

Efficient Enzymatic Enrichment of High-purity Nervonic Acid from *Malania oleifera* Seed Oil

Ximei Yang^{1#}, Sen Lin^{1#}, Ying Chen¹, Wen Chen¹, Dongming Lan^{1*}, and Yonghua Wang^{1,2*}

¹ School of Food Science and Engineering, South China University of Technology, Guangzhou 510640, CHINA

² Guangdong Youmei Institute of Intelligent Bio-manufacturing Co., Ltd, Foshan, Guangdong 528200, CHINA

Abstract: Nervonic acid (NA) is a monounsaturated fatty acid vital for brain health and is of emerging importance in various industrial applications, including therapeutics, food, and cosmetics. Given the growing demands of the food and pharmaceutical industries, there's a pressing need for high-purity NA. Previously, NA constituents in plant seed oils were chemically transformed into nervonic acid ethyl ester (NAEE) to facilitate extraction from seed oils. In this study, we present an enzymatic approach to convert NA constituents in *Malania oleifera* seed oil to NAEE. Combined with the utilization of the semi-preparative chromatography, we achieved a remarkable purity of 97.52% NAEE. Compared to conventional chemical preparations characterized by multiple steps, prolonged processing times, and low yields and purities, our enzymatic method stands out as a more efficient and advantageous alternative. On top of that, this innovative approach is environmentally friendly and circumvents health and safety issues associated with chemical processes.

Key words: nervonic acid, nervonic acid ethyl ester, enzymatic, semi-preparative chromatography, *Malania oleifera* seed oil

1 Introduction

Nervonic acid (NA; C24:1Δ15; cis-15-tetracosenoic acid) is an ultra-long-chain monounsaturated fatty acid that is widely present in the brain and other neural tissues¹. This pivotal acid not only contributes to the normal functioning of neural tissues in organisms, but also promotes neurocellular synthesis and brain development in infants^{2,3}. Decreased levels of NA are closely associated with several neurological diseases, such as demyelinating disorders, Alzheimer's disease, adrenoleukodystrophy, and depression⁴⁻⁷. Consequently, studies have demonstrated NA's potential in the treatment and prevention of neurodegenerative diseases. However, it is difficult for the body to synthesize NA. Emerging research underscores the benefits of dietary NA supplementation, leading to its incorporation as an active constituent in health foods⁸. Beyond its medical relevance, the versatile NA has found its niche in cosmetic formulations due to its skin-related effects⁹. Given its multifaceted potential across pharmaceutical, nutraceutical, and industrial domains, the demand for high-purity NA is surging, emphasizing the need for consistent and high-quality production.

NA was initially isolated from shark brain tissue and also

known as shark acid¹⁰. Obtaining NA from mammalian sources presents challenges due to its limited availability and high cost. Subsequent research identified NA in the seed oils of approximately 200 plant species, spanning 136 genera and 54 families¹¹. Notably, in these plant seed oils, NA predominantly exists as triacylglycerides (TAGs). Typically, seed oils comprise a complex mixture of components including TAGs, diacylglycerides (DAGs), monoacylglycerides (MAGs), free fatty acids (FFAs), phospholipids, sterols, tocopherols, and other minor constituents. The similar chemical structures and properties of TAGs with fatty acids more or less randomly distributed make the purification and isolation of individual NA-containing TAG from this mixture challenging¹².

Recently, various methods have been developed to extract high-purity NA from the seed oils. Early research endeavors focused on the chemical extraction methods, such as saponification of the oil using NaOH/KOH followed by acidification with HCl/H₂SO₄. These processes converted NA-containing TAGs into their corresponding fatty acid forms. Following this, purification techniques like molecular distillation (MD), urea inclusion (UI), and low-temperature crystallization (LTC) were applied to separate the fatty

These authors contributed equally

*Correspondence to: Dongming Lan, School of Food Science and Engineering, South China University of Technology, Guangzhou 510640, PR CHINA. Yonghua Wang, School of Food Science and Engineering, South China University of Technology, Guangzhou 510640, PR CHINA

E-mail: dmlan@scut.edu.cn (DL), yonghw@scut.edu.cn (YW)

ORCID ID: <https://orcid.org/0000-0002-8790-4357> (YW)

Accepted October 22, 2023 (received for review September 5, 2023)

Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online

<http://www.jstage.jst.go.jp/browse/jos/> <http://mc.manuscriptcentral.com/jjocs>



acid form of NA. However, the purity of the resultant NA was found to be very low as indicated in Table 1. Moreover, the fatty acid form of NA is characterized by unfavorable taste properties, leading to limited consumer acceptance¹³⁾. This form is also prone to oxidation, potentially compromising its quality¹⁴⁾. Additionally, its high melting point ($\sim 42^{\circ}\text{C}$), poses challenges in separation and formulation, thereby restricting its practical applications.

Recent advancements have centered on the production of the ethyl ester derivative, nervonic acid ethyl ester (NAEE), which can either be used directly as a food ingredient or be transformed into NA or its derivatives. Notably, the ethyl ester version of NA presents several advantageous properties: a more favorable taste, a reduced melting point, enhanced resistance to oxidation, and heightened thermal stability, in comparison to its fatty acid counterpart¹⁵⁾. These enhancements not only refine the product's flavor profile but also critically improve product stability and extend its shelf life. Nonetheless, current methods for obtaining NAEE predominantly utilize chemical transformations, followed by a multistep purification process. For instance, Wang *et al.*¹⁶⁾ used *Acer truncatum* Bunge seed oil, catalyzed by NaOH, to produce NAEE and employed a "one-stage UI–two-stage MD–five-stage UI" purification method. Similarly, Shi *et al.*¹⁷⁾ also extracted NAEE from the same seed oil source with NaOH catalysis and applied a six-stage UI separation technique. These methodologies achieved purities of 91.8% and 52.4%, respectively (Table 1). In these studies, the chemical synthesis of NAEE often demands energy-intensive processes, frequently relying on potentially harmful catalysts. Such dependencies raise questions about product safety and the consequent impacts on human health. Furthermore, the requisite multistep processes and extensive separation techniques, result in less efficient preparations and reduced yields. In light of these challenges, there's a burgeoning interest in the potential of biocatalysis through enzymes. Such enzy-

matic methods operate under milder conditions, offer improved substrate specificity, enhance catalytic efficiency, and produce fewer byproducts^{18, 19)}. As a result, the enzymatic strategy is being recognized as a more sustainable and health-centric alternative, capturing significant industrial interest^{20–22)}.

In this work, we have developed an efficient and sustainable method for the preparation of high-purity NAEE. The seed oils of *Malania oleifera*, an evergreen broad-leaved tree from Southwest China, were selected as the primary source owing to their high nervonic acid content. A screening of various lipases revealed that immobilized Lipase UM1 is the most effective enzyme that converts the NA-containing TAGs in *Malania oleifera* seed oil into NAEE. Semi-preparative chromatography was performed using an ethanol/water mixture as the "green" mobile phase for further purification of NAEE, achieving a remarkable purity of 97.52%. The final NAEE product was characterized using gas chromatography (GC) and high-performance liquid chromatography (HPLC). Its chemical structure was confirmed using nuclear magnetic resonance (NMR) spectroscopy and fourier transform infrared spectroscopy (FTIR). This research not only offers a solution to the challenges associated with producing high-purity NAEE but also broadens the potential avenues for nervonic acid deployment in various industrial domains.

2 Materials and Methods

2.1 Materials

Immobilized Lipase UM1 was obtained from Guangdong Youmei Institute of Intelligent Bio-manufacturing Co., Ltd. (Foshan, China). Novozym 435, Lipozyme TL IM and Lipozyme RM IM were obtained from Novo Nordisk (Bagsvaerd, Denmark). *Malania oleifera* seeds were obtained from Guangnan county, Yunnan province, China. Standards of

Table 1 Methods for producing high-purity NA products from plant seed oils.

