APPLICATIONS OF LIPASES FOR MODIFICATION OF NATURAL AND SYNTHETIC MATERIALS

WINRANATH KANPRAKOBKIT

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
for the Doctor of Philosophy in Chemistry- (Type 1.1)
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By Winranath Kanprakobkit

has been approved by the Graduate School as partial fulfillment of the requirements for the Doctor of Philosophy in Chemistry- (Type 1.1) of Naresuan University

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Title	APPLICATIONS OF LIPASES FOR MODIFICATION OF NATURAL AND SYNTHETIC MATERIALS
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ABSTRACT

Lipases (triacylglycerol acyl hydrolase, EC 3.1.1.3) are naturally occurring enzymes found in the stomach and pancreatic juice. The function of lipase is to digest fats and lipids, so they can be absorbed in the intestines. Lipases can be isolated from many species of plants, animals, bacteria, fungi, and yeast. Lipases are one of the important groups of biocatalysts used in biotechnological applications and is mainly used to hydrolyze ester bonds of triacylglycerols (oils and fats) into diglycerides, monoglycerides, fatty acids, and glycerol. Furthermore, lipases catalyze esterification, interesterification, acidolysis, and transesterification reactions. This ability makes lipases a popular choice for potential applications in the industry, such as uses in food, detergents, pharmaceuticals, leather, textiles, cosmetics, paper, and polymers. In addition, lipase has high specificity and selectivity. As a result, these enzymes have practically limitless application possibilities.

This work aims to present the potential of lipases to improve their structures in natural and synthetic materials. This research selected lipase B from *Candida Antarctica* immobilized on acrylic resin, Lipozyme TL IM, Novozym© 435, and Novozym 51032 to catalyze the reaction. The optimal condition was investigated using statistical methods. The transformation of the material was analyzed using NMR, GC, and MASS spectroscopy techniques.

This study is divided into three parts: In the first part, the fatty acid composition of coconut oil was modified using enzyme-catalyzed interesterification with the aim of obtaining a product more similar to commercial MCT oils. This modification was carried out with the aim of obtaining a product with some of the health benefits shown by MCT oils. The second part, capsaicin and dihydrocapsaicin were enzymatically hydrolyzed into fatty acids. These fatty acids, which have been shown to have potential health benefits, have been utilized for the modification of coconut oil to generate a suitable delivery vehicle for them. The third part, poly(ethylene terephthalate) (PET) from water bottles, was degraded with the help of enzymes. Then, the photooxidative degradation step carried out with the help of an iridium complex as a photocatalyst, would be used to partially degrade the structural integrity of the PET material with the aim of making it more readily susceptible to enzymatic degradation. These enzymes and light will act on PET and end its breakdown into monomeric units, subsequently resulting in this loss of weight.

It is clear from this work that the lipase enzyme has a significant potential as a biocatalyst because it can increase the rates and stereospecificity of reactions. Additionally, they can be reused in a continuous flow process, reducing the cost of production. Furthermore, this

study demonstrates the guide application for enzyme improvement, as well as the opportunities and limitations for technological processes.

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Winranath Kanprakobkit

TABLE OF CONTENTS

Page

LIST OF TABLE

Page

LIST OF FIGURE

Page

LIST OF ABBREVIATIONS

NMR	=	nuclear magnetic resonance
GC	=	gas chromatography
LC-MS-MS	=	liquid Chromatography – mass spectrometry and liquid chromatography – tandem mass spectrometry
Q-TOF LC/MS	=	liquid chromatography-quadrupole time-of-flight mass spectrometry
FC	=	flash chromatography
HPLC	=	high performance liquid chromatography
FTIR	=	fourier transform infrared
SEM	=	scanning emission microscopy
DSC	=	differential scanning calorimetry
TLC	=	thin layer chromatography
RSM	=	response surface methodology
DOE	=	design of Experiments
EIE	=	enzymatic interesterification
IO	=	oil interesterification
TRPV1	=	transient receptor potential cation channel subfamily V member 1
RNA	=	ribonucleic acid
DNA	=	deoxyribonucleic acid
TMS	=	tetramethyl silane
$CDCl_3$	=	deuterated chloroform
NaOH	=	sodium hydroxide
HCl	=	hydrochloric Acid
MeOH	=	methyl alcohol
H_2SO_4	=	sulfuric acid
Na_2CO_3	=	sodium carbonate
Na_2SO_4	=	sodium sulfate
H_2O	=	water
EtOAc	=	ethyl acetate

LIST OF ABBREVIATIONS (CONT.)

2-MeTHF	=	2-Methyltetrahydrofuran
Me	=	methyl
CS	=	cobalt stearate
CI	=	carbonyl index
R	=	substituted group
δ	=	chemical shifts
ppm	=	part per million
m/z	=	mass-to-charge ratio
MHz	=	megahertz
g	=	gram
mg	=	milligram
g/L	=	grams per liter
M	=	molar
L	=	liter
mL	=	milliliter
mM	=	milimolar
mm	=	millimeter
mbar	=	millibar
mmol	=	milimole
µ	=	micro
µl	=	microliter
µm	=	micrometer
µM	=	micromolar
Min	=	minute
h	=	hour
T	=	temperature
RT	=	room temperature
°	=	degree (angle)
°C	=	degrees Celsius
w/w	=	weight by weight

LIST OF ABBREVIATIONS (CONT.)

Conc.	=	concentration
WL	=	weight loss
Co.,Ltd.	=	corporation, Limited
®	=	registered
©	=	copyright
e.g.	=	exempli gratia, which means "for example."
i.e.	=	id est, which means “in other words.”
etc.	=	et cetera
viz.	=	videlicet used as a synonym for "namely"
TM.	=	trademark
et al	=	et alia, which means “and others.”
CO	=	coconut oil
MC	=	medium chain
FA	=	fatty acid
MCFA	=	medium-chain fatty acid
FFA	=	free fatty acid
MCG	=	medium chain glycerides
MCT	=	medium-chain triglycerides
FAME	=	fatty acid methyl ester
FAD	=	fatty acid distillates
TAG	=	triacylglycerol
MAG	=	monoacylglycerol
DAG	=	diacylglycerol
MLCT	=	medium- and long-chain triacylglycerol
PUFA	=	polyunsaturated fatty acid
LC-PUFA	=	long-chain polyunsaturated fatty acid
TFA	=	trans fatty acid
SL	=	structured lipid
C8:0	=	caprylic acid
C10:0	=	capric acid

LIST OF ABBREVIATIONS (CONT.)

C12:0	=	lauric acid
C14:0	=	myristic acid
C16:0	=	palmitic acid
C18:2	=	linoleic acid
C18:1	=	oleic acid
C18:0	=	stearic acid
BT	=	beef tallow
POL	=	palm olein
SDA	=	stearidonic acid
HMFS	=	human milk fat substitutes
DHA	=	docosahexaenoic acid
AA	=	arachidonic acid
CCSO	=	cinnamomum camphora seed oil
CBE	=	cocoa butter equivalent
hPS	=	hard palm stearin
RBO	=	rice bran oil
FHPO	=	fully hydrogenated palm oil
SFC	=	solid fat content
CLA	=	conjugated linoleic acid
FHSO	=	fully hydrogenated soybean oil
EFA	=	essential fatty acid
ARASCO	=	triglyceride oil that contains high amounts of ARA, arachidonic acid (20:4 n-6)
SI	=	small intestine
T2DM	=	type 2 diabetes mellitus
MLM	=	triacylglycerol (s) containing medium-chain fatty acid at sn-1 and sn-3 positions (M) and a long-chain fatty acid (L) at position sn-2
C	=	control, normal diet
HF	=	high-fat diet

LIST OF ABBREVIATIONS (CONT.)

VCO	=	virgin coconut oil
LLA	=	low lauric acid coconut oil
BW	=	body weight
PET	=	poly(ethylene terephthalate)
PEG	=	poly(ethylene glycol)
CTR	=	cyclic trimer
TPA	=	terephthalic acid
BHET	=	bis(2-hydroxyethyl) terephthalate
MHET	=	mono(2-hydroxyethyl) terephthalate
EG	=	ethylene glycol
PBR	=	packed bed reactor
PTT	=	poly(trimethylene terephthalate)
BHPT	=	bis(3-hydroxypropyl) terephthalate
MHPT	=	mono(3-hydroxypropyl) terephthalate
lc	=	low-crystallinity
bo	=	biaxially oriented
HiC	=	*Humicola insolens*
PmC	=	*Pseudomonas mendocina*
FsC	=	*Fusarium solani*
rROL	=	*Heterologous* ROL
ROL	=	*Rhizopus oryzae* lipase

CHAPTER I

INTRODUCTION

1.1 Introduction to the research

Lipases (triacyglycerol acylhydrolase, EC 3.1.1.3) are enzyme hydrolases that catalyze the hydrolysis of triacylglycerols to glycerol and fatty acids. Beyond this natural activity, lipases are biocatalysts used to catalyze a wide variety of reactions such as hydrolysis, alcoholysis, acidolysis, aminolysis, trans-esterification, inter-esterification, and esterification (1). Lipases are found in humans, animals, plants, and microorganisms such as yeast, fungi, and bacteria. Lipases present in the pancreatic juice and stomach of humans and animals can hydrolyze fats into glycerol and fatty acids. Each type of lipase has different specific properties such as source, cycle time, or optimal range of pH and temperature. Lipases from microorganisms are most interesting commercially because of their low production costs, high production yield, stability, varieties of catalytic activities, and ease of use (2). Moreover, lipases are relatively resistant to pH, temperature, and organic solvents, and they have specificities such as regio-specificity, stereo-selectivity, and fatty acid specificity. Nowadays, lipases extracted from micro-organisms are used in a variety of industrial applications such as food, detergent, textile, cosmetics, biodiesel, and pharmaceuticals. Lipases are available in many varieties and with various properties for catalysis of numerous reactions and yielding a high percentage of production. Therefore, we are interested to use lipases to perform selected chemical transformations: capsaicin hydrolysis, coconut oil modification, and PET decomposition.

1.2 Research objectives

1. To investigate lipases catalyzed acidolysis and interesterification of coconut oil with MCFAs or fatty acid alkyl ester.

2. To investigate and further improve lipases can catalyzed hydrolysis of capsaicin and dihydrocapsaicin into fatty acids.

3. To utilize fatty acids from capsaicin and dihydrocapsaicin hydrolysis for triglyceride modification.

4. To investigate the possibility of PET degradation using a combination of photooxidative and enzymatic methods.

1.3 Scope and limitation

The purpose of this research is to investigate the applications of lipases for 1) catalyzed acidolysis and interesterification of coconut oil with MCFAs or fatty acid alkyl ester; 2) hydrolysis of capsaicin and dihydrocapsaicin to yield medium-chain 8-methyl-6-nonenoic acid and 8-methyl nonanoic acid to carry out acidolysis or interesterification of coconut oil with MCFAs or fatty acid alkyl ester to medium-chain fatty acid (MFA); and 3). Decompose Poly(ethylene terephthalate) (PET). The reactions will be investigated using NMR and GC methods. Optimization of reactions in terms of reaction parameters such as temperature, reaction time, and enzyme recycling will be investigated. Finally, we will investigate the biological effects of the produced compounds.

1.4 Research hypothesis

Lipases are biocatalysts, which have unique properties such as good stability, selectivity, and substrate specificity position and which can catalyze a wide range of bioconversion reactions, such as hydrolysis, interesterification, esterification, alcoholysis, acidolysis, and aminolysis. These properties make them useful in a wide range of applications including industrial applications, in the pharmaceutical sector, the chemical industry, food industry, in fats and oil industry etc. Some of these applications as shown in Figure 1 (3).

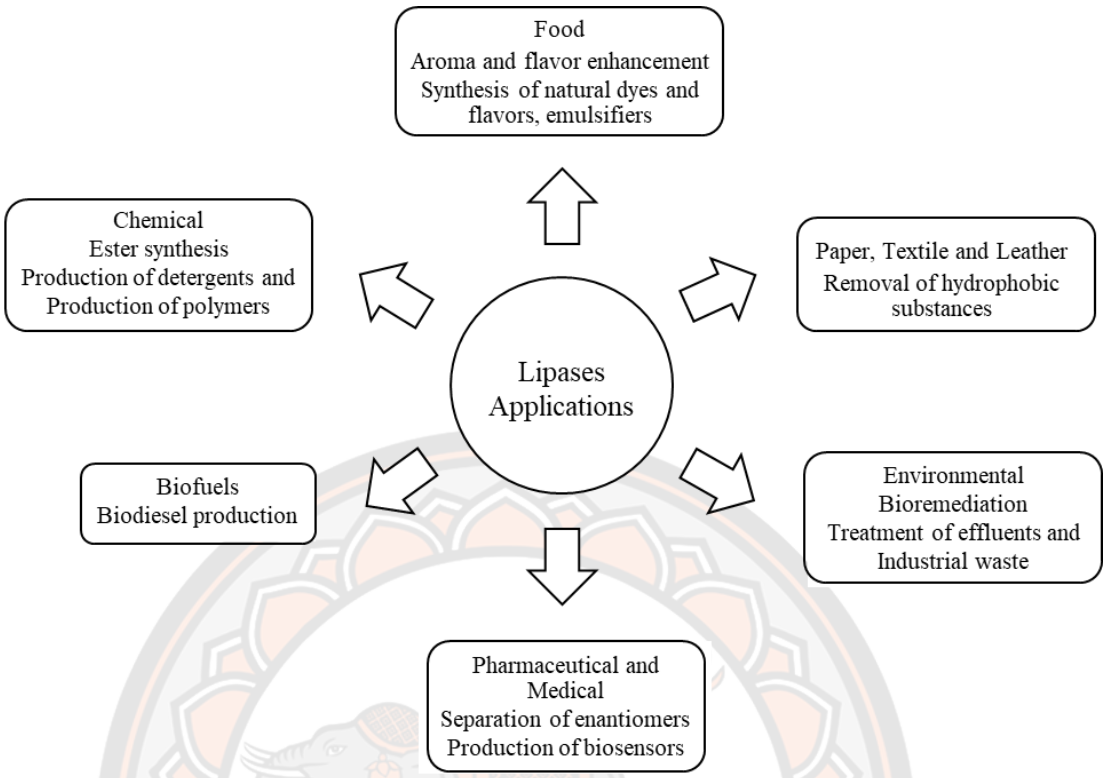


Figure 1 Some applications of lipases in the industrial sectors (3)

We are interested in utilizing lipases due to their peculiar characteristics such as the ability to utilize a wide range of substrates, high activity, stability in organic solvents, and effective reaction catalysis. In the process of this work, we are interested in using lipases to catalyze transformations of natural and synthetic materials, which include capsaicin and dihydrocapsaicin hydrolysis, coconut oil modification, and PET decomposition.

1.4.1 Enzymatic modification of coconut oil by acidolysis or interesterification with MCFAs or their alkyl esters

Coconut oil is relatively rich, in comparison to other vegetable oils, in short chain or medium chain fatty acids (6-12 carbons), which are readily digested and absorbed, transported to the liver and easily utilized as energy, and less likely to accumulate as body fat. Previous studies indicate that the beneficial properties of medium chain fatty acids (MCFAs) are related to their ability to exert protection against cardiovascular disease, autoimmune disorders, diabetes, arthritis, support of healthy growth and development, boosting cellular energy, and improve cognitive function for

those suffering from memory disorders. Another interesting effect of MCFAs is on weight control, lipid metabolism, and reducing cholesterol. Many previous reports have shown the potential of MCFAs for weight reduction and reduction of the risk of disease. However, one of the potential issues with considering coconut oil as a source of MCFAs is the somewhat contradicting evidence on the properties of its major fatty acid, dodecanoic acid (lauric acid), which shows properties that are on the border between those of medium chain and long chain fatty acids.

These reasons led us to hypothesize that the modification of the fatty acid composition of coconut oil is one of the ways to achieve the goal of obtaining a material richer in MCFAs, which could have properties more closely resembling those of medium chain triglycerides. The enzyme catalyzed reactions acidolysis and interesterification in which fatty acid residues of triglycerides are exchanged with added fatty acids or their alkyl esters, respectively, have been identified as the potential approaches to achieve this goal as they can be carried out at relatively mild conditions. Furthermore, it is hypothesized that this approach might also be simpler than the traditional synthesis of MCT oils from fatty acids (octanoic and decanoic acid and glycerol) where active removal of water from the reaction mixture is required to achieve good yield of triglycerides. Finally, it is expected that such product could have similar biological properties to an MCT oil given the 1,3-selectivity of the pancreatic lipase. Therefore, the aim of this part of the project will be an investigation of augmenting the content of MCFAs in coconut oil using enzymatically catalyzed acidolysis and interesterification reactions Figure 2. As part of this work, the search for optimal reaction conditions will be performed in a systematic approach using statistical methods such as response surface methodology (RSM).

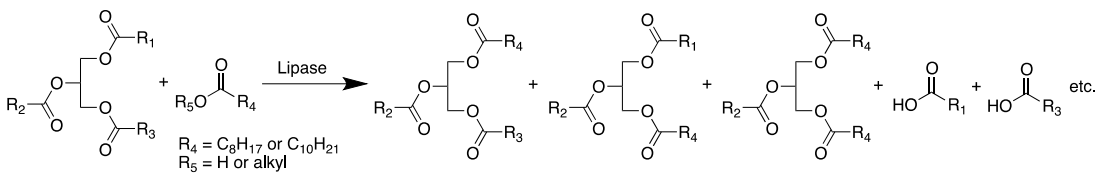


Figure 2 Lipases catalysed acidolysis/interesterification of coconut oil with capric and caprylic acids

As mentioned above, the acidolysis and interesterification reactions to be used for modification of coconut oil require medium chain fatty acids (octanoic, decanoic) or their alkyl esters as reagents, respectively. These can also be obtained from coconut oil, or waste products of coconut oil production (e.g., coconut oil fatty acid distillate). The methods of isolating these key starting materials will also be investigated as part of this work. Furthermore, both the isolation of the octanoic and decanoic acids as well as the isolation of the products of the enzymatic modification of coconut oil will produce significant amounts of interesting side products. These will be mainly the longer chain fatty acids, their alkyl esters, or mono and diglycerides, in the case of sourcing of octanoic and decanoic acids from coconut oil. Isolation of these potentially interesting materials will be investigated as well.

As mentioned above, in addition to using octanoic and decanoic acids and their esters as the reagents for coconut oil modification, the E-8-methyl-6-nonenoic acid obtained from capsaicin hydrolysis will be investigated as an alternative, unsaturated, medium chain fatty acid as well.

