacent to the C9–C10 hydroxylation site. The presence of two hydroxyl groups in this region likely enhances the relative stability of the fragments formed following carbon chain cleavage. The fragmentation pattern in the m/z 177–216 range is also consistent with the mass spectral characteristics of hydroxylated C18 fatty acids. Furthermore, dehydration of the molecular ion yields an ion at m/z 299, which can undergo further fragmentation to produce the ion at m/z 285 as well as smaller fragments. Overall, the presence of the molecular ion, the loss of water, and the fragmentation pattern associated with the hydroxyl group positions provide mass spectrometric evidence supporting the formation of DHOA. The mass spectrum and proposed fragmentation pathway are presented in Figure 9 and are generally consistent with the principles of fatty acid fragmentation reported by Christie (2003) and McLafferty and Tureček (1993).

3.7 The ^{13}C and ^1H NMR Analysis of DHOA

In NMR spectra with CDCl_3 as the solvent, we found residual solvent signals at δ 7.26 ppm in the ^1H NMR spectrum and δ 77.0 ppm in the ^{13}C NMR spectrum. These signals are used internally to determine the chemical shifts (δ , ppm) of the resonances. In the ^{13}C NMR spectrum of DHOA (Figure 9), we observed resonances of approximately δ 74 ppm, which are composed of carbon atoms C9 and C10 and are bonded to hydroxyl groups (C–OH). We believe that these resonances

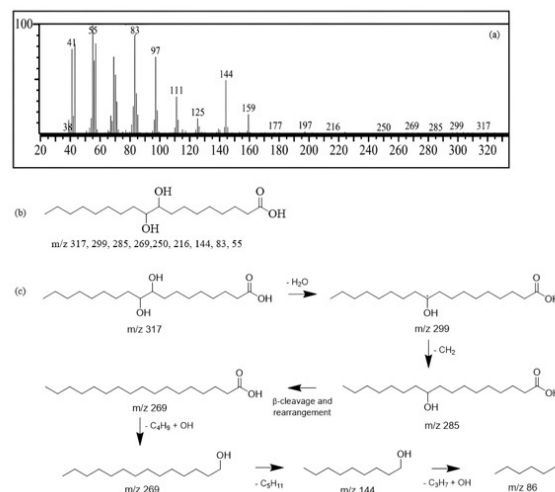


Figure 9. a) GC-MS chromatogram of DHOA; (b) Structure and m/z of DHOA; (c) Proposed fragmentation of DHOA

are evidence of the formation of a dihydroxyl group on the DHOA carbon chain. The results of Awang et al. (2001), which showed that resonances for hydroxylated carbons at positions C9 and C10 are around δ 75.6 and 75.5 ppm, respectively.

Resonances in the range of δ 54–57 ppm are attributed to carbons of the oxirane ring (C–O), so there are still some epoxide groups in the product. The low intensity of resonance in the range of δ 54–57 ppm indicates that the majority of oxirane rings were opened during the hydroxylation process, but some epoxide groups are still present (Derawi et al., 2014). Thus, the ^{13}C NMR spectrum indicates that high levels of epoxide conversion were achieved in the hydroxylation process, but it cannot be concluded that the process was complete. Low-intensity resonances in the range of δ 123–132 ppm also indicate that there are a small amount of olefinic carbons (C=C) in the product, which means that a few double bonds of UFA precursor were not fully converted during the reaction stages. The chemical shift values obtained in the ^{13}C NMR analysis are given in Table 10.

The ^1H NMR spectrum exhibits a resonance at approximately δ 3.3 ppm, attributable to the methine proton bonded to an oxygen atom (–CH–O–) within the DHOA structure (Figure 11). The presence of this resonance supports the formation of a hydroxyl group on the product's carbon chain and is consistent with the spectral characteristics of standard DHOA. These results align with the findings of Awang et al. (2001), who reported that the proton in the –CH–O– environment of DHOA appears at approximately δ 3.3 ppm.