Product	Reaction system			Separation techniques	Purity (%)	Reference
	Oil sources	Method	Specific processing			
NA	<i>Thlaspi arvense</i> Linn seed oil	Chemical	Saponification of oil with NaOH /KOH followed by acidification with HCl /H ₂ SO ₄	Two-stage distillation; UI	26.23	23)
	<i>Acer truncatum</i> Bunge seed oil			Triple-stage LTC	25.05	24)
	<i>Acer truncatum</i> Bunge seed oil			One-stage LTC	13.11	25)
NAEE	<i>Acer truncatum</i> Bunge seed oil	Chemical	Ethanolysis of oil with NaOH-ethanol	One-stage UI; Two-stage MD; Five-stage UI	91.8	16)
				Six-stage UI	52.4	17)

the 37 fatty acid methyl esters (C4-C24), dioleoyl glycerol (composed of 15% of 1,2-dioleoyl glycerol and 85% of 1,3-dioleoylglycerol), and trioleoyl glycerol were sourced from Sigma-Aldrich (Shanghai, China). Thin layer chromatographic (TLC) plate was purchased from Qingdao Haiyang Chemical Co., Ltd. (Shandong, China). HPLC-grade hexane, formic acid, ethanol, and isopropyl ethanol were obtained from Aladdin Reagent (Shanghai, China). All other chemicals used were of analytical grade unless otherwise stated.

2.2 Extraction of *Malania oleifera* seed oil

The dried *Malania oleifera* seeds were dehusked. 400 g of dehusked *Malania oleifera* seeds were weighed and then finely chopped with scissors before being placed into a laboratory-scale oil press. During oil extraction, the barrel preheating temperature of oil press was set at 150°C, and the oil outlet temperature was maintained at 50°C. Subsequently, the extracted oil was centrifuged at 10,000 rpm (approximately 24,200 × g) for 15 minutes at 4°C. The supernatant containing *Malania oleifera* seed oil was collected. The obtained *Malania oleifera* seed oil sample was transferred to amber-colored bottles and stored under refrigerated conditions (0–4°C) for subsequent physico-chemical analyses.

2.3 Enzymatic synthesis of NAEE

The enzymatic ethanolysis reactions were performed in a 25 mL conical flask containing 10 g of substrates (ethanol and *Malania oleifera* seed oil). The reactions were initiated by adding immobilized lipases and were conducted under constant magnetic agitation at 200 rpm for 24 hours. Under the same catalytic reaction conditions, Novozym 435, Lipozyme RM IM, Lipozyme TL IM, and Lipase UM1 were employed as biocatalysts to catalyze the ethanolysis of *Malania oleifera* seed oil. Novozym 435, Lipozyme RM IM, Lipozyme TL IM are commercially available lipases with a documented history of utilization in numerous catalytic

reactions^{26–30}. In contrast, Lipase UM1, also known as MAS1, is a novel enzyme, isolated and characterized in our laboratory, which has demonstrated superiority in various enzymatic aspects^{31–33}. The results of the enzymatic action of these distinct lipases showed that various fatty acids previously bound in the TAGs of the seed oils were liberated and simultaneously transformed into fatty acid ethyl esters (FAEEs)³⁴, which comprised the target product NAEE. Concurrently, several byproducts such as glycerol and water were generated, as illustrated in Fig. 1. The efficiency of these lipases was assessed by comparing the FAEE conversion rates. Subsequently, the effects of ethanol-to-oil molar ratio (1.5:1, 3:1, 6:1, 9:1), water content (0%, 2%, 4%, 6% w/w relative to total substrates), temperature (25, 35, 45, 55°C) and enzyme dosage (50, 75, 100, 125 U/g substrate) on the FAEE conversion rate were investigated. Each factor was optimized separately, and the selected values were used for subsequent optimizations. Samples were periodically withdrawn for HPLC analysis. After the completion of the reaction, the catalyzed mixture was centrifuged at 10,000 rpm (approximately 24,200 × g) for 10 minutes at 25°C to remove the lipase. The resulting FAEEs were collected. The reaction was then scaled up to a 150 mL reactor with mechanical stirring and conducted under optimized conditions using 60 g of substrates for industrial proof-of-concept validation.

2.4 Production of high-purity NAEE

Semi-preparative chromatography (Waters, USA) was employed to obtain high-purity NAEE. The chromatography system consisted of an XBridge Peptide BEH C18 XP Column (250 mm × 19 mm i.d., 10 μm particle size), an automatic sampler (Waters 2707), a Fraction Collector III and a photodiode array detector (Waters 2998). The mobile phase, composed of ethanol and ultrapure water in a volume ratio of 96:4, was pumped at a flow rate of 15.0 mL/min. The mixture after enzymatic catalysis (~2 g) was dissolved in 4 mL of the mobile phase, and 1 mL aliquot of the

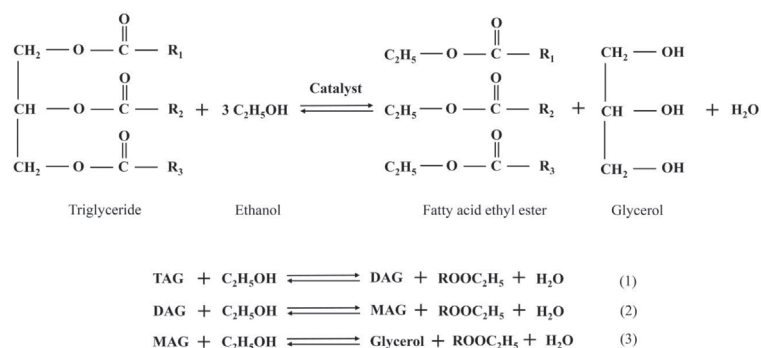


Fig. 1 Mechanism of lipase-catalyzed ethanolysis reaction. This schematic representation illustrates the step-by-step enzymatic process where lipase facilitates the breakdown of ester bonds in the presence of ethanol, leading to the formation of fatty acid ethyl esters, glycerol and H₂O.

mixed solution was used for each injection. The total run time was set to 10 minutes with isocratic elution, and absorbance signals at 205 nm were used for detection. Each chromatographic peak was systematically collected using an automated program, and the mobile phase associated with each component was evaporated through nitrogen blowing. The fatty acid composition of each fraction was verified through GC analysis and reported as the area percentage of NAEE.

The final products were subjected to comprehensive analyses to determine their compositions. HPLC was employed to determine the lipid compositions, while GC analysis was utilized to validate the fatty acid compositions (NAEE content). Additionally, NMR and FTIR spectroscopy analyses were carried out to further characterize the obtained compounds. The yield of NAEE was determined by calculating the mass of NAEE (m_{NAEE}) in the final product as a percentage of that in the original sample used for the synthesis and purification process (m_0):

$$\text{NAEE yield (\%)} = m_{\text{NAEE}}/m_0 \times 100$$

2.5 Lipid composition

The analysis of the reaction mixtures was performed using a normal-phase HPLC system equipped with a refractive index detector and a Phenomenex Luna column (250 mm $\times$ 4.6 mm i.d., 5 μ m particle size), following the method described by Li *et al.*³⁵⁾ with minor modifications. The mobile phase consisted of *n*-hexane, isopropanol, and formic acid (23:1:0.003, v/v/v) with the running rate at 1 mL/min. The identification of peaks in the HPLC analysis was based on a comparison of their retention times with those obtained from known standards. The retention times for TAGs, FAEEs, FFAs, 1,3-diacylglycerols (1,3-DAGs), 1,2 (2,3)-diacylglycerols [1,2 (2,3)-DAGs], 1 (3)-monoacylglycerols [1 (3)-MAGs], and 2-monoacylglycerols (2-MAGs) were 3.16, 3.43, 3.81, 4.82, 6.48, 33.17, and 37.39 minutes, respectively. The weight percentage of each acylglycerol species, FAEEs, and FFAs was calculated based on the peak areas and expressed as weight percentage. Peak area percentages were determined using Waters 2695 integration software. In this study, the FAEE conversion rate was calculated as follows:

$$\text{FAEE conversion rate (\%)} = \frac{\text{FAEEs (\%)}}{(\text{TAGs (\%)} + \text{DAGs (\%)} + \text{MAGs (\%)} + \text{FAEEs (\%)} + \text{FFAs (\%)})} \times 100\%$$