1.4.2 Enzymatic hydrolysis of capsaicin and utilization of the fatty acid product for triglyceride modification

Capsaicin (8-methyl-N-vanillyl-trans-6-nonenamide) is the major pungent compound found in chili peppers. Notably it is an agonist of the TRPV1 neuronal receptor employed in nociception. The structure of capsaicin consists of three regions: head group (A, aromatic region), and an aliphatic tail (C, hydrophobic side chain region) linked by a central amide bond (B, amide bond region). All these regions are contributing to the pharmacological activities of capsaicin. Literature reports indicate that the properties of capsaicin can be useful in treatment of pathologies and problems such as cancer, cardiovascular problems, gastrointestinal problems, obesity etc. While capsaicin has been shown to possess beneficial properties it also has its drawbacks as it causes burning and irritation. Furthermore, previous studies reported that the biological effects of capsaicin (e.g., effects on the energy metabolism) can be elicited by the products of its biodegradation. It is likely that one of the products of capsaicin biodegradation is E-8-methyl-6-nonenoic acid, which is a medium chain fatty acid (MCFA). MCFAs are known to possess effects on energy metabolism and have other

biological effects. For example, they are believed to have an effect on lipid handling and thus could exhibit potential for weight control applications. One of the potential approaches to obtaining this medium chain fatty acid is enzymatic hydrolysis of capsaicin.

We have therefore hypothesized that it would be of interest to investigate the possibility to develop a practical way to obtaining the medium chain fatty acid form enzymatic hydrolysis of capsaicin. The ability to do so, including the possibility of recycling the enzyme catalyst, has been established in our previous work. In the current project we aim to investigate this reaction further to improve its practicality and potential. For example, the aim of this work will be the shortening of the reaction time with the aim of obtaining faster reactor turnover. Furthermore, we also aim to utilize the fatty acid obtained from capsaicin hydrolysis in the second part of this work, which will focus on the modification of coconut oil with the aim of making it more MCT like. The E-8-methyl-6-nonenoic acid obtained from capsaicin hydrolysis will be used as an alternative unsaturated fatty acid to the octanoic and decanoic acids, which will also be utilized in this work.

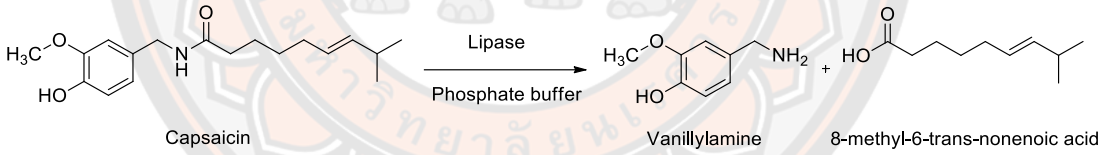


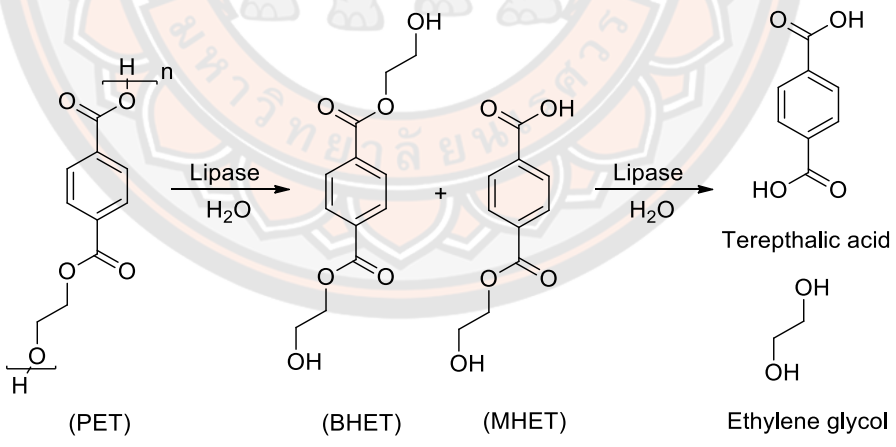
Figure 3 Enzymatic hydrolysis of capsaicin

1.4.3 Enzymatic degradation of poly(ethylene terephthalate) (PET)

Poly(ethylene terephthalate) (PET) is the co-polyester of ethylene glycol (EG) and terephthalic acid (TPA). The characteristics of PET are high strength and resilience, resistance to abrasion, and stretching. These properties make PET an important commercial polymer, which is having applications ranging from packaging, fabrics, films, etc. Furthermore, PET is widely used in plastic bottles (e.g., water bottle, soft drink containers). The amount of PET plastics made, utilized, and discarded is increasing day by day. Therefore, dealing with PET has become a significant issue. Previous reports indicate that some enzymes (lipases and cutinases) or microorganisms

are capable of degrading PET to its component monomers, which could potentially be recycled. Despite these promising results, full recycling of post-consumer PET remains a challenge. Therefore, in this part of the project we propose to combine photooxidative and enzymatic methods of PET degradation. The hypothesis of this combined approach is that the photooxidative degradation step carried out with the help of a photocatalyst, would be used to partially degrade the structural integrity of the PET material with the aim of making it more readily susceptible to enzymatic degradation. Specifically, we aim to utilize photoactive iridium complexes developed in our group in other research projects, and known to promote the formation of singlet oxygen, as the photocatalyst to be used.

In this part, we have hypothesized that it interests in degradation or recycling of poly(ethylene terephthalate) (PET) from water bottles using enzymes as catalysts. Which the optimal conditions will be key aspects that will be investigated. It should also be noted that the aim of this work is not only to effectively degrade PET but also to be able to recover the released monomers as potentially interesting starting materials.



PET: Polyethylene terepthalate
BHET: Bis(2-Hydroxyethyl) terephthalate
MHET: monomeric mono-2-hydroxyethyl terephthalate

Figure 4 Enzymatic poly(ethylene terephthalate) (PET) degradation

CHAPTER II

LITERATURE REVIEW

2.1 Biocatalysis

Biocatalysis refers to the use of biological systems to speed up (or catalyze) chemical reactions. The biological systems can be enzymes (Microbial cells or isolated enzymes), catalytic antibodies, non-enzymatic proteins (receptors/ion channels), or biopolymers (e.g. RNA). Chemical industries need to consider environmental and economic impacts when developing processes. In this sense, catalysis is significant for chemical reactions as it speeds them up and lowers their energy input requirements. Biocatalysis plays essential parts in both green chemistry and sustainable technology as it is less wasteful and environmentally and economically appealing in comparison to conventional chemical synthesis. Furthermore, biocatalysis processes are more environmentally friendly as reactions performed with biocatalysts can be carried out under mild reaction conditions in water with high catalytic activity and selectivity (4).

The key feature of biocatalysts is the use of special types of catalysts called biocatalysts. Biocatalysts are tools for synthesis in a wide range of applications. Currently, biocatalysts are being widely utilized in the chemical industries, food processing, pharmaceutical, detergent, cosmetic ingredient, textile, biofuels, and pulp and paper industries (5).

Biocatalysis has important advantageous features for chemical industry since enzymes can be used as tools for chemical synthesis. Enzymes are biocatalysts which exhibit high efficiency and have many properties that make them very interesting. For example, they are the most efficient catalysts in nature and can be active under very mild conditions (at low temperature, in aqueous media). Furthermore, enzymes are environmentally friendly and non-toxic. The use of enzymes in chemical processes can be cost effective especially if they can be reused again. Moreover, improved yields, higher quality products without contamination are other benefits of biocatalysis. However, limitations exist for the application enzyme catalysis in chemical processes. For example, there are limited quantities of enzymes available for general use and

enzymes show poor stability at high temperatures. However, these problems can be addressed by advances in modern biotechnology, such as recombinant DNA technology, which makes production of sufficient quantities of enzymes more feasible. It has also resulted in the reduction of enzyme prices.

Currently, enzymes can be used in a wide variety of industrial applications such as for the preparation of pharmaceuticals. The example below shows enzyme use for the synthesis of levetiracetam starting from a racemic 2-haloester, which was reported in 2018 by Zhejiang Changming Pharmaceutical Co., Ltd.. Their chemoenzymatic synthesis of the anti-epileptic drug levetiracetam (Keppra®, Elepsia®) starts from racemic methyl 2-bromo(chloro) butanoate. This material is resolved using the enzyme Novozym 435 (range of buffer pH during 6.5-7.5, temperature from 30°C to 35°C, reaction time ranging from 24 to 48 h), producing the (S)-acid and the desired ester (R)-2. This intermediate is then converted into levetiracetam via transformation of the (R)-ester into the α-aminoamide (S)-3 and a further cyclization with 4-chlorobutanoyl chloride, as shown in Figure 5 (6).

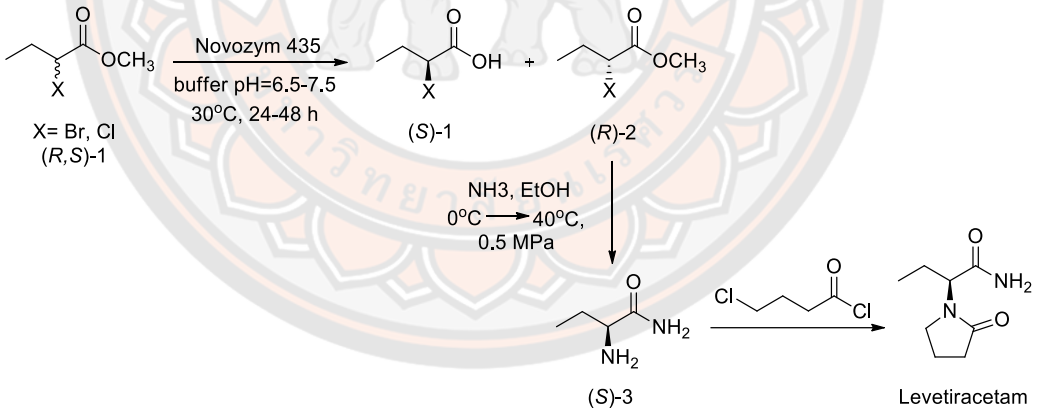


Figure 5 Chemoenzymatic preparation of levetiracetam starting from a racemic 2-haloester (7)

Another application of using enzymes for the synthesis of drug precursors can be found in a patent filed by Tiantai Yisheng Biochemical Technology Co., Ltd., (granted in 2019) for the synthesis of valsartan, which is a drug used in the treatment of hypertension, diabetic nephropathy, and congestive heart failure. Valsartan is

synthesized using an enantioselective hydrolysis of racemic esters and catalyzed by lipases shown in Figure 6 (6).

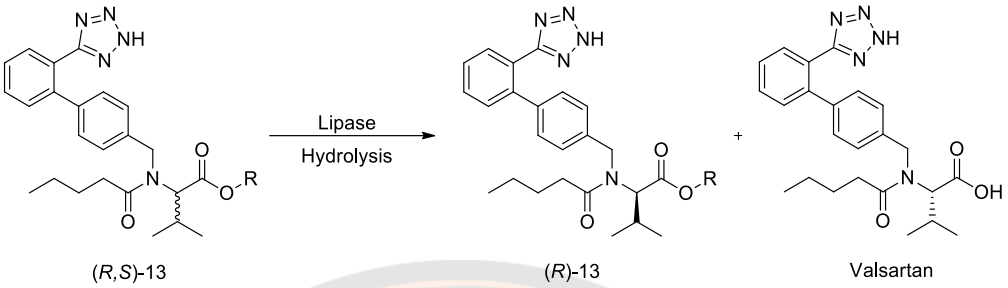


Figure 6 Enzymatic resolution of racemic esters of valsartan (8)

Recycling and recovery of enzymes in catalysis is of key importance for applications in chemical industry as it helps to reduce production costs. This can be achieved by enzyme immobilization. Immobilization can also improve the stability and recyclability of the biocatalyst. Furthermore, immobilized enzymes can perform chemical transformations in aqueous as well as organic solutions. An immobilized enzyme is an enzyme attached to solid support, which is an inert and insoluble material. This can increase the stability of enzyme for resistance to changes in conditions such as temperature, pH, or solvent. It also lets the enzyme to be easily separated from the products so that it may be used again. Thus, enzyme immobilization is an efficient process and is widely used in the chemical industry (9).

An example of application of immobilized enzyme for the catalysis of chemical reaction is shown in Figure 7. In Figure 7(a), synthesis of prodrug of Polydatin (36) (a pharmacological compound) using immobilized lipase from C. *antarctica* (Novozym 435) in 2-MeTHF and other organic solvents. The prodrug (36) shows high yield ranging 97%-100%. In Figure 7(b), Synthesis of C-6′-lauroyl monoester using geniposide with different immobilized enzyme (yield up to 78%) (6).

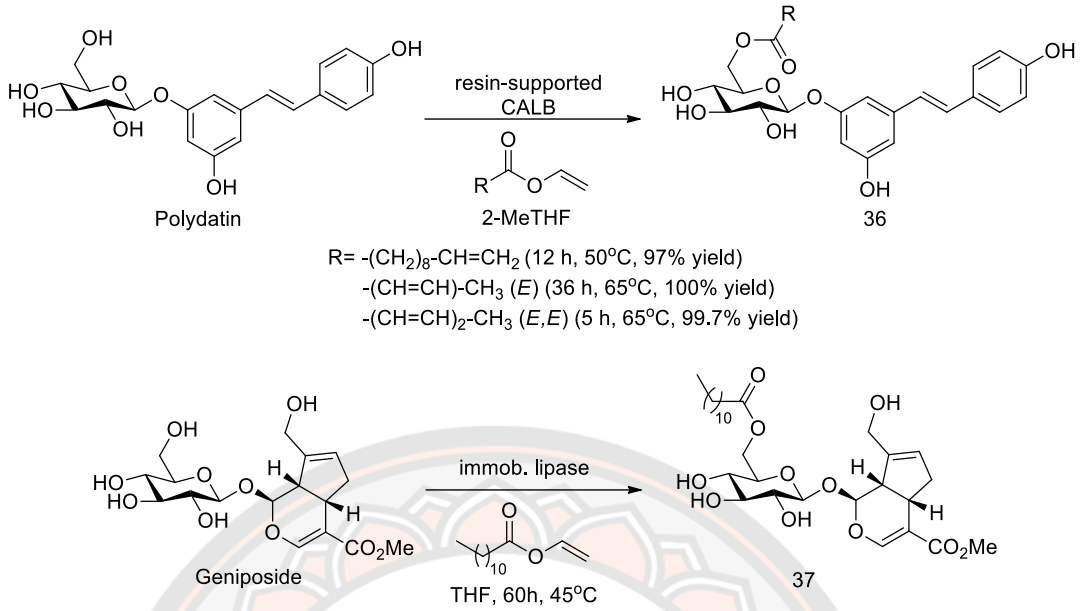


Figure 7 (a) Cont. (b) Regioselective monoacylation of glycosides

2.2 Lipases

Lipases (E.C. 3.1.1.3) are enzymes classified as a hydrolase group that can break down triglycerides into free fatty acids and glycerol. In addition, lipases can catalyze the synthesis reaction just as efficiently. Lipases are found in living organisms including plants, animals, and microorganisms (bacteria, yeast, or fungi). The properties of lipases depend on their sources with respect to positional specificity, fatty acid specificity, thermostability, pH optimum, etc. (Huang, 1984). Lipases from microorganisms are most interesting because these lipases can be produced in large quantities, with low costs of production, and with desired properties. Lipases have attracted significant amount of scientific attention as they can catalyze esterification, interesterification, and transesterification reactions in nonaqueous media. In addition, the properties of lipases include high stabilities, thermal stabilities, and stability in organic solvents. This versatility makes lipases a good choice for potential applications in many industries such as food, leather, detergent, polymers, food processing, medical and pharmaceutical, pulp and paper, cosmetics and perfumery, and biodiesel. Lipases catalyzed reactions are interesting in comparison to chemical processes due to high specificity, mild reaction conditions (temperature, pH, and pressure), reduced side

reactions, and ability of some enzymes to perform well in organic solvents. Furthermore, enzymatic processes are saving energy consumption and cause less environmental pollution.

1. Hydrolysis of ester

2. Synthesis of ester

3. Transesterification

3.1 Acidolysis

3.2 Alcoholysis

3.3 Interesterification

3.4 Aminolysis

Figure 8 Types of reaction catalyzed by lipases

Lipases catalyzed reactions may be classified in two main categories: Hydrolysis and ester synthesis. Ester synthesis includes esterification, interesterification,

alcoholysis, and acidolysis. The last three reactions are organized into transesterification group (1). But Yamane 1987, classified aminolysis into transesterification group as well, as shown in Figure 8 (10).

Application of lipases

Lipases are high performance biocatalysts. Beside their hydrolytic activity on triglycerides, lipases are capable to catalyze other reactions such as esterification, interesterification, acidolysis, alcoholysis, and aminolysis (Figure 8). These properties of some lipases led to their applications in different industrial fields such as dairy, pharmaceutical, detergent, leather, cosmetic, and paper. Houde et al. (2003) reported commercially available lipases and their industrial application on Table 1 (11).

Table 1 Commercially available lipases and their industrial applications (Houde , 2003)

Industry	Application	Trade name
Dairy	EMC production (cheddar-type flavors)	Lipase A "Amano" 6 (*Aspergillus niger*) Lipase M "Amano" 10 (*Mucor javanicus*) Lipase F-AP15 (*Rhizopus oryzae*) Lipase AY "Amano" 30 (*Candida rugosa*) Lipase G "Amano" 50 (*Penicillium camembertii*) Piccnate *(Mucor miehei)* Lipomod™ 187P-L187P (fungal lipases) Lipomod™ 224P-L224P (porcine pancreas)
Oil and fat	Cheese-flavor enhancement	Palatase® (*Rhizomucor miehei*) Lipase A "Amano" 6 (*Aspergillus niger*) Lipase M "Amano" 10 (*Mucor javanicus*) Lipase G "Amano" 50 (*Penicillium camembertii*) Lipase F-Ap15 (*Rhizopus oryzae*) Lipase AY "Amano" 30 (*Candida rugosa*) Newlase F (*Rhizopus niveus)*

Table 1 (Cont.)

Pharmaceutical	Interesterification of vegetable oil	Lipozyme® TL IM
	Pharmaceutical ingredient	Lipase MY (*Candida cylindracea* [rugosa])
	Synthesis of chiral compounds	Lipase ALC, Lipase ALG, Lipase PLC, Lipase PLG, Meito Sangyo, Lipase QLC, Lipase QLG (*Alcaligenes* sp.)
Detergent		Lipolase®, Lipolase® Ultra, Lipo Prime™, Lipex® (*Thermomyces lanuginosus*)
Baking	Improvement of dough texture and color	Lipomod™ 627P-L627P (*Rhizopus oryzae*)
	Emulsifier	Lipopan® F
Leather	Liming	NovoLime® (with protease)
	Fat dispersion	Greasex®, NovoCor® AD
Cosmetics	Production of isopropyl myristate	Novozym® 435 (*Candida antarctica B*)
Paper	Control of pitch	Resinase® (*Candida rugosa*)
Noodles/pasta	Improvement of quality of noodles	Noopazyme®
Miscellaneous	Dietary supplement	Lipase L036P-L036P (*Rhizopus oryzae*), Lipase F-DS (*Rhizopus oryzae*)
	Various uses	Lypolyve AN (*Aspergillus niger*), Lypolyve CC (*Candida cylindracea*)
	Delipidation of egg white	Lipomod™ 34P-L034P (*Candida cylindracea*)

- **Fat and oleochemical industry**

The current trend in the oleochemical industry is the use of lipases to catalyze the hydrolysis, esterification, interesterification, and transesterification reactions of oils and fats as an alternative to chemical processes due to energy saving and specificity of the reactions.