In addition, the absence of clear resonances in the δ 5.3–5.4 ppm region, which is a characteristic region of olefinic protons, indicates that most of the C=C bonds have been consumed during the reaction stage. However, the presence of a very low-intensity resonance, or one potentially obscured by signal overlap, cannot be entirely ruled out. No clear resonance

Table 9. Semi-quantitative FTIR Analysis

Sample	Functional group	Wavenumber (cm ⁻¹)	% Transmittance	Absorbance	Ratio to CH ₂
TC Seed oil	O–H	3468.38	45.79	0.34	0.44
TC Seed oil	CH ₂ (ref)	2920.73	47.88	0.32	1.00
TC Seed oil	C=C	3007.79	52.46	0.28	0.88
Epoxide	O–H	3418.67	4.70	1.33	0.99
Epoxide	CH ₂ (ref)	2927.35	4.48	1.35	1.00
Epoxide	C–O–C epoxy	935.09	39.89	0.40	0.30
DHOA	O–H	3432.84	2.16	1.67	0.73
DHOA	CH ₂ (ref)	2929.90	0.50	2.30	1.00

Table 10. ¹³C-NMR Analysis of DHOA

Chemical Shift δ (ppm)	Assignment	Functional Group
178.5	Carbonyl ester/acid	C=O
163.9	Conjugated carbonyl/ester	C=O
132–123	Unsaturated alkene/aromatic carbon	C=C
84–70	Oxygen-bound carbon (epoxide/hydroxyl)	C–O
57–54	Carbon epoxy residue	C–O–C
31–29	Long aliphatic chain methylene	–CH ₂ –
22–14	Terminal methyl	–CH ₃
77.0	CDCl ₃ solvent	–
75–72	Secondary carbon containing –OH	C–OH (C9 and C10)
70–68	Carbon C–O resulting from the opening of the epoxy ring	C–O

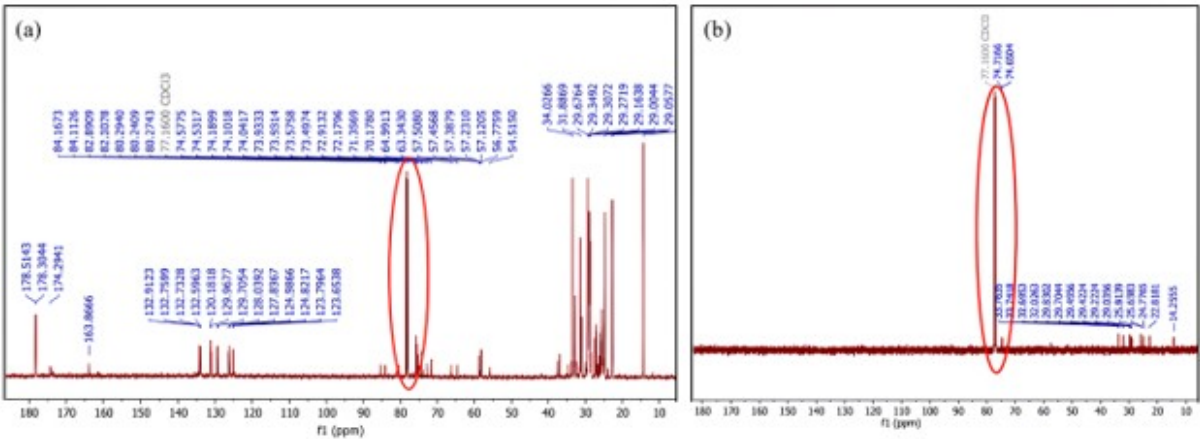


Figure 10. (A) ¹³C-NMR Spectrum of DHOA; (B) ¹³C-NMR Spectrum of Standard DHOA

for the carboxyl proton (–COOH) was observed; this may be attributed to its characteristically broad signal and rapid proton exchange, factors that can result in a low-intensity or poorly defined resonance in the ¹H NMR spectrum. The chemical shift profile obtained for the DHOA protons is summarized in Table 11.