2.6 Fatty acid composition

The ethanolysis product containing TAGs, DAGs, MAGs, FFAs, and FAEEs were separated by TLC (silica gel GF UV-254 plate, 10 cm $\times$ 20 cm) using *n*-hexane: ethyl ether: acetic acid (80:20:2, v/v/v) as the developing solvent. The

ethanolysis product (30 mg) was mixed with 170 μ L of developing solvent and then spotted onto the dried TLC plate, which was then placed into the TLC tank. The standards were spotted and run parallel with the samples for identification of the TAGs, DAGs, MAGs, FFAs and FAEEs after equilibration for approximately 60 min. The developed plates were air-dried, and a 0.2% solution of 2,7-dichlorofluorescein in ethanol was sprayed onto the plates for visualization under ultraviolet light. The bands of FAEEs were scraped off and extracted three times with 1 mL of diethyl ether each time. The diethyl ether was evaporated under a nitrogen stream, and then the FAEEs were dissolved in isooctane for GC analysis. The *Malania oleifera* seed oil was methylated to fatty acid methyl esters following the method described by Wang *et al.*³⁶⁾, and subsequent analysis was conducted using GC. The fatty acid composition of samples (ethanolysis product and *Malania oleifera* seed oil) was determined using a GC system (Agilent 7890A) equipped with a capillary column CP-Sil 88 (60 m $\times$ 0.25 mm $\times$ 0.2 μ m; Dikma Technologies, Beijing, China). The detailed analysis procedure was conducted as described by Qin *et al.*³⁷⁾.

2.7 FTIR analysis of the final product

10 mg of the final high-purity NAEE product was mixed with 1,000 mg KBr, and the mixture was thoroughly ground using a mortar. The resulting mixture was then pressed into a pellet. The obtained NAEE pellet was analyzed using a Nicolet 8210E FTIR spectrometer. The spectral measurement was performed over a wavelength range of 400 to 4000 cm^{-1} , with a total of 128 scans and a resolution of 2 cm^{-1} .

2.8 ^1H and ^{13}C NMR analysis of the final product

The final high-purity NAEE product was analyzed using a Bruker AVANCE III 600HD spectrometer equipped with a 5 mm BBO probe operating at 600 MHz for ^1H and ^{13}C NMR analysis. Prior to the analysis, the obtained NAEE (30 mg) was dissolved in 600 μ L of deuterated chloroform (CDCl_3). The ^1H NMR spectrum was acquired with a recycle delay of 1.0 s and 16 scans, while the ^{13}C NMR spectrum was obtained with a recycle delay of 2 s and 160 scans. The acquired spectra were analyzed using MestReNova software (Mestrelab Research SL, Santiago de Compostela, Spain).

2.9 Statistical analysis

All experiments were carried in triplicate. The data was presented as the means $\pm$ standard deviations. Statistical analysis was performed using Origin 2018.

3 Results and Discussion

3.1 Fatty acids and lipid composition of *Malania oleifera* seed oil

The lipid composition of the *Malania oleifera* seed oil was analyzed using HPLC. In parallel, the fatty acid composition was analyzed using GC. The lipid composition of *Malania oleifera* seed oil is listed in Table 2: TAGs account for 82.76%, DAGs account for 2.01%, MAGs account for 0.32%, and FFAs account for 13.91%. As shown in Table 3, *Malania oleifera* seed oil is primarily composed of 10 fatty acids, listed in a descending order of relative abundance: nervonic acid, oleic acid, erucic acid,

arachidonic acid, lignoceric acid, linoleic acid, behenic acid, palmitic acid, stearic acid, and linolenic acid. *Malania oleifera* seed oil stands out with a remarkable NA content of 40.56%, making it an excellent raw material for obtaining high-purity NA. For context, the majority of other plants exhibit a nervonic acid content of less than 2%.

3.2 Enzymatic preparation of NAEF

3.2.1 Lipase screening

We conducted a comparative analysis of the catalytic performance of four lipases: Lipase UM1, Novozym 435, Lipozyme TL IM, and Lipozyme RM IM, in the ethanolysis re-

Table 2 Lipid composition of the *Malania oleifera* seed oil, UM1-catalyzed product and final product purified by semi-preparative chromatography.

Lipid	<i>Malania oleifera</i> seed oil (%)	UM1-catalyzed product (%) ^a	Final product (%)
FAEEs	ND ^b	93.36 ± 0.21	99.81 ± 0.02
TAGs	82.76 ± 0.25	0.77 ± 0.06	ND
FFAs	13.91 ± 0.29	5.52 ± 0.15	ND
1,3-DAGs	1.88 ± 0.27	0.23 ± 0.06	0.15 ± 0.02
1,2-DAGs	1.13 ± 0.09	0.12 ± 0.03	0.04 ± 0.03
MAGs	0.32 ± 0.07	ND	ND

^a: Reaction conditions of ethanolysis catalyzed by Lipase UM1. (3:1 molar ratio of ethanol to oil, lipase dosage of 100 U/g substrate, 45°C for 12 h.)

^b: ND, Not Detected.

Table 3 Fatty acid composition of *Malania oleifera* seed oil, UM1-catalyzed product and final product purified by semi-preparative chromatography.

Fatty acids	<i>Malania oleifera</i> seed oil (%)	UM1-catalyzed product (%)	Final product (%)
C16:0	0.74 ± 0.05	0.65 ± 0.06	ND
C18:0	0.36 ± 0.02	0.24 ± 0.02	ND
C18:1	29.86 ± 0.22	31.85 ± 0.21	ND
C18:2	1.34 ± 0.09	1.55 ± 0.11	ND
C18:3	0.16 ± 0.01	0.19 ± 0.02	ND
C20:1	2.85 ± 0.12	2.87 ± 0.10	ND
C22:0	1.21 ± 0.05	1.10 ± 0.02	2.37 ± 0.13
C22:1	15.74 ± 0.16	14.21 ± 0.17	ND
C24:0	1.74 ± 0.13	1.76 ± 0.08	0.11 ± 0.04
C24:1	40.56 ± 0.17	41.96 ± 0.32	97.52 ± 0.12
SFAs	4.05 ± 0.24	3.75 ± 0.14	2.48 ± 0.13
MUFAs	89.01 ± 0.30	90.89 ± 0.16	97.52 ± 0.12
PUFAs	1.50 ± 0.08	1.74 ± 0.13	ND

SFAs, saturated fatty acids; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids.

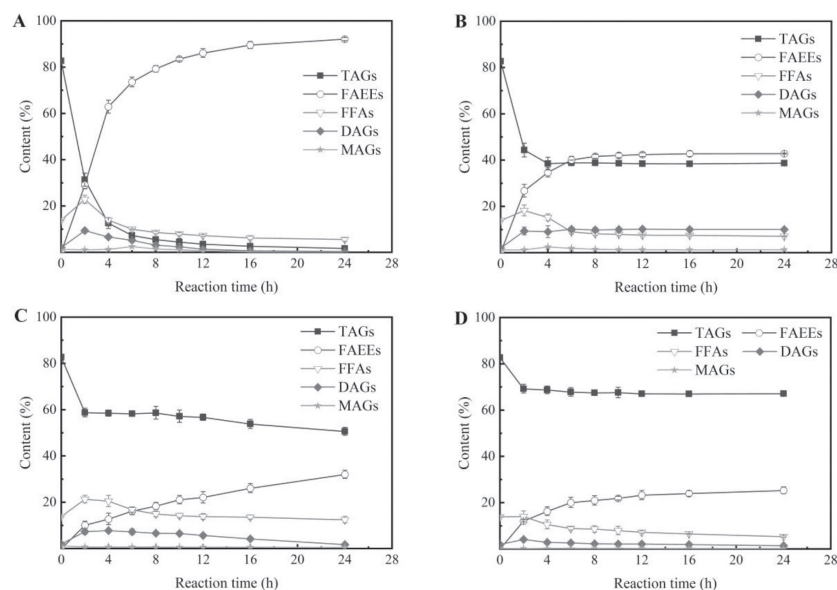


Fig. 2 Time course of enzymatic ethanolysis of *Malania oleifera* seed oil by Lipase UM1 (A), Lipozyme RM IM (B), Lipozyme TL IM (C), and Novozym 435 (D). The enzymatic reactions were operated under the conditions: 100 U/g substrate of lipase dosage, 3:1 molar ratio of ethanol to oil, 35°C for 24 hours, 2% water content (w/w, relative to total substrates). The reaction mixtures were periodically withdrawn for HPLC analyse over time. Samples were taken at 0, 2, 4, 6, 8, 10, 12, 16, and 24 h to assess the composition and content of TAGs, DAGs, MAGs, FAEEs, and FFAs.