Fatty acids and glycerol hydrolyzed from lipids, have important industrial applications. For instance, fatty acids are used in soap production. Lipases used for this purpose such as *Candida cylindracea* lipase (Miyoshi Oil & Fat Co., Japan), *Candida rogusa* and *Pseudomonas* fluorescens (12).

Mucor miehei (IM 20) and *Candida antarctica* (SP 382) lipases were used for esterification of free fatty acids in the absence of organic solvent or transesterification of fatty acid methyl esters in hexane with isopropylidene glycerols (13).

- **Textile**

Enzymes are used in the textile industry for the removal of large lubricants to provide a fabric with greater and improved levelness in dyeing and reduced frequency of streaks and cracks in the denim. Currently, the use of enzymes in textile processes has spread world- wide since they are environmentally friendly and non- toxic. Therefore, they are not harmful to the people and environment. The use of enzymes also leads to improved product quality and speeding up of the process. Other applications, which use lipases, in the textile processing are improving fading of denim and non-denim fabrics, bio-scouring, bio-polishing, wool finishing, peroxide removal, decolorizing dyestuff, and others. The main enzymes applied in the textile industry are hydrolases (amylases, cellulases, proteases, pectinases and lipases/ esterases) and oxidoreductases. Amylases are used to remove starch- based sizes from fabrics after weaving. Cellulases are used to remove fibrils and fuzz fibres. Esterases are used for improving their hydrophilicity and aiding further finishing. Oxidoreductases are used in various textile-processing steps for instance, catalases are used to remove H_2O_2 after bleaching, reducing water consumption etc. (14).

- **Detergent**

The most commercially important field of application of hydrolytic lipases are detergents, where they contribute to oil and fat stain and traces removal. The main types of detergent that use lipases are household and industrial laundry and in household dishwashers. Lipase enzymes can reduce the environmental load of detergent products as the chemicals used in conventional detergents are reduced and they are biodegradable, non- toxic and leave no harmful residues (15). Lipases are used in commercial detergents such as Lipolase® and Lipex® both examples of lipases sold to the detergent industry.

In 1988, Lipolase® was the first commercial lipase developed for the detergent industry. It is obtained from the fungus *T. lanuginosus* and was expressed in *A. oryzae*. Lipolase has an optimal pH of 10.5–11.0 and a temperature optimum at 40°C; is stable in proteolytic wash solutions; shows oxidation stability; and is stable toward several other detergent ingredients including surfactants; therefore, it has a potential for application in the detergent industry (11)

- **Pulp and paper**

In the paper and pulp process, lipases can be used to remove undesirable triglycerides. Wood contains hydrophobic components such as triglycerides and waxes. The most important application of lipases in paper and pulp processing is to increase the bleachability pre bleaching of kraft pulps. Xylanase enzymes have been found to be most effective for that purpose. The use enzymes for bleaching of kraft pulps can reduce the consumption of chlorine chemicals in the bleaching process and reduce releasing wastewater. Other applications such as removal of shives and slime, retting of flax fibers, and selective removal of xylanase etc. (16).

- **Polymers**

Lipases play an important role in the polymerization of a range of monomers. Lipases can catalyze the polymerization and can be used to assist the production of polymers, such as polyesters, PLA (polylactide), PCL (polycaprolactone), PEG (polyethylene glycol) and etc. Moreover, lipases are an alternative to chemically catalyzed polymerizations providing regio-, chemo-, and enantioselectivity that allows for the direct preparation of functional materials. Examples of lipases as catalysts

utilized in polymerizations are as follows. *Candida antarctica* lipase *B* (Novozyme 435) was used as the catalyst for the synthesis of quantitative end-functionalized poly(ethylene glycol)PEG (17). Amano lipase M was used to prepare thymine functionalized PEG by Michael addition of thymine to PEG diacrylate (18). PAA (Poly acrylic acid) backbone was modified through esterification with polyols in bulk using CALB as catalyst (19). Lipase catalysis has become an increasingly attractive alternative to conventional chemical catalysis as it has several advantages including high selectivity, ability to operate under mild conditions, catalyst recyclability, and biocompatibility. Therefore, lipases were selected for the synthesis of a variety of polymers such as polyesters, polyaromatics, polysaccharides, and various modified polymers. Moreover, using lipases as catalysts can lead to designation of the product as a green polymer (20).

- **Food processing**

In food processing, lipases have also been used for modification of fats and oils. Lipase mediated modifications are interesting in oil industry for tailoring structured lipids because lipases are regiospecific and fatty acid specific and the reactions can be carried out at moderate reaction conditions. Lipases were utilized in flavour development and improving quality (e.g. flavour and fragrance compounds) in the processing of other foods such as meat products, baked foods, bakery products, confectionery, and cheese flavouring, cocoa butter processing and others. Lipases can synthesize nutritionally important structured triacylglycerols like cocoa butter substitutes and can be used for converting cheap oils into nutritionally rich oils, low calorie tri-acylgylcerols PUFA, and oleic acid-rich oils (21).

For example, one application which uses lipases in the food processing is dairy industry. Lipases are used for hydrolysis of milk fat, used to enhance the flavor of cheeses, and the lipolysis of cream. In the baking industries, lipases are used to degrade wheat lipids to produce emulsifying lipids in situ and used to improve the flavor of bakery products by releasing short-chain fatty acids through esterification along with flavor enhancement. In beverage industries, lipases used to improve the aroma in alcoholic beverages by hydrolysis of the lipids in barley (22).

- **Medical and pharmaceutical sectors**

In the pharmaceutical industry, lipases have numerous advantages including enantioselectivity, regioselectivity, specificity, and mild reaction conditions that avoid unwanted processes such as isomerization, racemization, epimerization, and rearrangement reactions and reuse ability. Lipases have a high level of specificity and exhibit an ability to be used to produce active pharmaceutical compounds. For example, enantioselective or regioselective enzymes are used as an alternative technology for the preparation of alcohol, amine, amide, carboxylic acid, and ester intermediates in the synthesis of pharmaceuticals (23). These molecules are used in antiinflammatory drugs(ibuprofen, naproxen), anticancer drugs (Taxol®, spergualin), an antiviral drug (lobucavir), an antihypertensive drug (captopril), anticholesterol drugs (squalene synthase inhibitor), an anti-Alzheimer disease drug ([S]-2-pentanol), and vitamin A (11).

- **Cosmetics**

In cosmetic industry, lipases are used to catalyze esterification for the synthesis of several speciality esters. Application of lipases for the development in cosmetic industries such as skincare, lipases were used to catalyze the preparation of water-soluble retinol derivatives, as retinoids are useful agents in the treatment of photoaging, skin cancer, and numerous skin disorders. In general, retinoids (vitamin A and derivatives) are fat-soluble but water insoluble (24). Lipases have also been used as a component of topical antiobese creams and used in hair waving preparations. Also, a lipase (Immobilised *Rhizomucor meihei*) was used to catalyze the solvent free esterification for the production of isopropyl myristate, isopropyl palmitate, and 2-ethylhexylpalmitate to be used as an emollient in personal care products such as skin and sun-tan creams, bath oils etc. (Unichem International company) (15).

- **Biodiesel**

Currently, biodiesel is an interesting alternative fuel due to the limited amounts of fossil fuels and environmental consequences of exhaust gases from diesel engines. Biodiesel fuel from vegetable oils does not produce sulphur oxide and minimizes the soot particulates and emits less carbon monoxide in comparison with petroleum based diesel (25). The environmental advantages of biodiesel fuel have attracted attention to it as a potential renewable source and, which can be expected to act as a substitute for

petroleum-based diesel fuel. Lipases have become one of the most important groups of catalysts for the transesterification of triglycerides with alcohol in the formation of alkyl esters commercially known as biodiesel (26).

Lipases used in biodiesel fabrication, such as Novozym 435 and Lipozyme TL IM, can be used as catalysts to prepare fatty acid ethyl esters from castor oil (26), Immobilized *Pseudomonas cepacia* lipase was used to catalyse transesterification of soybean oil for biodiesel production oil in methanol and ethanol (25), PS30 lipase used to catalysed production of biodiesel fuel from two Nigerian lauric oils, palm kernel oil and coconut oil (28).

Lipases are commonly used in food processing, leather and textile manufacture, detergents and degreasing formulations, paper manufacture, synthesis of fine chemicals, and production of pharmaceuticals, production of polymer, cosmetics, and biodiesel.

Herein, we are interested in studying applications of lipases for hydrolysis capsaicin and dihydrocapsaicin to yield the medium chain 8-methyl-6-nonenoicacid, 8-methylnonanoic acid, and vanillyl amine, to catalyze acidolysis or interesterification of coconut oil with MCFAs or fatty acid alkyl ester to medium chain fatty acid (MFA), and to decompose Poly(ethylene terephthalate) (PET).

2.3 Capsaicin

2.3.1 Capsaicin: general information

Capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide) is a natural vanilloid isolated from various chili peppers. Capsaicin was discovered in the 19th century, and the therapeutic roles of capsaicin have been well characterized. Capsaicin is causing spicy flavor and pungency and has been used for a long time in foods and drugs. General characteristics of capsaicin is a spicy, pungent, hydrophobic, colorless, and odorless white crystalline powder. Apart from pungency, capsaicin is reported to have benefits in increasing metabolism by burning fats, relieving topical pain, and reducing inflammation of the muscles and joints. Furthermore, capsaicin also exhibits pharmacological activities such as an analgesic, anti-obesity, anti-pruritic, anti-inflammatory, anti-apoptotic, anti-cancer, antibacterial activity, reducing insulin spikes in diabetes, healing of gastric ulcers, antioxidant, and neuro-protective functions.

2.3.2 Capsaicin and the TRPV1 receptor

Capsaicin, as a member of the vanilloid family, binds to a receptor called the vanilloid receptor subtype 1 (TRPV1). TRPV1 is an ion channel-type receptor. TRPV1, which can also be stimulated with heat and physical abrasion, permits cations to pass through the cell membrane and into the cell when activated. The resulting "depolarization" of the neuron stimulates it to signal the brain (Figure 9). By binding to the TRPV1 receptor, capsaicin molecule produces the same effect that excessive heat or abrasive damage would cause, explaining why the spiciness of capsaicin is described as a warming, burning, stinging, or itching sensations. The action of capsaicin at the TRPV1 receptor is the source of both its pungent and analgesic properties. Analgesics are drugs that can relieve pain such as aspirin ibuprofen, or diclofenac (29).

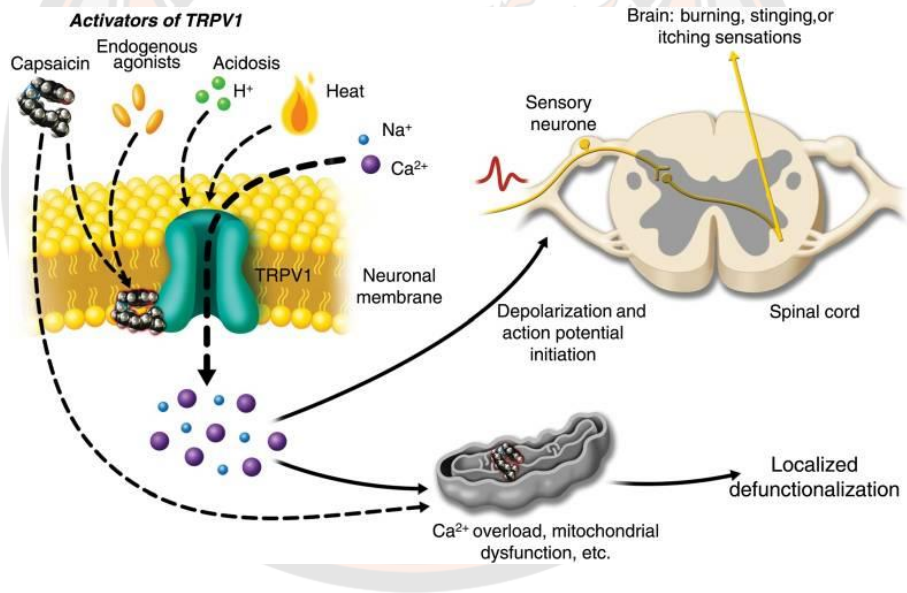


Figure 9 Activation of TRPV1 by capsaicin results in sensory neuronal depolarization, and can induce local sensitization to activation by heat, acidosis, and endogenous agonists (30)

For example, reports from 2009 and 2010 describe the use of capsaicin and its analogues in topical creams and patches to treat chronic pain syndromes such as post-herpetic neuralgia, musculoskeletal pain, diabetic neuropathy, osteoarthritis, and rheumatoid arthritis. These products have also been applied to treat pain from rashes, psoriasis, mastectomy, and bladder disorders. Furthermore, it was found that capsaicin

can reduce adipose tissue in rats by enhancing energy and lipid metabolism. Capsaicin reduced adipose tissue possibly by increasing catecholamine secretion from the adrenal medulla in response to activation of the sympathetic nervous system. These results provided supporting evidence of capsaicin's possible utilization in obesity treatment.

2.3.3 Structure of capsaicin

Capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide) is a natural vanilloid identified from various chili peppers. The structure of capsaicin consists of three regions: head group (A, aromatic region), and an aliphatic tail (C, hydrophobic side chain region) linked by a central amide bond (B, amide bond region) as depicted in Figure 10. This contribution of these regions is accountable for the pharmacological activities of capsaicin (31).

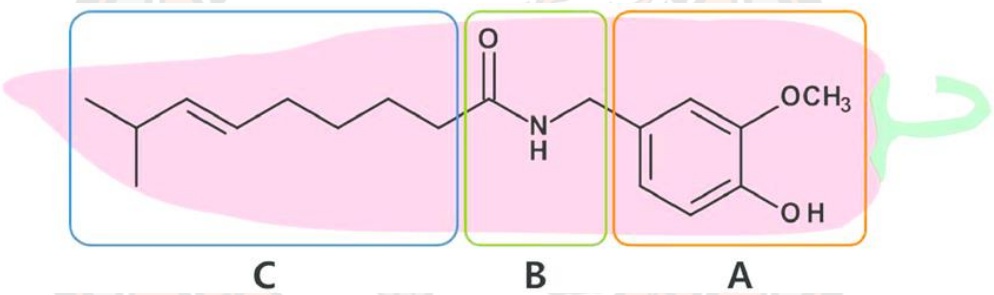


Figure 10 Chemical structure of capsaicin, the primary ingredient of chili pepper, and its three important regions, namely A (aromatic head), B (amide linkage) and C (hydrophobic tail)

In terms of the activity at the TRPV1 receptor, capsaicin molecular structure and capsaicin binding at the TRPV1 receptor as well as effects of their modifications on this activity have been extensively studied. However, these are beyond the scope of interest of this work and can be review elsewhere.

Herein, we are interested to use lipases for catalytic hydrolysis of capsaicin. The ability of the enzyme to perform this reaction, the effectiveness of isolation of the desired product (*E*-8-methyl-6-nonenoic acid), and ability to reuse the enzyme will be key aspects that will be investigated.

Example of reports relating to using enzymes for hydrolysis capsaicin into medium chain 8-methyl-6-nonenoic acid and vanillyl amine.

In 2000, the first report concerning amide hydrolysis by a lipase has been published. Duarte and coworkers reported the hydrolysis of capsaicin using lipase B from *Candida antarctica* into vanillyl amine and 8-methyl-6-trans-nonenoic acid (Figure 11). The authors used various enzymes and three media for the hydrolysis of the amide bond. It was found that lipase B from *Candida antarctica* (NOVOZYM435) was capable to hydrolyze the amide bond resulting in conversion of 70% after 72h at 70^{o}C (32).

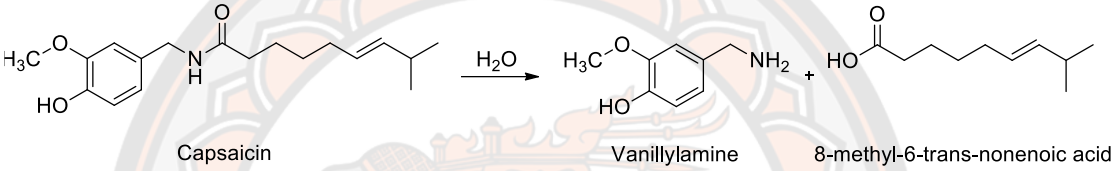
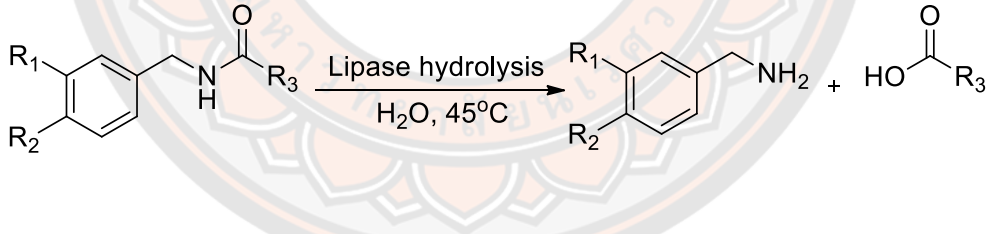


Figure 11 Enzyme hydrolysis of capsaicin



1: R_1 = -OMe R_2 = -OH
2: R_1 = -OMe R_2 = -H
3: R_1 = -H R_2 = -H

A: R_3 = $-CH_2CH_2CH_3$
B: R_3 = $-CH_2(CH_2)_3CH_3$
C: R_3 = $-CH_2(CH_2)_5CH_3$
D: R_3 = $-CH_2(CH_2)_7CH_3$
E: R_3 = $-CH_2(CH_2)_9CH_3$
F: R_3 = $-CH_2(CH_2)_{11}CH_3$
G: R_3 = $-CH_2(CH_2)_{13}CH_3$
H: R_3 = $-CH_2(CH_2)_3CH{=}CHCH_2(CH_3)_2$

Figure 12 Nature of amine substituents and fatty acid residues considered for hydrolysis by CAL-B

In 2006, Torres-Gavilan reported that the amide hydrolysis using lipase B from *Candida antarctica*. They used 15 amides with different linear saturated acyl

residues and different substituents in the aromatic ring. The reactions were performed in water at 45ºC (Figure 12). The authors reported that the amide hydrolysis using the enzyme lipase B from *Candida antarctica* strongly depended on the structure of the substrate (33).