During the epoxidation and hydroxylation stages, several

potential side reactions could occur, including excessive oxirane ring opening, further oxidation, and oxidative cleavage of the unsaturated carbon chain. However, characterization results using FTIR, NMR, and GC-MS indicate that the formation of by-products occurs at relatively low levels. This is supported by the absence of significant additional carbonyl bands or resonances and the predominance of signals associ-

Table 11. ^1H -NMR Spectra of DHOA

Chemical Shift δ (ppm)	Multiplet Type	Integration	Assignment/Functional Group
4.17–4.05	m	2H	Glycerol proton –CH ₂ OCO
4.02–3.90	m	1H	Glycerol proton –CHOCO
3.75–3.55	m	1–2H	Proton on hydroxylated carbon
3.45–3.33	m	1H	–CHOH resulting from opening the epoxy ring
2.35–2.25	t	2H	α -CH ₂ to carbonyl ester
2.10–1.95	m	2H	CH ₂ allylic/near oxygen group
1.70–1.50	m	2–4H	β -CH ₂ carbonyl
1.45–1.20	br m	20–24H	Aliphatic chain –(CH ₂) _n –
0.95–0.85	t	3H	Terminal methyl –CH ₃

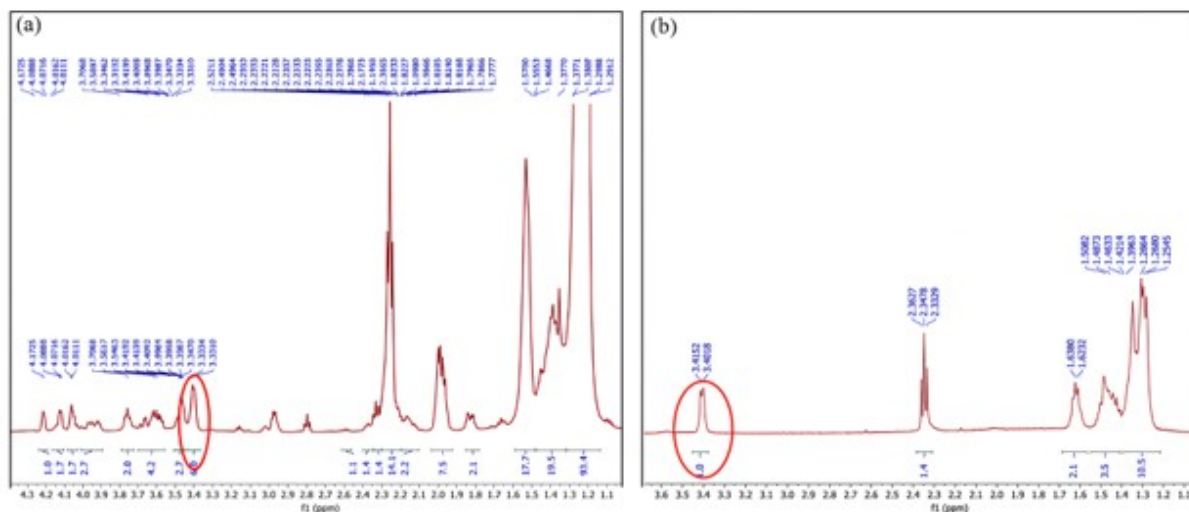


Figure 11. (A) ^1H -NMR Spectra of DHOA; (B) ^1H -NMR Spectra of DHOA Standard

ated with hydroxylated fatty acid components in the GC-MS analysis. Overall, the characterization data demonstrate that the primary transformation proceeds selectively toward the formation of the hydroxylated product.

3.8 DSC Analysis of DHOA

DSC analysis shows the DHOA undergoes a distinct endothermic transition that corresponds to 166.34–172.64 °C with an onset temperature of 166.34 °C, melting peak temperature of 168.62 °C, and a final temperature of 172.64 °C (Figure 12). The endothermic transition can be attributed to the melting transition of DHOA from the crystalline to liquid state. The relatively narrow melting range around 6.30 °C indicates the thermal transition is fairly well defined and the crystalline structure of the product is relatively uniform. A sharp, narrow melting peak correspondingly indicates a more homogeneous distribution of the components and higher purity, but the purity interpretation based on DSC should also be supported by other compositional analysis methods (Ghanbari et al., 2023).