action of *Malania oleifera* seed oil. The aim was to identify an enzyme that could efficiently catalyze the conversion of *Malania oleifera* seed oil into FAEEs. The results, depicted in **Fig. 2**, demonstrate that Lipase UM1 achieved a significantly higher FAEE conversion rate (92.52%) compared to Novozym 435 (24.98%), Lipozyme RM IM (42.96%), and Lipozyme TL IM (32.36%) after 24 hours of reaction. Additionally, **Fig. 2** provides insights into the dynamic changes in reaction products during the ethanolysis reaction catalyzed by Lipase UM1. The concentrations of TAGs and FFAs exhibited continuous reductions, while DAGs and MAGs initially increased before gradually decreasing. These findings indicate that during the reaction catalyzed by Lipase UM1, there is no accumulation of TAGs, FFAs, or DAGs. In contrast, the ethanolysis reactions catalyzed by the other three commercial lipases displayed distinct catalytic behaviors, leading to the accumulation of various intermediates. Specifically, the reaction catalyzed by Lipozyme RM IM showed a significant accumulation of DAGs, while Lipozyme TL IM exhibited a higher accumulation of FFAs. Novozym 435-catalyzed reactions demonstrated substantial TAG accumulation. These results highlight Lipase UM1 as the preferred choice for further optimization of the ethanolysis reaction.

3.2.2 Effect of substrate molar ratio

In this reaction, ethanol serves as both a reactant and a reaction medium. Increasing the amount of ethanol enhances the interaction between substrates, positively influencing FAEE synthesis. However, excessive use of ethanol

can decrease the activity of the lipase, thereby reducing the catalytic efficiency. In this study, four levels of substrate molar ratios (ethanol/*Malania oleifera* seed oil) were investigated: 1.5:1, 3:1, 6:1, and 9:1 (**Fig. 3A**). As the substrate molar ratio increased from 1.5:1 to 3:1, the FAEE conversion rate significantly increased from 49.29% to 92.52%. However, further increasing the substrate molar ratio (>3:1) resulted in an increase in the initial reaction rate but no further increase in the FAEE conversion rate. Instead, it decreased from 92.52% to 84.40%. This may be due to excessive ethanol in the reaction system, which adheres to the lipase, causing displacement of water molecules on the enzyme surface and resulting in an unstable protein structure, thereby reducing the catalytic activity of the lipase³⁸). Additionally, excessive ethanol dilutes the concentration of *Malania oleifera* seed oil in the reaction system, reducing the collision frequency between *Malania oleifera* seed oil and the lipase, thereby decreasing the formation of enzyme-substrate complexes and FAEEs. The substrate molar ratio of 3:1 was determined as the optimal value for subsequent experiments, to reach the maximum FAEEs conversion rate.

3.2.3 Effect of water content

The water content in the reaction system is an important factor that affects the lipase activity, reaction rate, and product yield. In this study, we investigated the effect of water content on the synthesis of FAEEs. As depicted in **Fig. 3B**, increasing the water content from 0% to 2% resulted in an increase in both the reaction rate and the con-

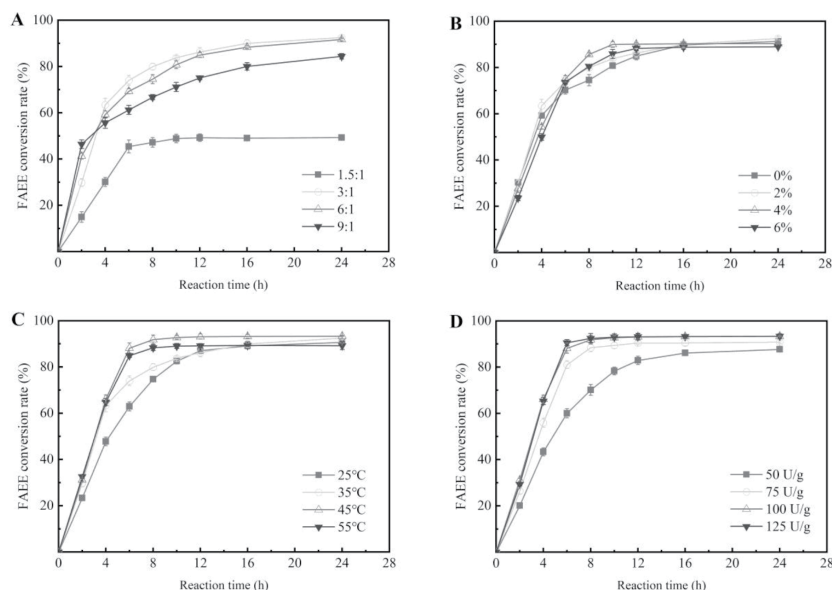


Fig. 3 Optimization of ethanolysis conditions.

- (A) Effect of ethanol to oil ratio on FAEE conversion rate. Reaction conditions: Lipase UM1 100 U/g substrate, 35°C for 24 hours, 2% water content (w/w, relative to total substrates).
- (B) Effect of water content on FAEE conversion rate. Reaction conditions: Lipase UM1 100 U/g substrate, 3:1 molar ratio of ethanol to oil, 35°C for 24 hours.
- (C) Effect of reaction temperature on FAEE conversion rate. Reaction conditions: Lipase UM1 100 U/g substrate, 3:1 molar ratio of ethanol to oil, 2% water content (w/w, relative to total substrates), 24 hours of reaction.
- (D) Effect of lipase dosage on FAEE conversion rate. Reaction conditions: 45°C for 24 hours, 3:1 molar ratio of ethanol to oil, 2% water content (w/w, relative to total substrates).

version rate of FAEEs from 91.28% to 92.52%. It has been reported that the oil/water interface area in the reaction system primarily influences the activation and conformational changes of the lipase³⁹⁾. The presence of an appropriate amount of water facilitates the formation of an effective oil/water interface and exposes the catalytic active sites of the lipase, thereby enhancing its catalytic activity. However, when the water content exceeded 2%, both the reaction rate and the FAEE conversion rate declined slightly. These findings suggest the coexistence of ester exchange, hydrolysis, and esterification reactions in this process, with excessive water favoring hydrolysis and resulting in the generation of more free fatty acids. Consequently, a water content of 2% was selected for further experiments.

3.2.4 Effect of reaction temperature

Temperature plays a crucial role in the activity and stability of lipase. Generally, enzyme-catalyzed reactions exhibit higher reaction rates with increasing temperature, as it raises average kinetic energy of the lipase and the substrate molecules, leading to accelerated reactions. However, maintaining a balance between temperature, enzyme activity, and stability is essential, as excessively high temperatures can deactivate the enzyme. In this study, we optimized the ethanolysis of *Malania oleifera*

seed oil catalyzed by Lipase UM1 at various temperatures, namely 25°C, 35°C, 45°C, and 55°C (**Fig. 3C**). The results demonstrated that higher temperatures correlated with faster initial reaction rates. The conversion rate of FAEEs significantly increased to 93.36% when the temperature was raised from 25°C to 45°C. This can be attributed to the fact that elevated reaction temperatures reduce the viscosity and mass transfer resistance of the reaction system⁴⁰⁾, facilitating better miscibility between the *Malania oleifera* seed oil and ethanol substrates. However, as the temperature further rose to 55°C, the conversion rate of FAEEs decreased to 89.25%. This decrease might be due to partial enzyme deactivation at higher temperatures, leading to a loss of enzymatic activity and impaired enzyme catalytic function⁴¹⁾. Therefore, the reaction temperature of 45°C was believed to be most suitable for Lipase UM1 catalyzed ethanolysis.