In 2017, Kanprakobkit and Kielar developed a practical enzymatic hydrolysis of capsaicin to the medium chain fatty acid using lipase B from *Candida antarctica* immobilized on acrylic resin. The optimization of the enzymatic reaction was performed focusing on the effects of temperature, pH, and buffer concentration. The reaction was investigated by ^{1}H-NMR and HPLC. The optimal conditions identified during this work were 45ºC, pH 7, and 80 mM phosphate buffer concentration. The results obtained in the content of vanillyl amine in the reaction mixture after 72 h reaches 77% and the desired product was successfully isolated in 54.8% yield (34).

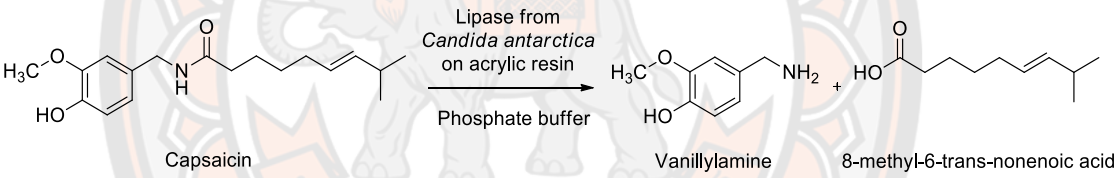


Figure 13 Enzymatic hydrolysis of capsaicin using lipase B from *Candida antarctica* immobilized on acrylic resin

2.4 Coconut oil

Coconut oil is the oil extracted from meat or kernel of a coconut. This oil is extracted through a variety of methods, which result in different types of coconut oil. In the traditional approach, coconut oil is produced from copra (i.e., the dried kernel of the coconut) through a process of refining, bleaching, and deodorizing. To make this process healthier, the production process was modified to result in coconut oil referred to as "virgin" coconut oil, which is made through a simpler process and has not been bleached, deodorized, or refined. Virgin coconut oil is instead made via a ‘wet process’, either being extracted from coconut milk or from fresh kernel which is not subjected to drying or chemical refining (35).

Figure 14 Coconut oil

The coconut oil is rich in short chain or medium chain fatty acids (Table 2) (36), meaning the fatty acids chains are between 6-12 carbons in length. This gives coconut oil a special place among other fats and oils as almost all other natural fats and oils are made predominantly from long-chain fatty acids (37).

Table 2 Fatty acid composition of virgin coconut oil and refined, bleached and deodorized (RBD) coconut oil

Fatty acid	RBD coconut oil	Virgin coconut oil
C6	n.d.-0.70	0.40-0.60
C8	4.60-10.0	5.00-10.00
C10	5.00-8.00	4.50-8.00
C12	45.10-53.20	43.00-53.00
C14	16.80-21.00	16.00-21.00
C16	7.50-10.20	7.50-10.00
C18:0	2.00-4.00	2.00-4.00
C18:1	5.00-10.00	5.00-10.00
C18:2	1.00-2.50	1.00-2.50
C18:3	n.d.-0.20	<0.5

The most common fatty acid in coconut oil is lauric acid (approximately 50%) containing 12 carbon atoms, which some sources consider as a medium chain fatty acid. Unfortunately, there is evidence to suggest its behavior in the body is more like the behavior of longer fatty acids (e.g. myristic, palmitic, or stearic) than that of the shorter ones (e.g. capric and caprylic).

Characteristics of medium-chain fatty acids (MCFAs) is that they are readily digested and absorbed, transported to the liver and easily utilized as energy, and less likely to accumulate as body fat. MCFAs can be quickly absorbed in the small intestine (SI) after the action of enzyme pancreatic lipase. The shorter chain length of the fatty acids results in different metabolism and absorption of MCFAs in the body in comparison to longer-chain fatty acids. This makes them less likely to be stored as fat. As several studies indicate the beneficial properties of MCFAs are related to their ability to exert protection against cardiovascular disease, autoimmune disorders, diabetes, arthritis, arrhythmia, cardiac disease, support of healthy growth and development, boosting cellular energy, and improve cognitive function for those suffering from memory disorders (38) such as Alzheimer's (39).

Another interesting effect of MCFAs is on weight control, lipid metabolism and reducing cholesterol. Ming-Hua Sung and coworkers reported that medium-chain triglycerides (MCTs) can exhibit protective effects on cardiovascular health in T2DM rats fed a high-fat diet, by improving serum lipid profiles and reducing hepatic total cholesterol (40). Many research reports have shown the potential of MCFAs and MCTs for weight reducing and reduce the risk of disease. For this reason, we are interested in enzymatic modification of coconut oil for increased amount beneficial properties of fats rich in MCT or MCFAs.

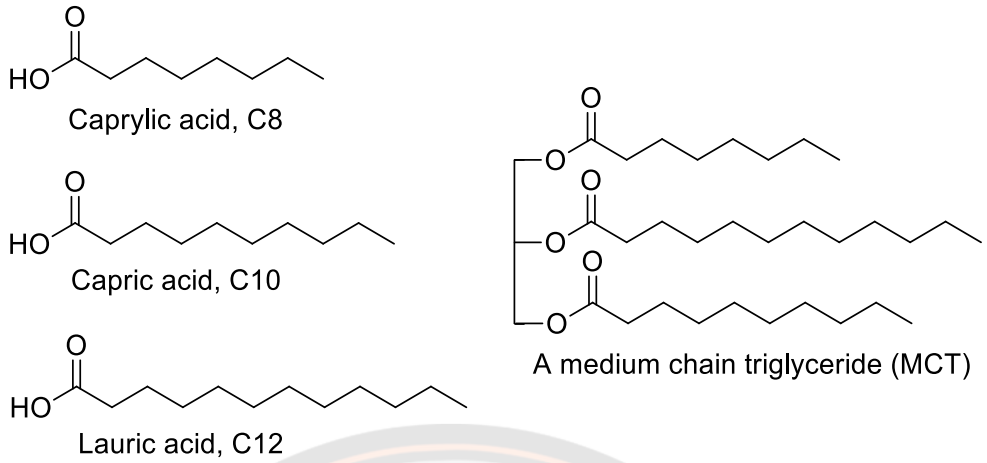


Figure 15 Examples of medium-chain fatty acids (MCFA) and medium-chain triglycerides (MCT) (41)

However, most of edible oils have limited applications as their natural triacylglycerol (TAG) structures cannot provide desirable characteristics to the products. Interesterification can be used to modify the TAG properties, thus expanding their applications.

Enzymatic interesterification (EIE) is increasing in popularity as a high-efficiency and eco-friendly process for lipid modification. The enzymatic process is advantageous because of lipase's high reaction efficiency, specificity, and fewer contamination problems.

2.4.1 Examples of enzymatic modifications of fat molecules

Enzymatic modification of lipids means that the lipid molecules have been enzymatically modified from their natural form. Application of enzymes in lipid modification is among the most established uses of biocatalysts where enzymes are used for the synthesis of structured triacylglycerols, fats, and margarine and for the release of flavoring fatty acids for food applications (42). In addition, lipid modifications are interesting because the resulting product can possess desired nutritional, physicochemical, or textural properties for various applications in the food industry. Therefore, many research activities have been aimed at their commercialization. Enzymatic modification of lipids has a great potential in the future

market because of the specificity of lipases and phospholipases used as the biocatalysts. Recently research reporting enzymatic modification of lipids is of commercial interest such as for the production of medium- and long-chain triacylglycerols, human milk fat substitutes, cocoa butter equivalents, trans-free or low-trans plastic fats (such as margarines and shortenings), low-calorie fats/oils, health-beneficial fatty acid-rich fats/oils, mono- or diacylglycerols, and structurally modified phospholipids (Byung Hee, 2015) (43). Recent studies on the enzymatic synthesis of medium- and long-chain triacylglycerol (MLCT).

In 2011, Nunes and coworkers (2011) synthesized triacylglycerols (TAG) containing a medium-chain fatty acid (M) at positions sn-1,3 and a long-chain fatty acid (L) at sn-2 position, i.e. TAG of MLM type, by acidolysis of virgin olive oil with caprylic (C8:0) or capric (C10:0) acids, catalyzed by 1,3-selective *Rhizopus oryzae* heterologous lipase (rROL) immobilized in Eupergit C and modified sepiolite. The reactions were performed in solvent-free set up or in n-hexane media with oil and free fatty acid. The best results were obtained with the rROL immobilized on Eupergit® C in a solvent-free system. The result of incorporation of these conditions was 21.6 % for caprylic acid and 34.8 % for capric acid. Good selectivity for incorporation to the sn-1 and 3 positions was observed. Furthermore, the efficiency of enzymes was investigated in terms of stability of the biocatalysts. The immobilized enzymes showed high stability for the 4 or 3 first consecutive reuses. This work has shown that immobilized enzymes are good biocatalysts and are interesting for industrial applications (44).

In 2014, Caballero and coworkers synthesized MLM-type structured lipids (SL) from avocado oil catalysed by commercial immobilised lipases (e.g. lipases from R. *miehei* (Lipozyme RM IM) and T. *lanuginosa* (Lipozyme TL IM)). This work was conducted to add value to avocado oil by producing dietary triacylglycerols (TAG) containing medium-chain fatty acids (M) at positions sn-1,3 and long-chain fatty acids (L) at position sn-2. The reaction was performed in solvent-free media with free fatty acid and commercial lipases Lipozyme RM IM and TL IM used as biocatalysts to transform avocado oil into SL product. The best results were obtained with Lipozyme TL IM in a solvent-free system. Under these conditions the degree of maximum incorporation of caprylic acid (CA) into sn-1,3 positions of TAG was 29.2% mol. This

work pointed to the utilization of biocatalysts for providing added value to products of avocado oil (45).

In 2014, Ebenezer and coworkers modified stearidonic acid soybean oil (SDASO) by immobilized *Rhizomucor miehei* lipase (RML) to incorporate caprylic acid (C8:0). In this work, the effect of biocatalyst was studied in terms of effects of reaction temperature on acidolysis and acyl migration. And the optimal level of the reaction was determined by response surface methodology (RSM). The optimal reaction was carried out using substrate molar ratio (C8:0 to SDASO), incubation time, and enzyme loading for SL synthesis by C-RML at 50 °C was determined by response surface methodology to be 6:1, 24 h, and 20 % weight of substrates, respectively. The best result of incorporation under optimal condition was obtained in Celite-immobilized RML (C-RML) maximized caprylic acid (C8:0) 17.0–32.5 mol% and minimized acyl migration. Moreover, the authors reported that the structured lipid contained 17.0–32.5 mol% of caprylic acid (C8:0). When digested, these SL may deliver caprylic acid (C8:0) for quick energy and stearidonic acid (SDA) for heart health making them potentially valuable for medical and nutraceutical applications (46).

Other interesting enzymatic modifications of lipids to improve dietary properties such as synthesis of human milk fat substitutes (HMFSs) have also been reported. Human milk is the best food for infants, providing energy and essential vitamins, polyunsaturated fatty acids, bioactive components, and other types of essential nutrients (47). The main components of human milk are triacylglycerols (TAG). TAG account for 98 % of HMF, which provide more than 50 % of the calories. Xiaoqiang Zou and coworkers (2016) reported that after being ingested by infants, these TAG can be hydrolyzed by gastric lipase (a sn-3 preferential lipase) and pancreatic lipase (a sn-1,3-specific lipase) to sn-2 palmitoyl monoacylglycerols (MAG). The MAG with palmitic acid at the sn-2 position could be directly absorbed by intestinal epithelial cells. Furthermore, HMF also contains some long-chain polyunsaturated fatty acids (LC-PUFA), such as docosahexaenoic acid (DHA, n-3) and arachidonic acid (AA, n-6), which are important for the development of visual and central nervous systems of infants (48). Due to economic reasons and the inability of some mothers to breastfeed their babies, human milk fat substitutes with a similar chemical composition to human milk are needed. Many researchers reported the synthesis of human milk fat

substitutes (HMFS). For example, Xian-Guo Zou and coworkers (2014) synthesized human milk fat substitute (HMFS) enriched in medium-chain fatty acids (MCFAs). Human milk fat was synthesized from *Cinnamomum camphora seed oil* (CCSO) with oleic acid in a solvent-free system through acidolysis catalyzed by lipozyme RM IM, from *Rhizomucor miehei* under the optimal reaction conditions, which were determined using response surface methodology (RSM) based investigation. The results show that the final product human milk fat substitute (HMFS) was prepared with more than 70% of MCFAs at sn-2 position and 78.69% oleic acid at sn-1,3 position. In addition, the authors studied the reusability of lipozyme RM IM in the acidolysis reaction and found that Lipozyme RM IM could be reused up to 9 times without significant loss of activity. This research indicated that the enzyme has an enhanced stability and selectivity to improve potential application in infant formula industry (49). In 2016, Xiaoqiang Zou and coworkers prepared human milk fat substitutes (HMFS) having similarity in (TAG) composition to human milk fat (HMF) by Lipozyme RM IM-catalyzed interesterification of lard blended with selected oils (viz., lard:sunflower oil:canola oil:palm kernel oil:palm oil:algal oil:microbial oil) in a packed bed reactor. The experimentally determined optimal conditions were as follows: residence time, 1.5 h; reaction temperature, 50 °C. The obtained product had high degree of similarity in the fatty acid profile and the similarity of TAG composition was increased from 58.4 (the oil blend) to 72.3 (the final product). This research indicated potential of enzymes as biocatalysts for modification in lipids and fat.

Furthermore, enzymes can catalyze the synthesis of cocoa butter equivalents (CBEs). Cocoa butter equivalents (CBE) have similar TAG composition to cocoa butter but are produced from low-cost plant oils. CBE are used as an alternative to natural cocoa butter during chocolate manufacturing because of its high cost. Example of enzymatic synthesis of cocoa butter equivalents (CBEs) was reported by Mohamed (2012). They prepared cocoa butter equivalent (CBE) by enzymatic acidolysis reaction of substrate consisting of refined palm mid fraction oil and palmitic-stearic fatty acid mixture (50). Kim and coworkers (2014) prepared CBE by lipase-catalyzed transesterification of high-oleic sunflower oil with mixtures of palmitic and stearic acid ethyl esters using commercial immobilized sn-1,3- specific lipases, such as Lipozyme RM IM (51). A further example published by Saeed M. Ghazani and

coworkers (2018) prepared CBE from a mixture of a commercial enzymatically synthesized shea stearin and palm mid fraction, catalyzed by immobilized Lipozyme RM IM. The authors reported that physical properties of the synthesized CBE were also very similar to those of CB (52). These research works have shown that it is possible to synthesize a CBE using commercial enzymes.

In addition, there are several studies on the enzymatic synthesis of low-trans or trans-free plastic fats. Normally, plastic fats (margarines and shortenings) contain high levels of trans fatty acids (TFAs), which are formed during partial hydrogenation of the plant oils that are used as the main ingredients of these products. It is well-known that trans fatty acids (TFAs) formed in foods may increase the risks of obesity, diabetes, cancer, and cardiovascular diseases (53), (54). This has led the food industry to seek alternative processes to reduce or completely remove trans fatty acids (TFAs) in their products, resulting in low-trans or trans-free fat foods with acceptable functional properties. One of the most interesting potential alternatives to obtain plastic fats is lipase catalyzed interesterification of highly-saturated fats with highly-unsaturated plant oils. For example, Pimwalan Ornla-ied and coworkers (2016) prepared trans-free interesterified fats from blends of hard palm stearin (hPS) and rice bran oil (RBO) using immobilized *Mucor miehei* lipase as a catalyst. After the reaction, the trans-free fats from the blends were investigated for their physical properties, crystallization and melting behaviors, and compared with commercial margarine fats. The results found that interesterified blend of 40:60 mixture of hard palm stearin (hPS) and rice bran oil (RBO) exhibited a slip melting point and solid fat contents (SFC) and crystallization and melting characteristics most similar to commercial margarine fats and also had small needle-like β' crystals. This research pointed out that the interesterified 40:60 blend is suitable for use as a trans free margarine fat. Moreover, enzymatic interesterification is an effective method for the modification of physical and chemical properties of fats and oils without creating undesirable TFAs (55).

Yuxi Xu and coworkers (2018) prepared functional shortening with trans-free fats and low calories using coix seed oil (CO), fully hydrogenated palm oil (FHPO) and *Cinnamomum camphora seed oil* (CCSO) by enzymatic interesterification. Trans-free lipid with low calories was synthesized using coix seed oil (CO), fully hydrogenated palm oil (FHPO), and *Cinnamomum camphora seed oil* (CCSO) in mass

ratios of 45:45:10, 50:40:10, and 55:35:10. The reactions were catalyzed by Lipozyme RM IM. After the reaction, physicochemical properties of these products were evaluated by fatty acid composition, triacylglycerol (TAG) composition, melting and crystallization profiles, and solid fat content (SFC). The result of SFC analysis revealed that interesterified fat had SFC ranging from 22.98% to 34.62% at 25 °C with most β' crystal forms, which was beneficial to improve the spreadability in terms of shortening. The studies have shown that plastic fat synthesized from CO, FHPO, and CCSO by enzymatic interesterification might be beneficial for food product (56).

Currently, enzymatic syntheses of monoacylglycerols (MAGs) and diacylglycerols (DAGs) are of interest due to their use as principal food-grade emulsifiers. Byung Hee Kim and Casimir C. Akoh (2015) reported that MAGs can occur as 2 different types of positional isomers: 1-MAGs and 2-MAGs. 2-MAGs, retaining particular FAs at the sn-2 position, can be used as an efficient carrier of the FAs because they are well absorbed through the intestinal wall. DAGs have been used by the food industry as emulsifiers in mixture with MAGs. Several studies have been published detailing the synthesis of 2-monoacylglycerols (2-MAG) through the enzymatic conversion of several different kinds of triacylglycerols (TAG) oils using enzymes as the biocatalysts. For example, Compton and coworkers published a report in 2013 describing the synthesis of soybean oil 2-monoacylglycerol (2-MAG) via ethanolysis of triacylglycerols using Novozym 435-catalysis and purification by conventional liquid–liquid extraction. The aim of this project was to synthesize 2-MAG from ethanolysis of soybean oil and developed a flash chromatography (FC) protocol for the purification of 2-MAG. The authors reported that 2-MAG was purified by flash chromatography (FC) using silica gel as the stationary phase with an acetone/hexane binary gradient. This method resulted in 60 % 2-MAG yield, no residual triacylglycerol, diacylglycerol, or glycerol coproducts (57).