The enthalpy of fusion (ΔH) of 417.19 J g⁻¹ shows the energy required to overcome interactions in the intermolecular materials and disturb the DHOA crystalline lattice during the melting process.

The thermal transition is attributed to the change in fatty acid molecular structure caused by the conversion of C=C double bonds to hydroxyl groups. The addition of hydroxyl groups helps molecules to form intermolecular interactions, especially hydrogen bonds, which can lead to different crystal regularity and melting properties of DHOA (Asaro et al., 2020)). This is in line with the findings of Koay et al. (2009) that the hydroxylation of unsaturated fatty acids into 9,10-dihydroxystearic acid can change crystal packing via intermolecular hydrogen bonding and yield different thermal properties of the product. The DSC profile shows that structural changes occur during DHOA formation and this product should be taken into account in further development and processing.

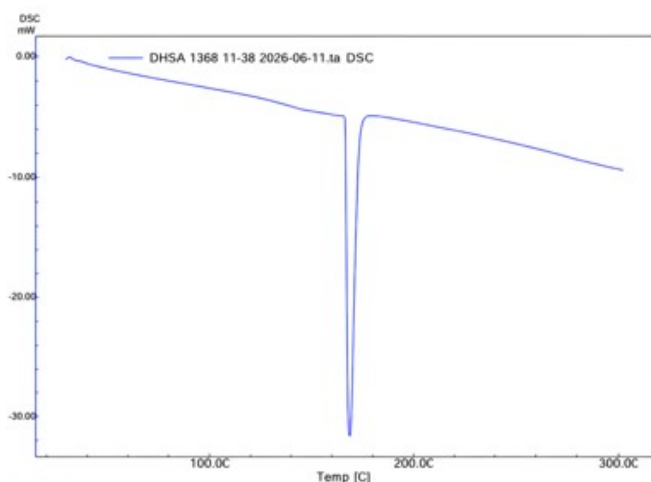


Figure 12. DSC thermogram of DHOA

4. CONCLUSIONS

UFAs were enriched from TC seed oil by urea crystallization at a urea-to-fatty acid ratio of 8.57:1 (w/w), resulting in a yield of $75.56 \pm 1.71\%$. The UFA fraction was then converted into 9,10-dihydroxyoctadecanoate (DHOA) by epoxidation and hydroxylation, leading to a yield of $53.57 \pm 0.39\%$. Structural transformation was confirmed by FTIR, GC-MS, and NMR in which hydroxyl groups were found along with the disappearance of epoxide groups and double bonds. FTIR showed a high O–H band at 3432.84 cm^{-1} and loss of the epoxide C–O–C band, while GC-MS showed a molecular ion at m/z 317. The ^{13}C NMR spectrum showed resonances for hydroxylated carbons at approximately δ 74 ppm, and ^1H NMR showed proton resonances at approximately δ 3.3 ppm. DSC characterization revealed a melting transition with an onset temperature of $166.34 \text{ }^\circ\text{C}$ and an enthalpy of fusion of 417.19 J g^{-1} , which is indicative of well-defined thermal properties. Overall, it is found that the combination of urea crystallization, epoxidation, and hydroxylation offers a promising approach to create TC seed oil-based DHOA, which may be seen as a bio-based precursor for the synthesis of oleochemical materials including polyols for bio-lubricant and polyurethane applications.

5. ACKNOWLEDGEMENT

The authors express their appreciation and gratitude to the Directorate of Research, Technology, and Community Service, Directorate General of Higher Education, Research, and Technology, Ministry of Education, Culture, Research, and Technology of the Republic of Indonesia, for the funding support provided for the conduct of this research under Research Contract Number SP DIPA 023.17.1.690523.

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