3.2.5 Effect of enzyme dosage

Enzyme dosage plays a significant role in the ethanolysis reaction, influencing the reaction rate. Higher enzyme dosage leads to shorter reaction times. In this study, the impact of different enzyme dosages (50 U/g substrate, 75 U/g substrate, 100 U/g substrate, 125 U/g substrate) on the catalytic preparation of *Malania oleifera* seed oil ethyl esters using Lipase UM1 was investigated. The results, de-

picted in Fig. 3D, showed that increasing the enzyme dosage from 50 U/g substrate to 100 U/g substrate resulted in an increase in the conversion rate of FAEEs from 87.72% to 93.36% and a reduction in the reaction equilibrium time from 24 h to 12 h. This improvement can be attributed to the availability of more active sites for the reaction at higher enzyme dosages, facilitating the formation of enzyme-substrate complexes and improving the initial reaction rate and conversion rate of FAEEs. Beyond 100 U/g substrate, further increasing the enzyme dosage had minimal effect on the conversion rate of FAEEs, primarily influencing the time required to reach equilibrium. This observation may be attributed to the saturation of some enzymes with the substrate at the early stage of the reaction which already reached equilibrium. An additional increase in enzyme dosage could lead to limited contact between enzymes and the substrate or incomplete suspension of enzymes, causing mass transfer limitations⁴²⁾. Therefore, subsequent experiments were conducted using an enzyme dosage of 100 U/g substrate.

Based on the experimental findings, the optimal process conditions were selected: The preferred enzyme was Lipase UM1, with an ethanol to *Malania oleifera* seed oil molar ratio of 3:1, a water content of 2%, an enzyme dosage of 100 U/g substrate, a reaction temperature of 45°C, and a reaction time of 12 h. Under these conditions, the conversion rate reached 93.36%. Furthermore, the lipid composition of the UM1-catalyzed product is as follows: TAG content was 0.77%, FAEE content was 93.36%, FFA content was 5.52%, and DAG content was 0.35% (Table 2).

3.3 Separation and identification of the high-purity NAEE

3.3.1 Separation of the high-purity NAEE

Semi-preparative chromatography is one of the most efficient and widely accepted techniques for separation and purification of compounds, but requires relatively large amounts of high-purity organic solvents (Such as methanol and acetonitrile) as mobile phase⁴³⁾. Unfortunately, although these solvents exhibit effectiveness in product separation, they are ranked hazardous due to their toxic effects. Therefore, the greening of mobile phases has received great interest among researchers. Ethanol is considered the greenest and desirable solvent due to its low toxicity and environmentally compatible waste⁴⁴⁾. In this context, we optimized the testing parameters of semi preparative chromatography using ethanol/water as the mobile phase to separate NAEE (data not shown). The optimized conditions are as follows: the mobile phase consists of ethanol-water (96:4, v/v), and the flow rate of the mobile phase is set at 15.0 mL/min. The obtained chromatogram was shown in Fig. 4. There are a total of seven chromatographic peaks. Each chromatographic peak was systematically collected using an automated program, and the

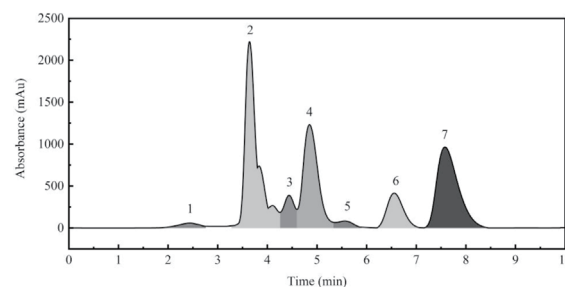


Fig. 4 Chromatogram of semi-preparative chromatographic separation of the UM1-catalyzed product. Separation was achieved using: mobile phase (ethanol-water 96:4, v/v); mobile phase velocity (15.0 mL/min); the numbers represent the collection fraction numbers.

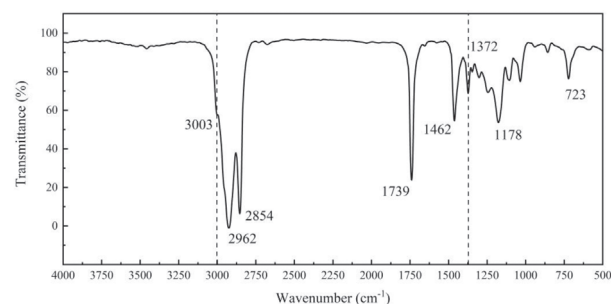


Fig. 5 FTIR spectrum of the high-purity NAEE product. The spectrum shows characteristic absorption peaks corresponding to specific functional groups, providing insight into the molecular composition and purity of the NAEE sample.

mobile phase associated with each component was evaporated through nitrogen blowing. The fatty acid composition of FAEEs in each component was analyzed using GC, and the results are presented in Table S1. Upon analyzing the fatty acid composition of each peak, peak 7 was found to have the highest NA purity, with content of 97.52%. Further, we analyzed the lipid composition of the isolated product. The results showed that the FAEE content of the product was 99.81% (Table 2). This result suggested that ethanol/water as a green mobile phase for the preparation of high purity NAEE was feasible.

3.3.2 Identification of the high-purity NAEE

The FTIR spectrum of the final NAEE product obtained is shown in Fig. 5. The ethoxy ester ($-\text{COOCH}_2\text{CH}_3$) exhibits a prominent stretching band of the strong C=O group at $1,739\text{ cm}^{-1}$. At 723 cm^{-1} , the rocking bending vibration of methylene groups $-\text{CH}_2-$ is observed. The C-H stretching vibration of the terminal methyl group ($-\text{CH}_3$) of the fatty acid chain and the methylene is represented by the peaks at $2,854\text{ cm}^{-1}$ and $2,926\text{ cm}^{-1}$, respectively. The stretching vibration of C-O is represented by a peak at $1,178\text{ cm}^{-1}$. Additionally, the bending vibrations of the CH_3 and CH_2 aliphatic groups are indicated by the peaks at $1,372\text{ cm}^{-1}$ and

1,462 cm^{-1} , respectively. The peak at 3,003 cm^{-1} represents the C–H stretching vibration of vinylic/cis olefinic C–H double bond ($=\text{C}-\text{H}$). Notably, the FTIR spectrum shows no significant absorption peak at 965 cm^{-1} , indicating the absence of a trans structure in the purified NAEF derived from *Malania oleifera* seed oil.

The ^1H NMR spectrum (Fig. 6A) of the final NAEF product was analyzed, and the major peaks are listed in Table S2. The presence of olefinic protons ($-\text{CH}=\text{CH}-$) bonded to carbon atoms is indicated by a multiplet signal at 5.34 ppm. At 2.01 ppm, a multiplet signal is observed, representing the α -methylene protons ($\alpha\text{-CH}_2$) of the double bond ($-\text{CH}_2-\text{C}=\text{C}-$). The peak at 2.01 ppm corresponds to the ethoxy protons ($-\text{OCH}_2-\text{CH}_3$) of ethyl ester ($-\text{COOCH}_2\text{CH}_3$). The triplet signals of the α -methylene protons ($\alpha\text{-CH}_2$) of ester ($-\text{CH}_2-\text{COO}-\text{CH}_2\text{CH}_3$) appear at 4.12 ppm. The β -methylene protons of ester ($-\text{CH}_2-\text{C}-\text{COOCH}_2\text{CH}_3$) exhibit a doublet of doublets signal at 1.62 ppm. The triplet signal at 0.88 ppm indicates the presence of the terminal methyl protons ($-\text{C}-\text{CH}_3$). The peaks at 1.27 ppm are assigned to the protons of the methylene ($-(\text{CH}_2)_n-$)

backbone present in long fatty acid chains.