Xiaosan Wang and coworkers (2013) synthesized 2-MAGs via enzymatic transesterification of vinyl palmitate with glycerol catalyzed by Novozym 435 lipase, and enzymatic esterification of 1,2-acetonide glycerol with palmitic acid catalyzed by Novozym 435 lipase. Moreover, this publication proved that Novozym 435 is very effective in catalyzing the transesterification between vinyl palmitate and glycerol. The

enzyme also catalyzed reaction between 1,2-acetonide glycerol and palmitic acid was simpler and safer than the typical method (58).

For DAGs, besides their use in food industry as emulsifiers, DAGs were developed for their physiological benefits, particularly hypotriglyceridemic and anti-obesity effects. In general, DAGs oils can be prepared via direct esterification of glycerol with free FAs using immobilized sn-1,3-specific lipases such as Lipozyme RM IM and Lipozyme TL IM. Several researchers studied the synthesis of DAGs. They prepared the DAGs using glycerolysis from plant oils (59) or by direct esterification of glycerol with free FAs (60), and Lipozyme RM IM and Lipozyme TL IM as catalyzed.

This project is specifically interested in enzymatic modification of coconut oil with MCFAs or fatty acid alkyl ester through acidolysis or interesterification, respectively. The reports relevant to the goals of this project will be discussed below.

In 2002, Rao and coworkers reported modification of coconut oil in hexane with free fatty acids (FFA) using immobilized lipase from *Rhizomucour miehei* (Lipozyme IM60) in an acidolysis reaction to produce n-3 or n-6 polyunsaturated fatty acids (PUFA). The aim of this project was the modification of the fatty acid composition of coconut oil in order for it to contain unsaturated fatty acids (e.g. essential linoleic acid) and investigated the influence of parameters (e.g. incubation time, temperature, and the substrate molar ratio, on the incorporation of n-3 or n-6 FA into coconut oil TAG) by using response surface methodology (RSM) to optimize reaction conditions to obtain statistically acceptable results (61).

In 2005, Nandi and coworkers produced medium chain glycerides (MCGs) containing C8:0 (caprylic acid) and C10:0 (capric acid) fatty acids from coconut and palm kernel fatty acid distillates by a combination of lipase-catalyzed hydrolysis and esterification reactions. For process to produce MCGs, coconut and palm kernel FADs were hydrolyzed by *Candida rugosa* lipase and then subjected to steam distillation under vacuum to get fractions rich in medium chain fatty acids (MCFAs). After that, the fraction from coconut and palm kernel FADs and MCFAs were esterified with *Rhizomucor miehei* (Lipozyme RM IM) lipase to produce MCGs, which is very much important for medicinal and nutritional applications (62).

In 2006, Hamam and Shahidi focused on the synthesis of structured lipids containing medium-chain and omega-3 fatty Acids. The authors have tested ability of

five lipases: lipase from *Candida Antarctica* (Novozyme-435), lipase from *Mucor miehei* (Lipozyme 1M), lipase from *Pseudomonas sp*. (PS-30), lipase from *Aspergillus niger* (AP-12), and lipase from *Candida rugosa* (AY-30) to incorporate omega-3 fatty Acids: eicosapentaenoic acid (EPA, C20:5n-3), docosapentaenoic acid (DPA, C22:5n-3), and docosahexaenoic acid (DHA, C22:6n-3) into high-laurate canola oil (Laurical 35). The optimal reaction conditions were investigated using response surface methodology (RSM). The results of this work indicated that the efficiency of the reaction depended on the combination of the enzyme and the fatty acid being incorporated (Table 3) and need for proper choice of enzyme to achieve optimal outcome (63).

Table 3 Percent incorporation of EPA, DPA, and DHA into high laurate canola oil by various enzymes

Enzyme source	EPA	DPA	DHA
C. antarctica	52.4	28.0	39.8
M. miehei	52.0	40.5	29.6
Pseudomonas sp.	62.5	30.5	42.5
C. rugosa	64.0	21.5	10.8
A. niger	53.9	17.5	7.73

In 2009, Maurelli and coworkers synthesized triacylglycerols containing medium-chain fatty acids and conjugated linoleic acid isomers by enzymatic acidolysis. The aim of this work was the preparation of triglycerides (TAGs) containing medium fatty acid (capric acid) in the sn-1- and sn-3- positions and conjugated linoleic acid isomers (CLA) and investigated the activity of different lipases in acidolysis reactions. The enzymes used to investigate the activity were: lipase from *Rhizomucor miehei* (Lipozyme IM), lipase from *Pseudomonas cepacia* (immobilized on ceramic particles), Lipase from *Candida Antarctica* (Novozyme 435), *Aspergillus niger* (Amano A), lipase from *Mucor javanicus* (Amano M), and lipase from *Pseudomonas cepacia* (Amano PS). The best results of capric acid incorporation in total triacylglycerides were obtained with Lipozyme IM, achieving 56.6% and incorporation of capric acid in sn-1(3)-

positions with Novozyme 435 and 61.8% with anhydrous Lipozyme IM, but the latter enzyme gave highest conjugated linoleic acid (CLA) percent content in sn-2- position, achieving up to 73.2% incorporation. This work indicates that the use of the selectivity of enzymes to achieve the capric acid incorporation into the sn-1 and sn-3 positions while the conjugated linoleic acid is mainly present in the sn-2 position (64).

The structured lipids (SLs) of the MLM type having long-chain fatty acids at sn-2 and medium-chain caprylic acid (CA, 8:0) at their sn-1,3-positions were prepared by Öztürk and coworkers in 2010. The aim of this study was to obtain the MLM-type structured lipids from corn oil enriched with caprylic acid. Structured lipids (SLs) of the MLM-type were prepared from acidolysis of corn oil with caprylic acid. Lipozyme TL IM from *Thermomyces lanuginosa* was used for the acidolysis of corn oil with caprylic acid in n-hexane and the reaction was optimized by response surface methodology (RSM). Under optimal conditions, caprylic acid incorporation into the triglycerides of up to 27.8 % was achieved (65).

In 2011, Chnadhapuram and Sunkired reported their work on the acidolysis of palm olein enriched with medium chain fatty acids by lipase acidolysis. The aim of this work was to incorporate medium chain fatty acids, caprylic and capric acids into palm olein by 1,3-specific lipase acidolysis. The reaction was performed by acidolysis of palm olein with mixtures of capric and caprylic acids using Lipozyme RM IM. The results found that the medium chain (MC) fatty acids, caprylic (C8:0) and capric (C10:0) were incorporated into palm olein by 1,3-specific lipase acidolysis, achieving up to 36% and 43% incorporation, respectively. These results indicate that Lipozyme RM IM has good selectivity for incorporation into the sn-1 and 3 positions (66).

Kavadia and coworkers reported their work on the acidolysis of fully hydrogenated soybean oil (FHSO) with caprylic acid, a medium chain fatty acid in 2018. The aim of the work was the modification of FHSO to produce a new fat with improved physicochemical and functional properties. The catalyst used in this work was immobilized lipase PyLip, which is known to be sn-1,3-specific. Under optimal conditions, incorporation of up to 45.16 % of caprylic acid in FHSO was observed. The authors report that this work is the efficient green enzymatic single step process for carrying out acidolysis reaction for bulk production of designer fats catalyzed by stable

low-cost indigenously immobilized lipase PyLip in shortened reaction time, without by-product formation making the overall process clean and environment friendly (67).

Currently, applications of enzymatic interesterification of fats and oils has gained increasing interest among scientists and technologists over the recent past. So, the modifications in TAGs of lipids are a method for synthesizing lipids to widen their applications. Since Interesterification catalyzed by enzymes is a process of rearrangement of fatty acids on the glycerol backbone resulting in structured lipids with modified physico-chemical and nutritional properties. Enzymatic interesterification has advantages such as milder processing conditions, fewer by-products, and easier product recovery than chemical interesterification. Moreover, enzymatic interesterification is used commercially to produce a variety of modified lipids such as human milk fat substitutes, zero-trans margarine and shortenings, cocoa butter substitutes and cocoa butter equivalents, low-calorie structured lipids, and edible films and coatings (68). With these advantages and applications, the enzymatic interesterification is popular today. For example, in 2018, Korma and coworker used enzymatic interesterification to make infant formula with structured lipids rich in medium- and long-chain triacylglycerols (MLCTs). Arachidonic acid (ARA) is an essential fatty acid (EFAs) which is important for an infant's growth and development. Medium chain triacylglycerols (MCTs) stimulate residual glyceride lipolysis, which improves fat absorption and hence decreases baby fat accumulation. The benefits of combined medium- and long-chain triacylglycerols (MLCTs) structured lipids (SLs) is that they easily deliver nutrients to infants. This study synthesized MLCTs-rich, structured lipids (SLs) by lipase-catalyzed interesterification of ARASCO with medium-chain triacylglycerols (MCTs) in a solvent-free system. The authors used commercial immobilized lipases from different sources and compared their efficiency in producing MLCTs. The results found that Lipozyme 435 from C. antarctica produced the highest yield of MLCTs under optimal conditions. Furthermore, the contents of MLCTs was 53.75%, and MLCTs-rich SLs melted below body temperature of 37 ◦C, which indicates that it is a nutritional and functional fat analogue with potential application in the infant formula industry (69).

In the same year, Huaitian Cuia and colleagues produced a superior margarine base stock from beef tallow (BT) blended with coconut oil (CO) by

enzymatic interesterification. This research was conducted to produce a margarine base stock that could be used in healthier margarine formulations and to ameliorate the problems of grainy and waxy mouthfeel associated with BT-based margarines. The oil interesterification (IOs) reaction processed into margarines was carried out with BT with three different concentrations of CO: 20, 30, or 40% in a solvent-free system with immobilized lipase, Lipozyme TL IM. The authors reported that at 40% CO the resulting product has the best properties for margarine production, with a low content of high melting-point triacylglycerols, which should minimize the "waxy" mouthfeel of BT-based margarines. Moreover, most of the fat in IO-based margarine (M-IO) was in the β'crystal form and the crystals were distributed more evenly than in BT-based margarine (MBT). A sensory and textural study revealed that the texture of M-IO was more compact, and that its color and flavor were better than M-BT. These results indicate that the EIE of BT and CO has the potential to be used as a means of producing base oils to replace BT in margarine and has potential applications in the margarine industry (70).

In 2020, Almeida and coworkers developed a biocatalyst for enzymatic transesterification of crude coconut oil and ethanol. They reported guava seed biochar appearing as a new alternative for the effective support for the immobilization of *Burkholderia cepacia* lipase (BCL) by physical adsorption. This research aimed to assess the immobilized biocatalyst's capability in the transesterification process of crude coconut oil and ethanol. The optimal condition of *Burkholderia cepacia* lipase (BCL) was determined to be 0.15 g enzyme/g support with 260 U/g hydrolytic activity and a 54% immobilization yield. The transesterification reaction was carried out at 40°C under different reaction conditions. At 24h, a substrate molar ratio of oil:ethanol of 1:7 was used to convert the oil to monoacylglycerols (MAGs) resulted in yields of up to 59%. At 96h, the molar ratio of 1:7 obtained the highest ethyl esters yield of 48%, and with the molar ratio of 1:6 at 24h gave a maximum conversion to diacylglycerols (DAGs) of up to 30%. Moreover, the authors studied Molecular Docking to clarify the mechanisms of transesterification reaction at the molecular level. This research has shown the potential of biocatalysts for efficient synthesis of ethyl esters (48%), monoacylglycerides (59%), and diacylglycerols (DAGs) (30%). It should be noted that MAGs and DAGs are compounds with excellent emulsifying properties used in

industrial production of several bioproducts such as cosmetics, pharmaceuticals, foods, and lubricants (71).

In 2022, Zhang and coworkers used immobilized lipase (Lipozyme ® TL IM) to modify the structure of the fat from palm olein. They studied the effect of process parameters on the properties of fats and Lipozyme ® TL IM. Enzymatic interesterification of palm olein (POL) occurred in a continuous packed bed reactor (PBR) on parameters (flow rates of 4.5–40 g/min and temperatures of 50–90ºC). The authors report that enzymatic interesterification (EIE) reaction, temperature, and flow rate resulted in acyl migration, which altered the sn-2 fatty acid compositions. Both a high temperature and a low flow rate increased acyl migration. The TAG composition of the products obtained was distinctive at different run times and temperatures. Furthermore, the enzymatic activity and SFCs decreased over time. The concentration of lipase protein was changed insignificantly, showing that the reaction did not change the protein content. This research shows that specific immobilized lipase can produce lipids with distinctive properties, and immobilized lipase in a packed bed reactor (PBR) for EIE to produce structured lipids has the advantage of preventing the damage of immobilized lipase, enhancing reaction efficiency, simplifying operating procedures, enabling the realization of continuous production, and reducing manufacturing costs (72).

2.5 Plastics: Poly(ethylene terephthalate) (PET)

Plastics are long chain polymeric molecules exhibiting high molecular mass, which are made from inorganic and organic raw materials, such as carbon, silicon, hydrogen, nitrogen, oxygen, and chlorine. The basic materials used for making plastics are extracted from crude oil, coal, and natural gas (Seymour, 1989) (73).

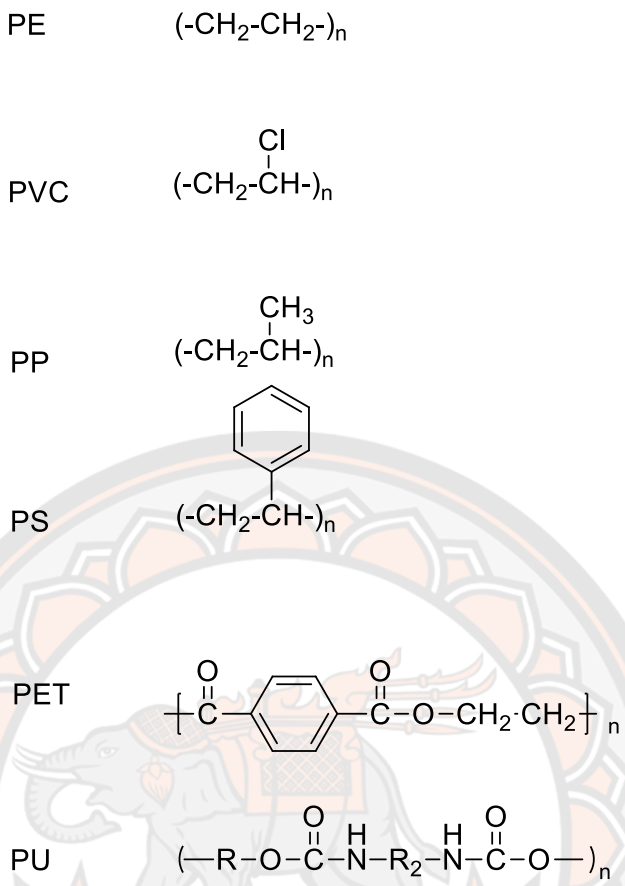


Figure 16 Structures of conventional petrochemical plastics (Polyethylene (PE), Polyvinyl chloride (PVC), Polypropylene (PP), Polystyrene (PS), Poly(ethylene terephthalate) (PET), Polyurethane (PU)).

Plastics can be easily molded into any shape and form using a degree of combination of polymerization and melting. The ability to design or engineer polymers provides plastics incredible versatility, with unique characteristics such as high strength, flexibility, durability, stress resistance, shock resistance, lightweight, and thermal and electrical insulation. Synthetic plastics are extensively used in packaging of products like food, pharmaceuticals, cosmetics, detergents, and chemicals. Furthermore, plastics are used worldwide for packaging applications such as beverage and food product bottles, microwaveable food trays, and ovenproof plastic wraps (Figure 16 and Table 4) (74).

Table 4 Uses of synthetic plastics

Plastic	Use
Polyethylene	Plastic bags, milk and water bottles, food packaging film, toys, irrigation and drainage pipes, motor oil bottles
Polystyrene	Disposable cups, packaging materials, laboratory ware, certain electronic uses
Polyurethane	Tyres, gaskets, bumpers, in refrigerator insulation, sponges, furniture cushioning, and life jackets
Poly(vinyl chloride)	Automobile seat covers, shower curtains, raincoats, bottles, visors, shoe soles, garden hoses, and electricity pipes
Polypropylene	Bottle caps, drinking straws, medicine bottles, car seats, car batteries, bumpers, disposable syringes, carpet backings
Poly(ethylene terephthalate) (PET)	Used for carbonated soft drink bottles, processed meat packages peanut butter jars pillow and sleeping bag filling, textile fibers
Nylon	Polyamides or Nylon are used in small bearings, speedometer gears, windshield wipers, water hose nozzles, football helmets, racehorse shoes, inks, clothing parachute fabrics, rainwear, and cellophane
Polycarbonate	Used for making nozzles on paper making machinery, street lighting, safety visors, rear lights of cars, baby bottles and for houseware. It is also used in skylights and the roofs of greenhouses, sunrooms and verandahs. One important use is to make the lens in glasses
Polytetraflouroethylene (PTFE)	PTFE is used in various industrial applications such specialized chemical plant, electronics, and bearings. It is met with in the home as a coating on non-stick kitchen utensils, such as saucepans and frying pans

Nowadays, many plastic materials based on synthetic polymers have suitable material properties that enable their widespread use. Unfortunately, these materials are resistant to environmental influences. Most synthetic plastics are very stable and cannot be easily degraded or recycled. As a result, the amount of plastic waste in the environment is continually increasing. Therefore, environmental pollution from synthetic plastics is a large problem. Although the problem of plastics still remains unsolved, different ways are being considered to reduce their impact on the environment. One of them is to use enzymes capable of degradation of plastic.

The biodegradation process of synthetic plastic is the most effective and best way for plastic degradation compared with other degradation processes due to its non-polluting mechanism, environmental friendliness, and cost-effectiveness. The biodegradation process of synthetic plastics is relatively slow due to environmental factors and the action of microbial species. Fungi play a key role in plastic degradation by secreting certain degrading enzymes such as cutinase, lipase, and protease. In addition, lignocellulolytic enzymes in the presence of some pro-oxidant ions may also cause efficient degradation. Moreover, the oxidation or hydrolysis by the enzyme creates functional groups that improve the hydrophilicity of polymers and consequently degrade the high molecular weight polymer into low molecular weight fragments which facilitates the rapid breakdown of polymers (75).

Recently, several reviews reported that several enzymes can attack ester bonds in some aromatic and aliphatic polyesters and can depolymerize such materials (76), (77). For example, in 2008, Eberl and coworkers reported the hydrolysis of polyester poly(trimethylene terephthalate) (PTT) and oligomer using enzymes. This work focused on enzymatic hydrolysis of the aromatic polyester poly(trimethylene terephthalate) using enzymes belonging to the class of polyesterases. Oligomers and polymers (film, fabrics) of the linear aromatic polyester poly(trimethylene terephthalate) (PTT) were treated with polyesterases from *Thermomyces lanuginosus*, *Penicillium citrinum*, *Thermobifida fusca*, and *Fusarium solani pisi*. The results indicated that cutinase from *Thermobifida fusca* was able to hydrolyse the aromatic polyester poly(trimethylene terephthalate) and release the highest amounts of hydrolysis products from PTT materials. In addition, T. *fusca* was able to open and hydrolyze a cyclic PTT dimer according to RP-HPLC–UV detection, whereas the lipase

from T. *lanuginosus* was only able to hydrolyze the fibers on bis(3-hydroxypropyl) terephthalate (BHPT) but was not able to hydrolyze the polymer film, mono(3-hydroxypropyl) terephthalate (MHPT) and the cyclic dimer of PTT. These results indicated that this enzyme has a high potential for PTT surface modification and removal of cyclic dimers (78).

This study is interested in the degradation and recycling of poly(ethylene terephthalate) (PET). Poly(ethylene terephthalate) (PET) is the copolyester of ethylene glycol (EG) and terephthalic acid (TPA or its esters). PET has many favorable characteristics, such as high strength and resilience, resistance to abrasion, stretching, shrinking, and wrinkling, as well as resistance to many chemicals. Thus, PET is an important commercial polymer and is having applications ranging from packaging, fabrics, films, molded parts for automotive industry, electronics, etc. PET plastics are very useful in our day-to-day life to meet our desired needs. Due to its good quality, its use is increasing day by day, however its degradation is becoming a significant problem. Objects made from PET, such as plastic bottles, soft drink containers, water bottles, soda bottles, containers, or certain specialty bottles, such as those designated for beer containment are omnipresent in our daily lives. The amount of these plastics is increasing and lack of suitable methods of disposal of plastic waste create a substantial environmental accumulation and pollution problem. Recently, numerous microorganisms were reported to produce degrading enzymes that can degrade plastic and be non-poisonous. This can be considered as an interesting and relatively safe technique of PET breakdown and recycling.

Therefore, this project is interested in the degradation or recycling of Poly(ethylene terephthalate) (PET) from water bottles using enzymes as catalysts. The reports relevant to the goals of this project will be discussed below.

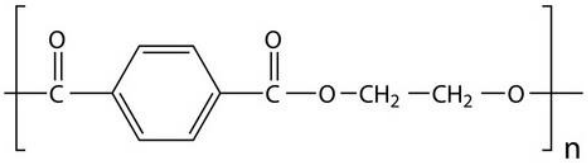


Figure 17 Poly(ethylene terephthalate) (PET)

Several enzymes were reported to be able to degrade synthetic aromatic polyesters such as PET are cutinases or lipases isolated from various fungi and bacteria including T. *fusca*. (Hookerand et al. 2002; Vertommen et al. 2005; Müller et al. 2005; Chen et al. 2008 and Ronkvist et al. 2009)

In 2003, an unidentified cutinase completely hydrolyzed cyclic PET trimers within 24 h and predominantly formed terephthalic acid and MHET as reported by Hooker and coworkers. The authors reported a method for the efficient removal of cyclic PET trimer by enzyme catalyzed hydrolysis (Figure 18). The project was focused on the development of a method for the removal of CTR by cutinase-catalyzed hydrolysis. The complete hydrolysis of the trimer was accomplished at pH 8 and 60°C, which can be considered as mild conditions and constitute a simple and effective procedure. The three significant products from enzymatic hydrolysis of CTR are TA, BHET, and MHET. TA and MHET were the predominant products, and BHET was found in trace amounts (Figure 19). In addition, the authors have studied effects of time, agitation, surfactant, and enzyme and trimer concentrations on the efficiency of hydrolysis (79).

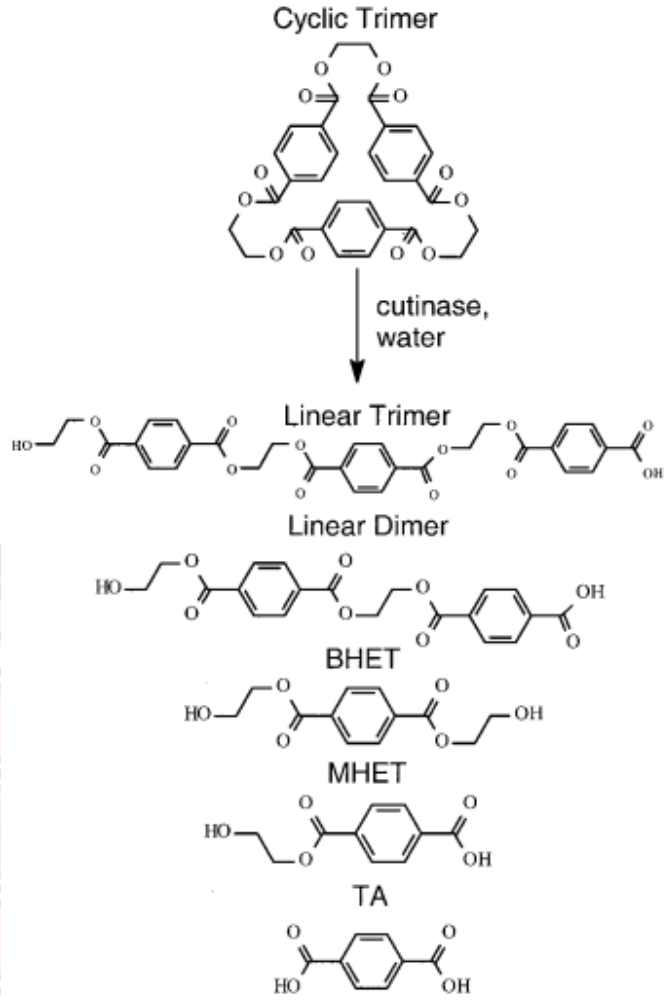


Figure 18 Potential cutinase-catalyzed hydrolysis products of CTR (BHET, bis(hydroxyethylene)terephthalate; MHET monohydroxyethylene terephthalate; TA terephthalic acid)

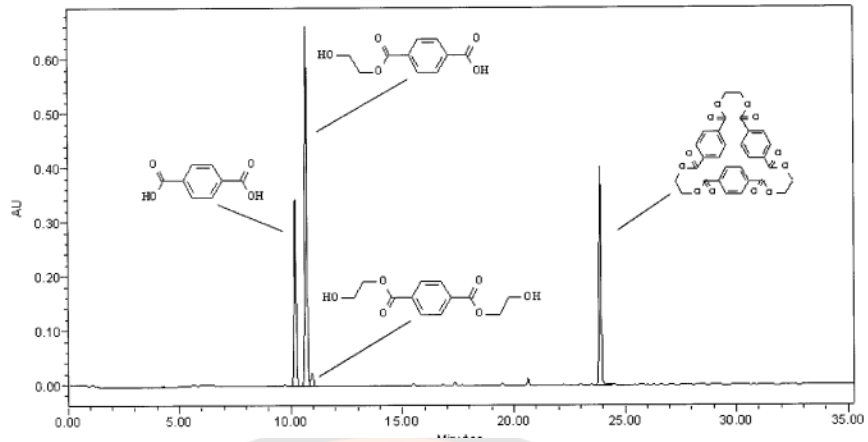


Figure 19 Typical HPLC chromatograms of CTR cutinase-catalyzed hydrolysis reaction (0.19232 mM CTR, 100 LU/mL cutinase, 60°C, pH 8, 150 rpm, 24 h)

In 2005, Vertommen and coworkers confirmed that cutinase from F. *solani pisi* is capable to hydrolyze the synthetic polymer poly(ethylene terephthalate). This research was intended as a fundamental study of enzyme action on synthetic substrates. The authors reported that cutinase from *F. solani pisi* shows significant hydrolytic activity towards amorphous regions of PET and shown high selectivity towards the soluble product MHET. In addition, crystallinity greatly affects the capability of the enzyme to hydrolyze the ester bonds on amorphous polyester film (80).

In 2005, Muller and coworkers published a report about hydrolase enzymes, which can degrade polyesters containing aromatic constituents. This work focuses on the depolymerization of the aromatic polyester PET by a hydrolase from the actinomycete *Thermobifida fusca*. The reactions were performed in phosphate buffer with PET film (commercial PET from beverage bottles) and hydrolase from the actinomycete *Thermobifida fusca* under optimal conditions. The results have shown that the weight loss of PET film increased continuously and film thickness (erosion rate) of the PET films decreased between 8 and 17 µm/week (per film side) (Figure 20). Therefore, this result pointed out that commercial PET can be effectively hydrolyzed by an enzyme (81).

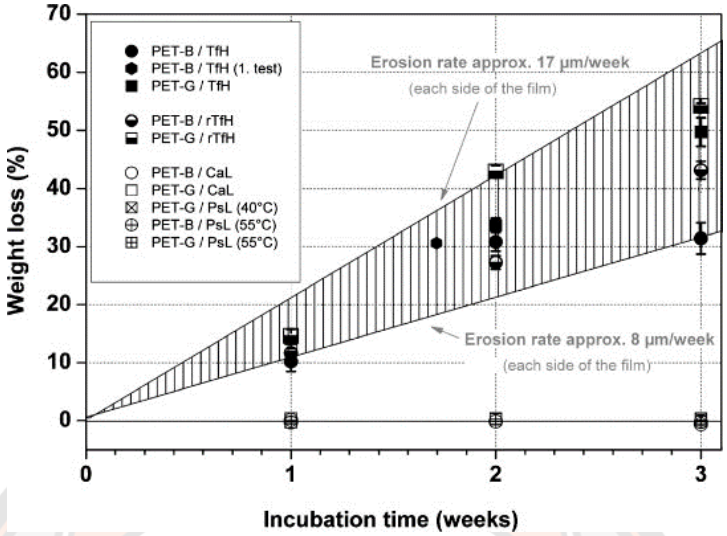


Figure 20 Degradation of melt-pressed PET films by a hydrolase from *Thermobifida fusca* (TfH) and other lipase (CaL and PsL) at 55 ºC in phosphate buffer at pH 7. Average of weight loss of three 12 mm circular films per test (except for 'PET-B/TfH 1. test' with only one film in the test). Erosion rates listed are calculated from weight loss data assuming a homogeneous surface erosion and a PET density of 1.3 g/cm^{-3}

A further study on cutinases capable to degrade PET was published by Ronkvist and coworkers (2009). The aim of this study was a comparison of the catalytic activities of cutinases from three different organisms, using low-crystallinity (lc, 7%) and biaxially oriented (bo, 35%) poly (ethylene terephthalate) (PET) films as model substrates. Three different cutinases used for the comparison of the catalytic activities included *Humicola insolens* (HiC), *Pseudomonas mendocina* (PmC), and *Fusarium solani* (FsC). The authors reported that, the initial hydrolysis rate of HiC is 7-fold higher than PmC and FsC (Table 5). Moreover, the results from incubations of 96 h duration at 70^{o}C of lcPET with HiC resulted in 3% film weight loss.

Table 5 Kinetic parameters for PET hydrolysis using HiC, PmC, and FsC at 70, 50, and 40°C, respectively

	$[S]_0$ (μM)	K(μM^{-1})	K_2(μmol/cm^2/h)
HiC	13	0.64	0.62
PmC	13	0.76	0.08
FsC	13	0.41	0.09

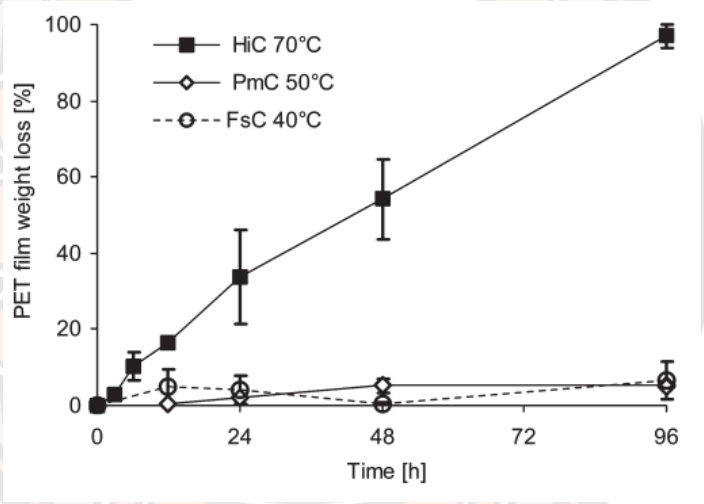


Figure 21 Degradation study of lcPET (2.25 cm^2/mL) as a function of incubation time in 1 M Tris-HCl with 10% glycerol, at pH 7.5 and 10 nmol/mL of either HiC, PmC, or FsC at 70, 50, and 40°C, respectively. Error bars represent the standard deviation method based on triplicate repeats

Whereas 96 h incubations of lcPET with PmC and FsC at 50 and 40°C, respectively, resulted in a 5% weight loss (Figure 21). Furthermore, all three cutinases degrade PET to TPA. These results indicated that this cutinase may be able to convert commercial PET materials of low crystallinity to TPA and ethylene glycol under mild conditions such as bottle transparency (water bottle) (82).

In 2011, a paper published from Ribitsch et al. reported that p-nitrobenzylesterase from *B. subtilis*. can hydrolyze poly(ethylene teraphthalate) (PET). The authors screened bacterial PET-hydrolases on agar plates with bis(benzoyloxyethyl) terephthalate (3PET), a Bacillus subtilis p-nitrobenzylesterase (BsEstB) was isolated and demonstrated to hydrolyze polyethyleneterephthalate (PET). This research demonstrates that the capability to hydrolyze polyethyleneterephthalate (PET) is not restricted to extracellular lipases and cutinases (83).

The enzymatic PET hydrolysis can be considerably enhanced by the fusion of hydrophobins to cutinases, which was reported by Ribitsch et al in 2015. The aim of this paper was to study the influence of hydrophobins on PET-hydrolyzing cutinase by comparing the effects of free hydrophobins to those of hydrophobins that are genetically fused to a cutinase. PET-hydrolyzing activity of a bacterial cutinase from *Thermobifida cellulosilytica* (Thc_Cut1) was investigated by fusion to one of three *Trichoderma hydrophobins*, i.e., the class II hydrophobins HFB4 and HFB7 and the pseudo-class I hydrophobin HFB9b. The authors reported that for the enzyme, which was fused to HFB4 or HFB7, the hydrolysis of PET was enhanced >16-fold over the level with the free enzyme. On the other hand, a mixture of the enzyme and the hydrophobins led only to a 4-fold increase at most. In contrast, fusion with the non-class II hydrophobin HFB9b did not increase the rate of hydrolysis over that of the enzyme-hydrophobin mixture, but HFB9b performed best when PET was preincubated with the hydrophobins before enzyme treatment. This result indicated that the cutinase-hydrophobin fusion proteins can increase the rate of PET modification and recycling (84).

In 2015, Barth and coworkers published a report that poly(ethylene terephthalate) (PET) was hydrolyzed by the polyester hydrolase in an aqueous reaction system contained in an ultrafiltration membrane reactor. The aim of this study was to degrade PET films with a polyester hydrolase TfCut2 from *Thermobifida fusca* KW3 in an ultrafiltration membrane reactor to minimize the product inhibition of the enzyme. In addition, parameters affecting the enzymes efficiency such as the buffer and the mechanical agitation of the reaction mixture were also investigated. The authors reported that the hydrolysis by the ultrafiltration membrane increased the efficiency of

the biocatalytic hydrolysis of PET by 70 % over a reaction time of 24 h compared to a hydrolysis performed in batch mode. This study showed that the membrane reactor could be applied to optimize the reaction conditions for hydrolysis of PET films (85).

A recent report from Yoshida et al. (2016) described a discovery of a bacteria that showed the ability to digest poly(ethylene teraphthalate) (PET). The authors reported that the bacterium (Ideonella sakaiensis 201-F6) secretes two hydrolytic enzymes to degrade the poly(ethylene teraphthalate) (PET) into monomers as shown in Figure 22. First, bacteria adhere to the PET surface and secrete two enzymes. One enzyme, PETase breaks down PET into the intermediate compound, mono(2-hydroxyethyl) terephthalic acid (MHET). Second enzyme, MHETase breaks down mono(2-hydroxyethyl) terephthalic acid (MHET) into the two starting monomers, terephthalic acid and ethylene glycol. Finally, the monomers are catabolized by the bacterium as its sole carbons source. These published results are interesting due to their environmental friendliness. The bacterium used PET as its major energy and carbon source. When grown on PET, this strain produces two enzymes capable of hydrolyzing PET into its two monomers, terephthalic acid and ethylene glycol (86).

In the same year, a dual enzyme reaction system composed of a polyester hydrolase and a carboxyl esterase was used for biocatalytic PET hydrolysis as reported by Barth and coworkers (2016). The aim of this project was to use a dual enzyme reaction system for inhibition of intermediate MHET from poly(ethylene terephthalate) (PET) hydrolysis reaction.