The ^{13}C NMR spectrum (Fig. 6B) of the final NAEF product was analyzed, and the major peaks are listed in Table S3. The ester carbonyl carbon ($-\text{COO}-$) is represented by a peak at 173.94 ppm. The signal 129.89 ppm indicates the presence of olefinic carbons ($-\text{C}=\text{C}-$). The ethoxy carbons of ethyl esters appear a singlet signal at 60.15 ppm ($-\text{OCH}_2\text{CH}_3$). Peaks ranging from 14.12 to 14.26 ppm correspond to the terminal carbons of methyl groups. The methylene carbons of long carbon chains appear in the range of 22.69–34.41 ppm. These results provide further evidence for the successful preparation and separation of high-purity NAEF using the enzymatic method combined with semi-preparative chromatography.

3.4 Scale-up experiment verification

To validate the operational stability of this method, we scaled up the reaction to 60 g of substrates and conducted it under the optimized conditions. The results, as depicted in Fig. 7, show that the initial reaction rate and the final conversion rate in the scaled-up reaction were similar to

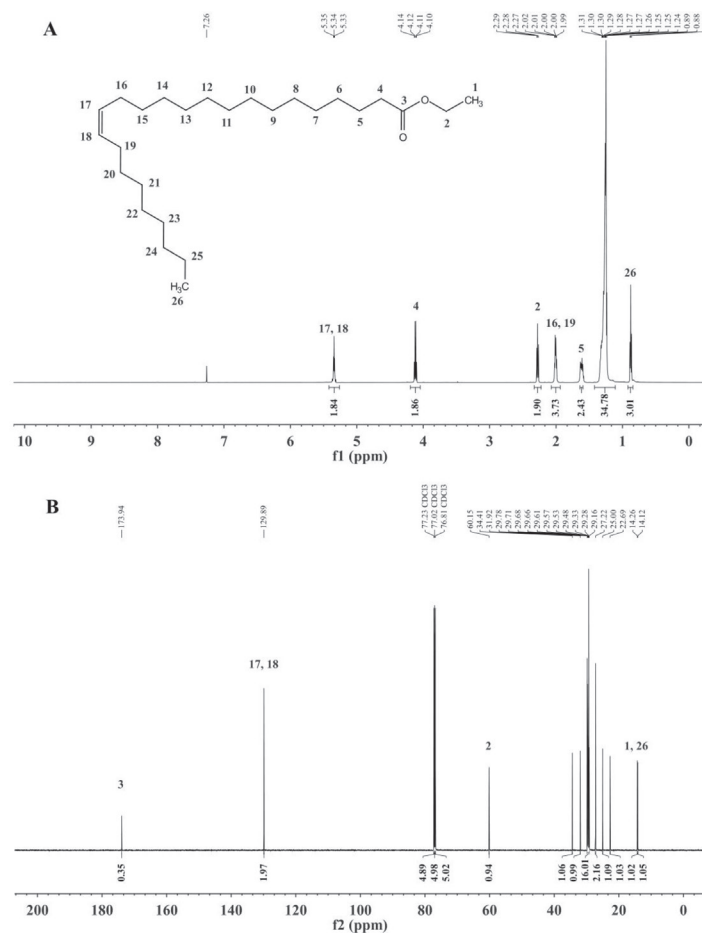


Fig. 6 NMR spectra of the high-purity NAEF product. (A) ^1H NMR spectrum and (B) ^{13}C NMR spectrum. Both spectra were recorded at 600 MHz with 160 scans in CDCl_3 as the solvent.

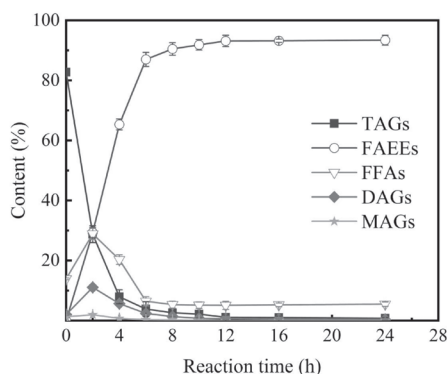


Fig. 7 Time course of ethanolsis of *Malania oleifera* seed oil in a scale-up experiment. Reaction conditions: 100 U/g substrate of lipase dosage, 3:1 molar ratio of ethanol to oil, 45°C for 24 hours, 2% water content (w/w, relative to total substrates). Samples were taken at 0, 2, 4, 6, 8, 10, 12, 16, and 24 h to assess the composition and content of TAGs, DAGs, MAGs, FAEs, and FFAs using HPLC analyse.

those obtained with 10 g of substrates. Following semi-preparative chromatography separation, the final high-purity NAEF reached a purity of 97.48% with a yield of 73.26%. Therefore, the high-purity NAEF preparation method developed in this study has proven its feasibility for upscaling. However, transitioning to an industrial scale necessitates further research and development.

Despite the successful implementation of our high-purity NAEF preparation method, we still face several potential challenges in its industrial application. Enzymatic processes are well-recognized for their environmental advantages, such as biodegradability and reduced toxicity in industrial wastewater. However, enzymes come at a higher cost, necessitating the search for cost-effective production methods to ensure viability in industrial-scale production⁴⁵. Moreover, enzymes need to maintain their activity for extended periods to achieve high yields and economic efficiency at an industrial scale^{46, 47}. Therefore, a comprehensive assessment of economic implications and enzyme stability is necessary for practical industrial applications⁴⁸.

4 Conclusion

This study focuses on producing high-purity NAEF from *Malania oleifera* seed oil using a combination of enzymatic ethanolsis and semi-preparative chromatography with ethanol/water as the “green” mobile phase. Among the lipases screened, Lipase UM1 exhibited the highest catalytic efficiency for the ethanolsis of *Malania oleifera* seed oil. Under the optimized conditions (Lipase UM1 at 100 U/g substrate, substrate molar ratio of 3:1, water content of

2%, temperature of 45°C, and reaction time of 12 h), the conversion rate of FAEs reached 93.36%, with a NAEF content of 41.96%. Subsequently, NAEF was isolated from FAEs through semi-preparative chromatography, resulting in a purity of 97.52%. This advancement not only positions the procedure for potential upscaling to industrial applications but also underscores the industrial value for *Malania oleifera* seed oil. Such utilization contributes to the economic development of impoverished mountainous regions rich in *Malania oleifera* seed oil resources. Collectively, this work serves as a significant reference for the development of a sustainable, efficient, and effective methodology for high-purity NAEF synthesis, addressing safety issues and reducing byproduct generation observed in prior studies.

Author Contributions

Yang X.M. contributed to conceptualization, data curation, formal analysis, methodology, software, validation, and wrote the original draft. Lin S. provided resources, contributed to methodology, and supervised the study. Chen Y. and Chen W. were involved in methodology, contributed to writing, and provided editing and supervision. Lan D.M. and Wang Y.H. were responsible for funding acquisition and project administration. All authors read and approved the final manuscript.

Acknowledgement

This study was funded by the National Key R&D Program of China (2022YFC2805104, 2022YFC2104905), Key Program of Natural Science Foundation of China (grant number 31930084).