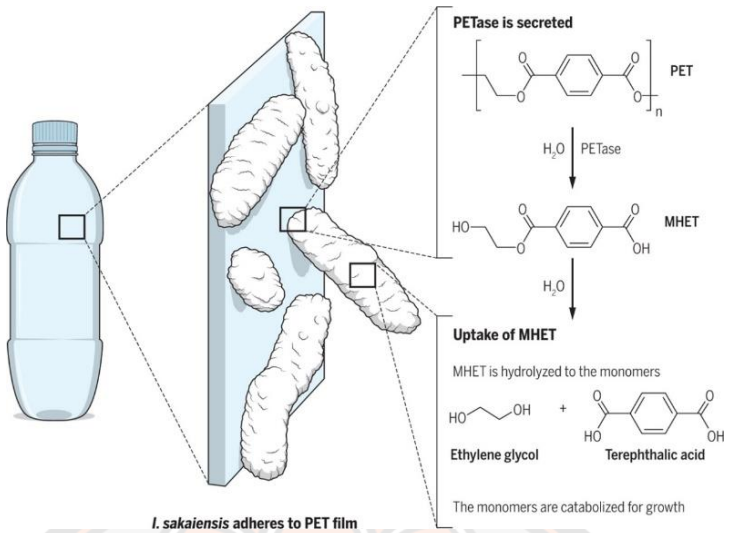


Figure 22 Enzymatic PET degradation by PETase and MHETase

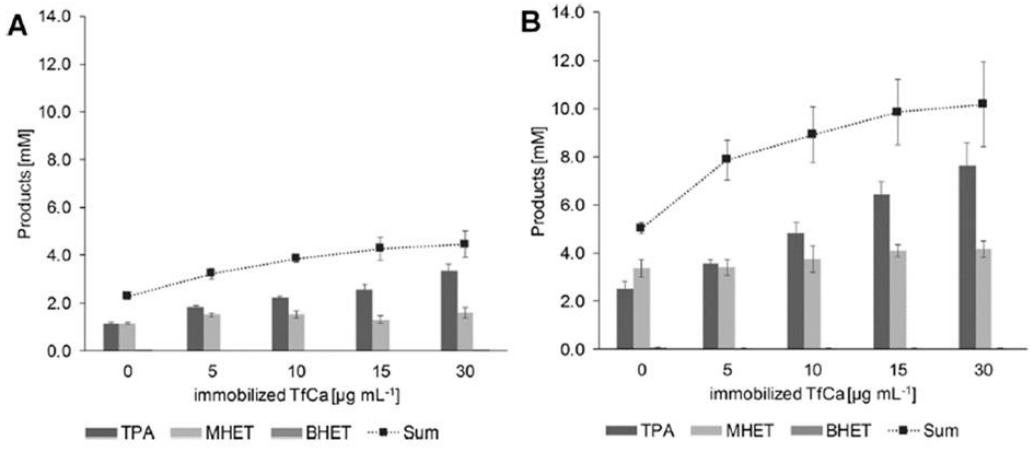


Figure 23 Total amounts of the hydrolysis products TPA, MHET, and BHET released from PET films by the dual enzyme reaction systems over a reaction time of 24 h. The reactions were performed at 60°C with 10 µg mL^{-1} TfCut2 (A) or LCC (B) and 0 and 30 µg mL^{-1} immobilized TfCa. Error bars show the standard deviations based on triplicate determinations)

The dual enzyme system consisted of TfCut2 from *Thermobifida fusca* KW3 (TFCut2) and the metagenome-derived LC-cutinase, which are bacterial polyester hydrolases (LCC). Both enzymes were used in PET hydrolysis reaction with immobilized TfCa at optimal conditions (potassium phosphate buffer pH 8.0, 60^{o}C, 24h). The resulting amounts of products from PET hydrolysis produced in the dual enzyme system (TFCut2 and LCC) with different concentrations of immobilized TfCa are shown in Figure 23. At a concentration of immobilized TfCa of 30 µg the dual system shows higher total amount of products compared to TfCut2 alone. This research indicates that the dual enzyme could be an interesting candidate for use in biocatalytic PET recycling processes. Moreover, the dual enzyme reaction system also prevents an inhibition by the reaction intermediate MHET thereby further increasing the efficiency of a biocatalytic PET hydrolysis system (87).

Further studies about enzymatic degradation of poly(ethylene terephthalate) (PET) were reported by Carniel and coworkers in 2017. The aim of this work was to enable monomer recycling by hydrolysis of poly(ethylene terephthalate) (PET) using a biocatalyst. The authors reported that lipase from *Candida antarctica* (CALB) and cutinase from *Humicola insolens* (Hic) act as efficient enzyme catalysts, able to convert poly(ethylene terephthalate) (PET) to its monomers. The studies of PET hydrolysis investigated various parameters such as the effect of PET pretreatment, temperature, enzyme loading, and mixture enzyme. The effects of the investigated parameters have shown that the performance of each enzyme is dependent on PET source and properties. Moreover, optimization of reaction conditions such as temperature, pH, and concentration of enzyme can increase the efficiency of depolymerization of PET (88).

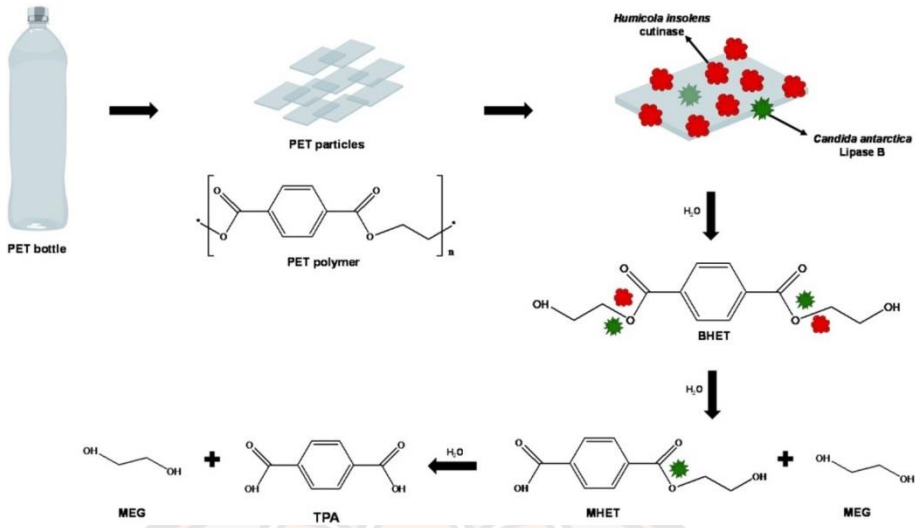


Figure 24 Lipase from *Candida antarctica* (CALB) and cutinase from *Humicola insolens* act synergistically for PET hydrolysis to terephthalic acid

In 2018, a further report of opportunities for enzyme-catalyzed PET recycling was published by Carniel and coworkers. The aim of the work was the depolymerization PET via glycolysis (EG as liquid phase and reagent) or hydrolysis (water as liquid phase and reagent) using enzyme catalysis. The authors selected Hic as the optimal catalyst for enzyme-catalyzed hydrolysis-glycolysis reactions. PET was depolymerized by simultaneous hydrolysis-glycolysis reactions with varied water and ethylene glycol concentrations. The reaction progress was monitored by the time course of total products formation using oligomers as substrates and combining different water/EG proportions for simultaneous hydrolysis-glycolysis reaction of PET catalyzed by HiC as shown in Figure 25. When EG concentration in the system increased, the proportion of the products has changed, increasing the content of MHET and BHET, which indicates that glycolysis reaction was taking place instead of hydrolysis. The best reaction was obtained with moderate EG concentrations (20–40%) and 20% EG was shown to lead to highest conversions of PET to TPA and to total end products. This result indicated that using EG as a co-solvent can aid depolymerization PET into its monomer (89).

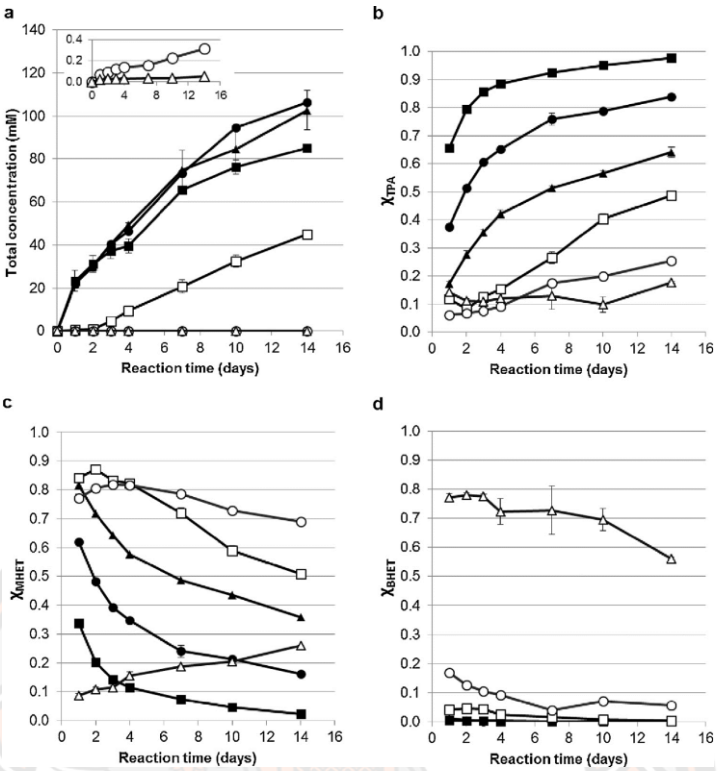


Figure 25 Time course of total products concentration (a) and TPA (b), MHET (c) and BHET (d) molar fractions (x) during simultaneous hydrolysis-glycolysis reactions of PC-PET catalyzed by HiC. Reactions were carried out at 62.6ºC and 0.02 protein/g PET. Different added EG contents were investigated: 0% (closed squares), 20% (closed circles), 40% (closed triangles), 60% (open squares), 80% (open circles) and 100% (open triangles). Small graph window shown in the up-left corner of figure (a) represents a closer view of conditions with 80% and 100% EG

In 2019, de Castro and coworkers use enzymes as catalysts for post-consumer PET (PC-PET) depolymerization into its monomers, terephthalic acid (TPA) and ethylene glycol (EG). In this work, they focused on improvements of both titer and productivity in TPA release, resulting from the depolymerization of post-consumer PET (PC-PET) sample, where these processes are catalyzed by *Humicola insolens* cutinase. They designed a procedure comprised of fractional factorial and central composite rotatable

designs for the improvement of PET depolymerization via enzymatic hydrolysis catalyzed by HiC. The highest terephthalic acid concentration and productivity during PC-PET hydrolysis were obtained in 100.9 mM (16.8 g/L) and 14.4 mM/day, corresponding to overall improvements of 10-fold and 20- fold, respectively.

In comparison with the previous results reported in Castro et al., the authors reported that this project is the best results described so far for enzyme-catalyzed hydrolysis of used PET packages. Moreover, this research uses a single enzyme system, instead of multiple biocatalysts to achieve final conversion of PET to its monomers, thus reducing process complexity and costs.

There are also reports that Poly(ethylene terephthalate) can degrade by photo and/ or oxidation processes. When the Poly(ethylene terephthalene) (PET) is exposed to terrestrial sunlight, it will gradually lose its chemical and physical properties. The deterioration in properties is attributed to photochemical reactions initiated by the absorption of near-ultraviolet radiation. It can be assumed that photo-oxidation has an important role in the decomposition process.

A researcher from Day and coworkers in 1972 reported that the effect of irradiation wavelength has an important part on the rate and the nature of photodegradation of PET and found that the wavelengths smaller than 315 nm effect on the rate of photodegradation of the polymer. They studies degradation of PET by monitored measuring the tensile strength, molecular weight, carboxylic acid endgroups, and fluorescence emission of the polymer (90).

Further reported in 2002, Fechine and coworkers studied effects of photodegradation on the structure of poly(ethylene terephthalate) (PET). The aim of this research was to investigate the changes in morphology during laboratory exposure of PET films with and without an ultraviolet absorber stabilizer. The PET films will be investigated by differential scanning calorimetry (DSC), X-ray diffraction and size exclusion chromatography. They prepared PET films in the form of semi-crystalline, bioriented films produced by extrusion, have been exposed in the laboratory for periods of up to 1020 hours. The PET films were test using Q-Panel UVA fluorescent lamps under the optimal weathering cycle. Then, the specimens are submitted to a combination of photo-, thermal and hydrolytic degradation, offering very harsh conditions to the sample deterioration. After that, the samples were tested in a Shimadzu

DSC 50 differential scanning calorimeter and X-ray diffraction. Size exclusion chromatography were carried out in a Shimadzu Class LC10 operating with an UV detector. The results from DSC indicated the appearance of a cold crystallization peak during DSC heating scans was noted for exposed samples and this considered to be a result of released molecules in the amorphous region that could rearrange into a crystalline phase. From X-ray analysis, a loss of crystalline orientation was observed after degradation and an interpretation was given based on relaxation in the mesophase region (91).

Another way of interesting for biodegradation of Poly(ethylene terephthalate) (PET) is use pro-degradant additives. These additives are transition metal ion complexes of carboxylic acids and dithiocarbamates which accelerates the cleavage of long molecular plastic chains due to oxidation reaction and thereby leads to fragmentation of the polymer to facilitate biodegradation process (92).

In 2017, Chelliah and coworkers attempt to biodegradable Poly(ethylene terephthalate) (PET) through oxidative reaction using cobalt stearate (CS) as transition metal ion complexes. The aim of this project was to study the effect of different loading amounts of cobalt stearate (CS) on the polymeric matrix of poly(ethylene terephthalate) (PET) based on FTIR and scanning emission microscopy (SEM) analysis. The formulated compounds (PET and Cobalt (II) Stearate) were melt blended using a twin-screw extruder. Then, the formulated compounds were pelletized prior to molding into dumb-bell shapes specimens using an injection molding machine following ASTM D 638 standard. The effect of CS on PET was evaluated by monitoring the changes in FTIR spectroscopy and SEM analysis. The results of adding varied loading amount of CS on PET found that the CS influences its tensile strength and elongation at break (%). The results from Fourier Transform Infrared Spectroscopy (FTIR) spectra, carbonyl index (CI) and SEM measurements indicate that blends' consisting of 0.25CS contributes to thermal degradation of PET (92).

In 2023, Sariah Giraldo-Narcizo and coworkers reported that the pre-treatment with alkali modifies the surface of the plastic and reduces its crystallinity and enables the enzyme to readily hydrolyze chemical bonds. Postconsumer PET bottles were pretreated with alkali (NaOH, 10 M) at 25^{o}C for 24 h and subsequently hydrolyzed with enzyme PETase at 30^{o}C. The results are shown in Figure 26. It can be observed that

untreated PET is transparent and colorless while PET pretreated with NaOH is opaque and white in color. After that, PET plastic samples surface was examined by Scanning Electronic Microscopy (SEM) (Figure 27). The untreated PET sample shows a very smooth surface with the presence of small crystalline granules embedded into the polymer matrix (Figure 27A). While the NaOH treated PET sample shows a rough surface with texture in the range of tens of microns (Figure 27B). This work points out guidelines for the accessibility of the enzyme to the hydrolysable bonds leading to the degradation of PET to its monomer, representing a promising method for its sustainable recycling (93).

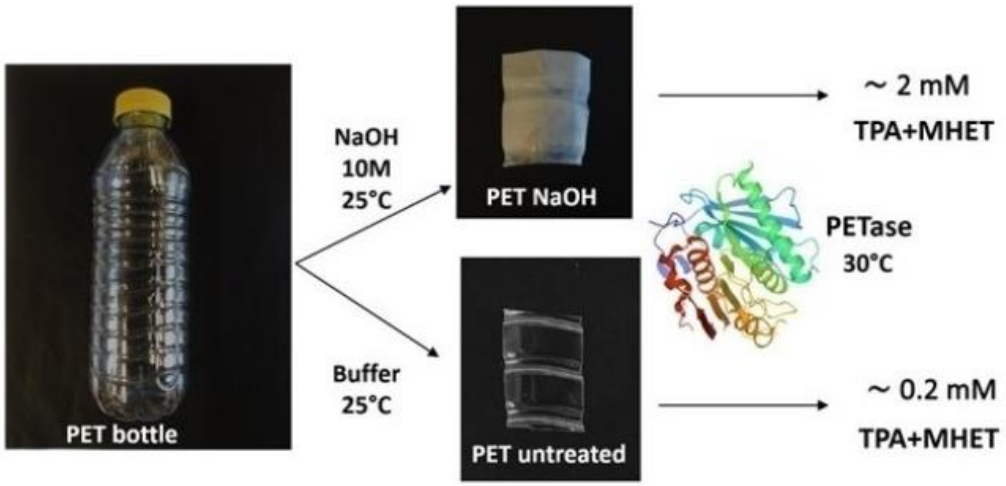


Figure 26 Schematic diagram of experimental design comparing PETase catalytic efficiency with or without alkaline pre-treatment of the PET substrate

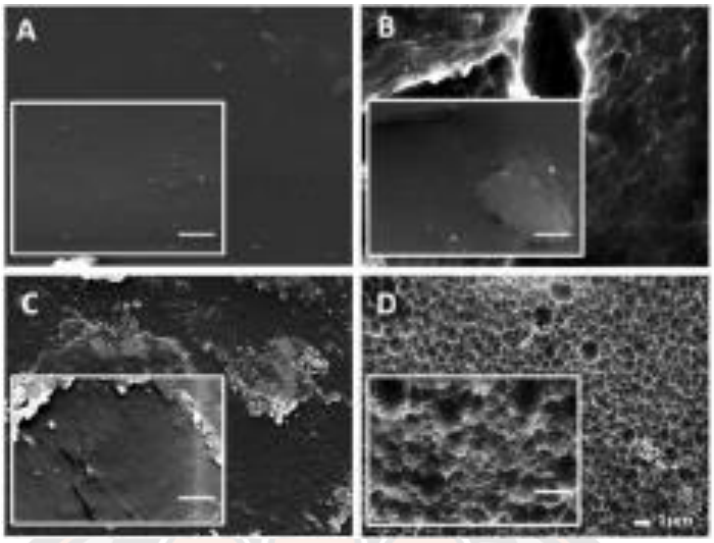


Figure 27 SEM measures of post-consumer PET water bottle surface. (A) PET bottle untreated. (B) PET bottle pretreated with 10 M NaOH. (C) Untreated PET bottle incubated with PETase. (D) PET bottle pretreated with alkali followed by PETase incubation. Scale bar: A-D and insets 1 μm

As mentioned above, the use of degrading enzymes for poly(ethylene terephthalate) (PET) hydrolysis is considered as an eco-friendly alternative. Many hydrolases from several fungi and bacteria have been discovered and successfully evaluated for their activity towards PET. In addition, it is found that PET can degrading by photo-oxidation reaction. Therefore, in this part of the project we are interests to combine photooxidative and enzymatic methods of PET degradation. In photooxidative degradation step, enzyme would be use as catalyst for making it more readily susceptible to enzymatic degradation.

CHAPTER III

RESEARCH METHODOLOGY

Lipases (triacylglycerol acyl hydrolase) are enzymes that are present in many different organisms, including animals, plants, and microorganisms. In synthetic organic chemistry, lipases are the enzymes that are utilized the most due to their ability to catalyze a wide variety of processes, including esterification, transesterification, and hydrolysis reactions of triglycerides, and their versatility. In addition, lipases can be widely used in different industrial applications. In this study, we were interested in applying lipases to catalyze transformations of natural and synthetic materials through various processes such as esterification, acidolysis, hydrolysis, and transesterification. The reactions will be investigated using NMR, GC, and Mass spectroscopy. Optimization of reactions in terms of reaction parameters such as temperature, reaction time, enzyme loading, and enzyme recycling will be investigated. This study was divided into three experiments as follows:

Part I: Enzymatic modification of coconut oil by acidolysis or interesterification with MCFAs or their alkyl esters.

Part II: Enzymatic hydrolysis of capsaicinoids and utilization of the fatty acid product for triglyceride modification

Part III: Degradation of poly (ethylene terephthalate) (PET) by photooxidative and enzymatic catalysis.

3.1 Part I Enzymatic modification of coconut oil by acidolysis or interesterification with MCFAs or their alkyl esters

3.1.1 Material and Chemicals

Coconut oil (Naturel brand/Lam Soon public company, Thailand) was purchased at a local supermarket. Coconut oil fatty acid distillate was received as a free sample from Lam Soon public company, Thailand. Methyl octanoate and methyl decanoate were purchased from ACROS Organic (New Jersey, USA). Lipozyme® TL

IM and NOVOZYM® 435 were obtained from Strem Chemicals. Lipase B from *Candida antarctica* immobilized on acrylic resin was obtained from Sigma. Ethanol, acetone, sulfuric acid, hydrochloric acid, methanol, and phenolphthalein were obtained from Carlo Erba. Hexane was purchased from RCl Lab scan limited. Sodium hydroxide and sodium carbonate were obtained from UNIVAR. Sodium sulfate anhydrous and tri-Sodium orthophosphate dodecahydrate were obtained from LOBA CHEMIE. Chloroform-D was from Cambridge isotope laboratories, Inc. Silica was from Silicycle.