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Supporting Information

This material is available free of charge via the Internet at doi: 10.5650/jos.ess23170

References

- 1) Umemoto, H.; Sawada, K.; Kurata, A.; Hamaguchi, S.; Tsukahara, S. *et al.* Fermentative production of ner-

- vononic acid by *Mortierella Capitata* RD000969. *J. Oleo Sci.* **63**, 671-679 (2014). doi: 10.5650/jos.ess14029
- 2) Yu, J.; Yuan, T.; Zhang, X.; Jin, Q.; Wei, W. *et al.* Quantification of nervonic acid in human milk in the first 30 days of lactation: influence of lactation stages and comparison with infant formulae. *Nutrients* **11**, 1892 (2019). doi: 10.3390/nu11081892
- 3) Ntoumani, E.; Strandvik, B.; Sabel, K.G. Nervonic acid is much lower in donor milk than in milk from mothers delivering premature infants—Of neglected importance? *Prostaglandins Leukot. Essent. Fatty Acids* **89**, 241-244 (2013). doi: 10.1016/j.plefa.2013.06.005
- 4) Xue, Y.; Zhu, X.; Yan, W.; Zhang, Z.; Cui, E. *et al.* Dietary supplementation with *Acer truncatum* oil promotes remyelination in a mouse model of multiple sclerosis. *Front. Neurosci.* **16**, 860280 (2022). doi: 10.3389/fnins.2022.860280
- 5) Wang, X.; Li, Z.; Li, X.; Liu, X.; Ying M. *et al.* Integrated metabolomics and transcriptomics reveal the neuroprotective effect of nervonic acid on LPS-induced AD model mice. *Biochem. Pharmacol.* **209**, 115411 (2023). doi: 10.1016/j.bcp.2023.115411
- 6) Terluk, M.R.; Tieu, J.; Sahasrabudhe, S.A.; Moser, A.; Watkins, P.A. *et al.* Nervonic acid attenuates accumulation of very long-chain fatty acids and is a potential therapy for adrenoleukodystrophy. *Neurotherapeutics* **19**, 1007-1017 (2022). doi: 10.1007/s13311-022-01226-7
- 7) Amminger, G.P.; Schäfer, M.R.; Klier, C.M.; Slavik, J.-M.; Holzer, I. *et al.* Decreased nervonic acid levels in erythrocyte membranes predict psychosis in help-seeking ultra-high-risk individuals. *Mol. Psychiatry* **17**, 1150-1152 (2012). doi: 10.1038/mp.2011.167
- 8) Fan, Y.; Yuan, C.; Jin, Y.; Hu, G.R.; Li, F.L. Characterization of 3-ketoacyl-coA synthase in a nervonic acid producing oleaginous microalgae *Mychonastes afer*. *Algal Res.* **31**, 225-231 (2018). doi: 10.1016/j.algal.2018.02.017
- 9) Satoto, G.; Fernandes, A.S.; Saraiva, N.; Santos, F.; Neng, N. *et al.* An overview on the properties of Ximania oil used as cosmetic in Angola. *Biomolecules* **10**, 18 (2019). doi: 10.3390/biom10010018
- 10) Wang, K.; Lin, L.; Wei, P.; Ledesma-Amaro, R.; Ji, X.J. Combining orthogonal plant and non-plant fatty acid biosynthesis pathways for efficient production of microbial oil enriched in nervonic acid in *Yarrowia lipolytica*. *Bioresour. Technol.* **378**, 129012 (2023). doi: 10.1016/j.biortech.2023.129012
- 11) He, X.; Lu, T.Q.; Li, J.Y.; Mao, P.; Zhang, L. *et al.* Germplasm resources of three wood plant species enriched with nervonic acid. *Plant Divers.* **44**, 308-315 (2022). doi: 10.1016/j.pld.2022.01.004
- 12) Montañés, F.; Catchpole, O.J.; Tallon, S.; Mitchell, K.; Lagutin, K. Semi-preparative supercritical chromatography scale plant for polyunsaturated fatty acids purification. *J. Supercrit. Fluids* **79**, 46-54 (2013). doi: 10.1016/j.supflu.2012.11.018
- 13) Fernie, C.E.; Dupont, I.E.; Scruel, O.; Carpentier, Y.A.; Sébédio, J. *et al.* Relative absorption of conjugated linoleic acid as triacylglycerol, free fatty acid and ethyl ester in a functional food matrix. *Eur. J. Lipid Sci. Technol.* **106**, 347-354 (2004). doi: 10.1002/ejlt.200300924
- 14) Xie, D.; Chen, Y.; Yu, J.; Yang, Z.; Wang, X. *et al.* Progress in enrichment of n-3 polyunsaturated fatty acid: A review. *Crit. Rev. Food Sci. Nutr.* 1-17 (2022). doi: 10.1080/10408398.2022.2086852
- 15) Litwinienko, G.; Kasprzycka-Guttman, T. Study on the autoxidation kinetics of fat components by differential scanning calorimetry. 1. saturated C₁₂-C₁₈ fatty acids and their esters. *Ind. Eng. Chem. Res.* **39**, 13-17 (2000). doi: 10.1021/ie990552u
- 16) Wang, J.; Zheng, S.; Gao, Y.; Shi, J.; Zhou, Z. *et al.* Enrichment of high-purity nervonic acid ethyl ester from *Acer truncatum* Bunge seed oil by combination method of urea inclusion and molecular distillation. *Can. J. Chem. Eng.* **101**, 5977-5991 (2023). doi: 10.1002/cjce.24875
- 17) Shi, J.; Wang, J.; Gao, Y.; Tian, S.; Zhou, Z. *et al.* Extraction of nervonic acid from *Acer truncatum* oil by urea inclusion. *Chem. Pap.* **77**, 5701-5711 (2023). doi: 10.1007/s11696-023-02890-5
- 18) Li, D.; Wang, W.; Qin, X.; Li, X.; Yang, B. *et al.* A novel process for the synthesis of highly pure n-3 polyunsaturated fatty acid (PUFA)-enriched triglycerides by combined transesterification and ethanolysis. *J. Agric. Food Chem.* **64**, 6533-6538 (2016). doi: 10.1021/acs.jafc.6b02675
- 19) De Sousa Junior, P.G.; Do Nascimento Camara, A.G.; Oliveira, A.R.T.; De Castro Lima, G.; Lima, G.V. *et al.* Optimization and theoretical analysis of lipase-catalyzed enzymatic esterification of glycerol for efficient glycerides synthesis. *Biochem. Eng. J.* **198**, 109033 (2023). doi: 10.1016/j.bej.2023.109033
- 20) Da Silva, J.L.; Sales, M.B.; De Castro Bizerra, V.; Nobre, M.M.R.; De Sousa Braz, A.K. *et al.* Lipase from *Yarrowia lipolytica*: prospects as an industrial biocatalyst for biotechnological applications. *Fermentation* **9**, 581 (2023). doi: 10.3390/fermentation9070581
- 21) De Sousa, I.G.; Mota, G.F.; Cavalcante, A.L.G.; Rocha, T.G.; Da Silva Sousa, P. *et al.* Renewable processes of synthesis of biolubricants catalyzed by Lipases. *J. Environ. Chem. Eng.* **11**, 109006 (2023). doi: 10.1016/j.jece.2022.109006
- 22) Da S. Moreira, K.; Barros De Oliveira, A.L.; Saraiva De Moura Júnior, L.; Germano De Sousa, I.; Luthierre Gama Cavalcante, A. *et al.* Taguchi design-assisted co-immobilization of Lipase A and B from *Candida ant-*

- arctica* onto chitosan: Characterization, kinetic resolution application, and docking studies. *Chem. Eng. Res. Des.* **177**, 223-244 (2022). doi: 10.1016/j.cherd.2021.10.033
- 23) Li, J.; Zhu, M.; Wu, M. Enrichment and purification of nervonic acid from *Thlaspi arvense* Linn seeds using a combination of molecular distillation and urea inclusion. *Food Research and Development* **43**, 82-87 (2021). doi: 10.12161/j.issn.1005-6521.2022.02.012
- 24) Li, Y.; Kong, F.; Zan, M.; Peng, L.; Wang, Z. *et al.* Optimization technology and kinetic studies of *Acer truncatum* seed oil saponification and crystallization separation of nervonic acid. *J. Food Sci.* **87**, 3925-3937 (2022). doi: 10.1111/1750-3841.16262
- 25) Ge, Z.; Jin, W.; Huang, N.; Zhou, L. Purification of nervonic acid in *Acer truncatum* Bunge seed oil by low temperature crystallization. *China Oils and Fats* **046**, 99-102 (2021). doi: 10.1016/j.molcatb.2015.01.009
- 26) Chen, W.; Sun, S.; Liang, S.; Peng, L.; Wang, Y. *et al.* Lipase-catalyzed hydrolysis of linseed oil: Optimization using response surface methodology. *J. Oleo Sci.* **63**, 619-628 (2014). doi: 10.5650/jos.ess13189
- 27) Utsugi, A.; Kanda, A.; Hara, S. Lipase specificity in the transacylation of triacylglycerin. *J. Oleo Sci.* **58**, 123-132 (2009). doi: 10.5650/jos.58.123
- 28) Awasthi, N.P.; Singh, R.P. Lipase-catalyzed synthesis of fatty acid amide (erucamide) using fatty acid and urea. *J. Oleo Sci.* **56**, 507-509 (2007). doi: 10.5650/jos.56.507
- 29) Monteiro, R.R.C.; Neto, D.M.A.; Fachine, P.B.A.; Lopes, A.A.S.; Gonçalves, L.R.B. *et al.* Ethyl butyrate synthesis catalyzed by Lipases A and B from *Candida antarctica* immobilized onto magnetic nanoparticles. Improvement of biocatalysts' performance under ultrasonic irradiation. *Int. J. Mol. Sci.* **20**, 5807 (2019). doi: 10.3390/ijms20225807
- 30) Stojanović, M.; Veličković, D.; Dimitrijević, A.; Milosavić, N.; Knežević-Jugović, Z. *et al.* Lipase-catalyzed synthesis of ascorbyl oleate in acetone: Optimization of reaction conditions and Lipase reusability. *J. Oleo Sci.* **62**, 591-603 (2013). doi: 10.5650/jos.62.591
- 31) Wang, X.; Qin, X.; Li, D.; Yang, B.; Wang, Y. One-step synthesis of high-yield biodiesel from waste cooking oils by a novel and highly methanol-tolerant immobilized lipase. *Bioresour. Technol.* **235**, 18-24 (2017). doi: 10.1016/j.biortech.2017.03.086
- 32) Wang, X.; Li, D.; Wang, W.; Yang, B.; Wang, Y. A highly efficient immobilized MAS1 lipase for the glycerolysis reaction of n-3 PUFA-rich ethyl esters. *J. Mol. Catal. B: Enzym.* **134**, 25-31 (2016). doi: 10.1016/j.molcatb.2016.09.012
- 33) Wang, X.; Li, D.; Qu, M.; Durrani, R.; Yang, B.; Wang, Y. Immobilized MAS1 lipase showed high esterification activity in the production of triacylglycerols with n-3 polyunsaturated fatty acids. *Food Chem.* **216**, 260-267 (2017). doi: 10.1016/j.foodchem.2016.08.041.
- 34) Pasha, M.K.; Dai, L.; Liu, D.; Du, W.; Guo, M. Biodiesel production with enzymatic technology: Progress and perspectives. *Biofuels Bioprod. Biorefin.* **15**, 1526-1548 (2021). doi: 10.1002/bbb.2236
- 35) Li, D.; Qin, X.; Wang, J.; Yang, B.; Wang, W. *et al.* Hydrolysis of soybean oil to produce diacylglycerol by a lipase from *Rhizopus oryzae*. *J. Mol. Catal. B: Enzym.* **115**, 43-50 (2015). doi: 10.1016/j.molcatb.2015.01.009
- 36) Wang, Y.H.; Mai, Q.Y.; Qin, X.L.; Yang, B.; Wang, Z.L. *et al.* Establishment of an evaluation model for human milk fat substitutes. *J. Agric. Food Chem.* **58**, 642-649 (2010). doi: 10.1021/jf903048p
- 37) Qin, X.L.; Huang, H.H.; Lan, D.M.; Wang, Y.H.; Yang, B. Typospecificity of crude *Geobacillus* sp. T1 lipase fused with a cellulose-binding domain and its use in the synthesis of structured lipids. *J. Am. Oil Chem. Soc.* **91**, 55-62 (2014). doi: 10.1007/s11746-013-2347-4
- 38) Matassoli, A.L.F.; Corrêa, I.N.S.; Portilho, M.F.; Veloso, C.O.; Langone, M.A.P. Enzymatic synthesis of biodiesel via alcoholysis of palm oil. *Appl. Biochem. Biotechnol.* **155**, 44-52 (2009). doi: 10.1007/s12010-008-8424-8
- 39) Choi, N.; Kim, Y.; Lee, J.S.; Kwak, J.; Lee, J. *et al.* Synthesis of fatty acid ethyl ester from acid oil in a continuous reactor via an enzymatic transesterification. *J. Am. Oil Chem. Soc.* **93**, 311-318 (2016). doi: 10.1007/s11746-016-2786-9
- 40) Aguiéiras, E.C.G.; De Abreu, K.E.A.; Oliveira, R.A.; Almeida, J.M.A.R.; Freire, D.M.G. Fatty acid ethyl esters production from distillers corn oil by enzymatic catalysis. *J. Am. Oil Chem. Soc.* **99**, 393-405 (2022). doi: 10.1002/aocs.12582
- 41) Wang, X.; Qin, X.; Li, X.; Zhao, Z.; Yang, B.; Wang, Y. Insight into the modification of phosphatidylcholine with n-3 polyunsaturated fatty acids-rich ethyl esters by immobilized MAS1 lipase. *Molecules* **24**, 3528 (2019). doi: 10.3390/molecules24193528
- 42) Yasvanthrajan, N.; Sivakumar, P.; Muthukumar, K.; Murugesan, T.; Arunagiri, A. Production of biodiesel from waste bio-oil through ultrasound assisted transesterification using immobilized lipase. *Environ. Technol. Innovation* **21**, 101199 (2021). doi: 10.1016/j.eti.2020.101199
- 43) Tobiszewski, M.; Namieśnik, J. Greener organic solvents in analytical chemistry. *Curr. Opin. Green Sustainable Chem.* **5**, 1-4, (2017). doi: 10.1016/j.cogsc.2017.03.002
- 44) Yabré, M.; Ferey, L.; Somé, I.; Gaudin, K. Greening reversed-phase liquid chromatography methods using alternative solvents for pharmaceutical analysis. *Mole-*

- cules* **23**, 1065 (2018). doi: 10.3390/molecules23051065
- 45) Melo, R.L.F.; Sales, M.B.; De Castro Bizerra, V.; De Sousa Junior, P.G.; Cavalcante, A.L.G. *et al.* Recent applications and future prospects of magnetic biocatalysts. *Int. J. Biol. Macromol.* **253**, 126709 (2023). doi: 10.1016/j.ijbiomac.2023.126709
- 46) Abdelmoez, W.; Mustafa, A. Oleochemical industry future through biotechnology. *J. Oleo Sci.* **63**, 545-554 (2014). doi: 10.5650/jos.ess14022
- 47) De Oliveira, U.M.F.; Lima De Matos, L.J.B.; De Souza, M.C.M.; Pinheiro, B.B.; Dos Santos, J.C.S. *et al.* Effect of the presence of surfactants and immobilization conditions on catalysts' properties of *Rhizomucor miehei* lipase onto chitosan. *Appl. Biochem. Biotechnol.* **184**, 1263-1285 (2018). doi: 10.1007/s12010-017-2622-1
- 48) Santos, J.C.S.D.; Dhenadhayalan, N.; Li, Y.; Pinilla, J.L. Editorial: Chemical reactions and catalysis for a sustainable future. *Front. Chem.* **11**, 1228591 (2023). doi: 10.3389/fchem.2023.1228591

CC BY 4.0 (Attribution 4.0 International). This license allows users to share and adapt an article, even commercially, as long as appropriate credit is given. That is, this license lets others copy, distribute, remix, and build upon the Article, even commercially, provided the original source and Authors are credited.