3.1.2 Apparatus

GC analysis was performed on a Shimadzu gas chromatography system (GC-2014) equipped with a flame ionization detector and a capillary fused silica column (Rtx-Wax, length: 30 m, inner diameter: 0.25 mm, film thickness: 0.25 mm). The initial temperature was set to 120^{o}C and was held for 7 minutes. This was followed up by a linear gradient up to 240^{o}C over the next 10 minutes, with the final temperature being held for further 50 minutes.

^{1}H-NMR analysis was recorded by using $CDCl_3$ as a solvent on a Bruker Advance NMR spectrometer operating at 400 MHz Chemical shifts (δ) are given in ppm relative to tetramethylsilane (TMS).

MS analysis was obtained on an Agilent 6540 ultra-high definition (UHD) accurate mass Q-TOF LC/MS system and Mass Hunter workstation data mining.

3.1.3 NMR analysis

The scope of modification of coconut oil during the interesterification reaction was evaluated using ^{1}H-NMR spectroscopy. The critical signal for this evaluation is the broad multiplet between 1.1 and 1.3 ppm. The integral of the proton at 5.25 ppm (the sn-2 position on the glycerol backbone) was set to 1 to standardize the integrals. This signal corresponds to overlapping signals from the internal majority part of the fatty acid residues alkyl chains. This signal's integral (Int) was used as the observed variable to determine the scope of coconut oil modification. The ^{1}H-NMR

spectrum of the coconut oil used as the starting material in this work can be found in Appendix A Figure 94.

3.1.4 GC analysis of the fatty acid composition

1. Analysis of coconut oil original

Coconut oil was initially submitted to a transesterification procedure in order to convert the triglyceride into fatty acid methyl esters (FAME). For this, Coconut oil (200 mg) and methanolic NaOH solution (2mL, 0.5 M) were heated in a round bottom flask to 55°C for 5-10 minutes. After cooling, 1.5 mL of hexane and 1 mL of water were added to the mixture and extracted (3 times). The hexane layer was combined and then treated with 2N HCl (1 mL) for 5 minutes, and the layers were separated. The hexane solution was dried in a vacuum, and the resulting residue was quantified using GC analysis by dissolving 10 mg in methanol (200 µl). The GC spectrum of the coconut oil used as the starting material in this work can be found in Appendix A Figure 95

2. Analysis of modified coconut oil

The fatty acid composition of modified oils was analyzed using GC after methylation in a MeOH/H_2SO_4 (98:2) mixture. Modified oil sample 100 mg was heated in a sealed tube at 80°C for 90 minutes with 5 mL MeOH/H_2SO_4. The reaction mixture was let to cool down, and 1 mL of water was added to the mix. Then the methyl esters were extracted with 5 mL of hexane and quantified using GC analysis.

The GC analysis was performed on a Shimadzu gas chromatography system (GC-2014) equipped with a flame ionization detector, and a capillary fused silica column (Rtx-Wax, length: 30 m, inner diameter: 0.25 mm, film thickness: 0.25 µm). The temperature program was as follows: injector and detector temperatures were 250°C; the initial column temperature was set to 120^{o}C and was held for 7 minutes. Followed by a programmed rise to 240^{o}C at 10 minutes and then maintained temperature held for 50 minutes. The injection volume was 1.0 µl. The percent values reported below for individual fatty acids represent the percentages of their corresponding GC peak areas relative to the total peak areas of all reported fatty acids.

3.1.5 Experimental

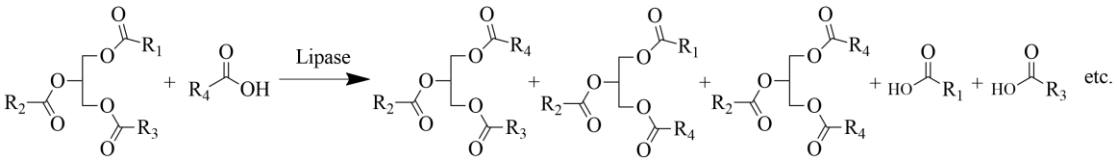


Figure 28 Triglyceride acidolysis and interesterification reactions

3.1.5.1 Coconut oil acidolysis

1. Initial experiments for coconut oil acidolysis with octanoic acid (C8:0)

Coconut oil (1.00 g), octanoic acid (0.64 g), and lipase enzyme (0.16 g) were dissolved in hexane (5.00 mL). The reaction was stirred at room temperature for 24 h. The enzyme catalyst was removed by filtration using filter paper. Ethanol (15 mL) and acetone (15 mL) were added to the filtrate together with a small amount of phenolphthalein indicator. The solution was titrated with 0.5 M NaOH until the solution turned pink. The solution was extracted with hexane (30 mL). The hexane layer was washed with 5% sodium carbonate solution (Na_2CO_3) (2x30 mL) and water (2x30 mL), dried over sodium sulfate (Na_2SO_4), filtered, and evaporated. The residue was analyzed by ^{1}H-NMR.

2. Adjusted conditions of initial experiments for coconut oil acidolysis with octanoic acid (C8:0)

Coconut oil (1.00 g), octanoic acid (0.64 g), and lipase enzyme (0.16 g) were dissolved in hexane (5.00 mL). The reaction was stirred at 45^{o}C for 72 h. The enzyme catalyst was removed by filtration using filter paper. Ethanol (15 mL) and acetone (15 mL) were added to the filtrate together with a small amount of phenolphthalein indicator. The solution was titrated with 0.5 M NaOH until the solution turned pink. The solution was extracted with hexane (30 mL). The hexane layer was washed with 5% sodium carbonate solution (Na_2CO_3) (2x30 mL) and water (2x30 mL),

dried over sodium sulfate (Na_2SO_4), filtered, and evaporated. The residue was analyzed by ^{1}H-NMR.

3. Select suitable enzyme for coconut oil acidolysis with octanoic acid (C8:0)

Coconut oil (1.00 g), octanoic acid (0.64 g), and 5 different lipases enzyme (0.16 g): lipase B from *Candida antarctica* acrylic resin, lipase from *Pseudomonus cepacia* immobilized on immobead 150, lipase from *Rhizopus oryzae* immobilized on immobead 150, lipase from *Candida rugosa* immobilized on immobead 150, and Lipozyme TL IM were dissolved in hexane (5.00 mL). The reaction was stirred at 45^{o}C for 72 h. The enzyme catalyst was removed by filtration using filter paper. Ethanol (15 mL) and acetone (15 mL) were added to the filtrate together with a small amount of phenolphthalein indicator. The solution was titrated with 0.5 M NaOH until the solution turned pink. The solution was extracted with hexane (30 mL). The hexane layer was washed with 5% sodium carbonate solution (Na_2CO_3) (2x30 mL) and water (2x30 mL), dried over sodium sulfate (Na_2SO_4), filtered, and evaporated. The residue was analyzed by ^{1}H-NMR.

4. Investigation of the effect of temperatures on coconut oil acidolysis with octanoic acid (C8:0)

Coconut oil (1.00 g), octanoic acid (0.62 g), lipase enzyme (different lipase enzyme: lipase *Candida antarctica immobilized* on acrylic resin, and Lipozyme TL IM enzyme) (0.16 g) were added to the vial. The reaction mixture was agitated for 72 h at different temperatures (45^{o}C, 50^{o}C, 55^{o}C, 60^{o}C, 65^{o}C, 70^{o}C, and 75^{o}C). After the reactions, the lipase enzyme was removed by filtration using filter paper and washed with hexane. The enzyme catalyst was removed by filtration using filter paper. Ethanol (15 mL) and acetone (15 mL) were added to the filtrate together with a small amount of phenolphthalein indicator. The solution was titrated with 0.5 M NaOH until the solution turned pink. The solution was extracted with hexane (30 mL). The hexane layer was washed with 5% sodium carbonate solution (Na_2CO_3) (2x30 mL) and water (2x30 mL), dried over sodium sulfate (Na_2SO_4), filtered, and evaporated. The residue was analyzed by ^{1}H-NMR.

5. Investigation of the potential for enzyme recycling on coconut oil acidolysis with octanoic acid (C8:0)

Coconut oil (1.00 g), octanoic acid (0.62 g), lipase enzyme (different lipase enzyme: lipase *Candida antarctica immobilized* on acrylic resin, and Lipozyme TL IM enzyme) (0.16 g) were added to the vial. The reaction mixture was agitated at 45^{o}C for 72 h. After the reactions, the lipase enzyme was removed by filtration using filter paper and washed with hexane. The enzyme catalyst was removed by filtration using filter paper. Ethanol (15 mL) and acetone (15 mL) were added to the filtrate together with a small amount of phenolphthalein indicator. The solution was titrated with 0.5 M NaOH until the solution turned pink. The solution was extracted with hexane (30 mL). The hexane layer was washed with 5% sodium carbonate solution (Na_2CO_3) (2x30 mL) and water (2x30 mL), dried over sodium sulfate (Na_2SO_4), filtered, and evaporated. The residue was analyzed by ^{1}H-NMR. After completion of the reaction, the catalyst was recovered, and used again under the same reaction conditions.

6. Investigation of the potential to decrease the enzyme loading on coconut oil acidolysis with octanoic acid (C8:0)

Coconut oil (1.00 g), octanoic acid (0.62 g), lipase enzyme (different lipase enzyme: lipase *Candida antarctica immobilized* on acrylic resin, and Lipozyme TL IM enzyme) (0.16, 0.08, or 0.04 g) were added to the vial. The reaction mixture was agitated at 45^{o}C for 72 h. After the reactions, the lipase enzyme was removed by filtration using filter paper and washed with hexane. The enzyme catalyst was removed by filtration using filter paper. Ethanol (15 mL) and acetone (15 mL) were added to the filtrate together with a small amount of phenolphthalein indicator. The solution was titrated with 0.5 M NaOH until the solution turned pink. The solution was extracted with hexane (30 mL). The hexane layer was washed with 5% sodium carbonate solution (Na_2CO_3) (2x30 mL) and water (2x30 mL), dried over sodium sulfate (Na_2SO_4), filtered, and evaporated. The residue was analyzed by ^{1}H-NMR.

7. Reaction scale-up of coconut oil acidolysis with the fatty acid (octanoic acid, C8:0)

Coconut oil (40.00 g), octanoic acid (40.0 g), and lipase enzyme (3.2 g) were dissolved in hexane (200.0 mL). The reaction was stirred at 45^{o}C for 7 days. The enzyme catalyst was removed by filtration using filter paper. Ethanol (60 mL) and acetone (60 mL) were added to the filtrate together with a small amount of phenolphthalein indicator. The solution was titrated with 0.5 M NaOH until the solution turned pink. The solution was extracted with hexane (120 mL). The hexane layer was washed with 5% sodium carbonate solution (Na_2CO_3) (2x120 mL) and water (2x120 mL), dried over sodium sulfate (Na_2SO_4), filtered, and evaporated. The residue was analyzed by ^{1}H-NMR.

8. Coconut oil acidolysis by octanoic acid (C8:0) and decanoic acid (C10:0)

Coconut oil (5.00 g), octanoic acid (1.8 g), decanoic acid (1.2 g), and lipase enzyme (0.4 g) were dissolved in hexane (25.00 mL). The reaction was stirred at 45^{o}C for 72 h. The enzyme catalyst was removed by filtration using a filter paper. Ethanol (15 mL) and acetone (15 mL) were added to the filtrate together with a small amount of phenolphthalein indicator. The solution was titrated with 0.5 M NaOH until the solution turned pink. The solution was extracted with hexane (30 mL). The hexane layer was washed with 5% sodium carbonate solution (Na_2CO_3) (2x30 mL) and water (2x30 mL), dried over sodium sulfate (Na_2SO_4), filtered, and evaporated. The residue was analyzed by ^{1}H-NMR and GC.

3.1.5.2 Coconut oil interesterification

Coconut oil (5.00 g), octanoic acid (3.0 g), decanoic acid (2.0 g), and lipase enzyme (0.4 g) were dissolved in hexane (25.00 mL). The reaction was stirred at 45^{o}C for 72 h. The enzyme catalyst was removed by filtration using a filter paper. Ethanol (15 mL) and acetone (15 mL) were added to the filtrate together with a small amount of phenolphthalein indicator. The solution was titrated with 0.5 M NaOH until the solution turned pink. The solution was extracted with hexane (30 mL). The hexane layer was washed with 5% sodium carbonate solution (Na_2CO_3) (2x30 mL) and water

(2x30 mL), dried over sodium sulfate (Na_2SO_4), filtered, and evaporated. The residue was analyzed by ^{1}H-NMR and GC

1. Initial experiments for coconut oil interesterification by methyl octanoate

Coconut oil (1.00 g), methyl octanoate (0.62 g), and lipase enzyme (Lipase enzyme used: lipase B from *Candida antarctica* immobilized on acrylic resin, and lipozyme TL IM enzyme) (0.16 g) were dissolved in hexane (5.00 mL). The reaction mixture was agitated for 72 h at 45^{o}C. After the reactions, the lipase enzyme was removed by filtration using a filter paper, and hexane was removed from the reaction mixture using a rotary evaporator. The fatty acid esters were removed by vacuum distillation using an oil pump. The residue was analyzed by ^{1}H-NMR spectroscopy.

2. Investigation of the effect of temperature for coconut oil interesterification by methyl octanoate

Coconut oil (1.00 g), methyl octanoate (0.62 g), lipase enzyme (Lipase enzyme used: lipase B from *Candida antarctica* immobilized on acrylic resin, and Lipozyme TL IM enzyme) (0.16 g) were added to the vial. The reaction mixture was agitated for 72 h at different temperatures (45^{o}C, 50^{o}C, 55^{o}C, 60^{o}C, 65^{o}C, 70^{o}C, and 75^{o}C). After the reactions, the lipase enzyme was removed by filtration using filter paper and washed with hexane. Then, hexane was removed from the reaction mixture using a rotary evaporator. The methyl esters were removed by vacuum distillation using a vacuum oil pump at reduced pressure. The residue was analyzed by ^{1}H-NMR spectroscopy. The experiments were carried out twice.

3. Investigation of the potential for enzyme recycling

Coconut oil (1.00 g), methyl octanoate (0.62 g), lipase enzyme (Lipase enzyme used: lipase B from *Candida antarctica* immobilized on acrylic resin, and Lipozyme TL IM enzyme) (0.16 g) were added to the vial. The reaction mixture was agitated at 45^{o}C for 72 h. After the reactions, the lipase enzyme was removed by filtration using filter paper and rinsed with hexane. The lipase enzyme was reused five times to set up a repeated reaction. Hexane was removed from the reaction mixture using a rotary evaporator. The residue was collected and subjected to vacuum

distillation using a vacuum oil pump to remove the methyl esters. The residue was analyzed by ^{1}H-NMR spectroscopy. The experiments were carried out twice.

4. Optimization by Response Surface Methodology

Design of Experiments (DOE) and response surface methodology (RSM) were used to optimize the processing conditions. The optimization work was carried out to investigate the combined effects of three factors; 1) Reaction time (h) (X1), 2) Ratio of methyl octanoate and coconut oil (w/w) (X2), and 3) Loading of enzyme catalyst (% w/w) (X3). Three levels of each of the three independent variables were chosen for study, which was -1, 0, and +1. The experimental design pattern of the three independent variables is summarized in Table 6.

Table 6 Values of parameters selected for optimization experiments

Independent Variable	**symbol**	**Levels**		
		-1	**0**	**+1**
Reaction time (h)	X_1	12	24	36
Reagent ratio (w/w)	X_2	0.6	0.9	1.2
Enzyme loading (%w/w)	X_3	1	5	10

The experiments of optimization by response surface methodology were carried out as follows. Coconut oil (1.0 g) mixed with methyl octanoate and Lipozyme TL IM enzyme at the desired ratio. The reaction mixture was agitated at 45^{o}C for the specified amount of time. Afterward, the lipase enzyme was removed by filtration and washed with hexane. Hexane was removed from the reaction mixture by a rotary evaporator, and the methyl esters present in the reaction mixture were removed by vacuum distillation at 140 ^{o}C. The residue was then purified through a silica gel plug in a Pasteur pipette using hexane as the mobile phase. The hexane solvent was removed

using a rotary evaporator, and the residual product was analyzed by ^{1}H-NMR. The integral value for the intermediate methylene units at 1.1-1.3 ppm was used directly as the observed variable (Y) to represent the length of the alkyl chains of the fatty acids. The analysis of the data was carried out using Design Expert 10.0.8. The response surface methodology-based analysis was carried out using a full factorial (3^3) Box-Behnken design with 15 runs summarized in Table 7. Integral of the peak between 1.1 and 1.3 ppm in the ^{1}H-NMR spectra of the product was used as the independent variable (Y). Regression analysis was performed to fit the response variable as a function of the independent variables.

Table 7 Level values for the Box-Behnken design of the response surface methodology experiments

Number of times	Reaction time	Ratio of methyl octanoate: coconut oil	Loading of enzyme
1	0	0	0
2	-1	-1	0
3	0	1	1
4	-1	0	-1
5	1	0	1
6	1	1	0
7	-1	0	1
8	0	-1	1
9	0	-1	-1
10	0	0	0
11	0	1	-1
12	-1	1	0
13	1	0	-1
14	0	0	0
15	1	-1	0

5. Interesterification of coconut oil with methyl octanoate at optimized conditions

The Interesterification reaction was carried out with coconut oil (1.0 g), methyl octanoate (1.16 g), and Lipozyme TL IM (0.12 g) as a catalyst. The reaction was stirred at 45^{o}C for 34.4 h. After the end of the reaction, the enzyme catalyst was removed by filtration using filter paper and washed with hexane. Next, hexane was removed by evaporation, and the fatty acid esters were removed by vacuum distillation at 140^{o}C. The residue was then purified through column chromatography on silica using hexane as the mobile phase. Finally, the hexane solvent was removed using a rotary evaporator. The residual product was analyzed by ^{1}H-NMR and GC techniques.

6. Reaction scale-up of interesterification of coconut oil with methyl octanoate

Coconut oil (20.0 g), methyl octanoate (23.2 g), and Lipozyme TL IM (2.4 g) were mixed. The reaction was stirred continuously at 45^{o}C for 34.4 h. Then, the lipase enzyme was removed by filtration and washed with hexane. A rotary evaporator removed the hexane from the reaction mixture, and the methyl esters present in the reaction mixture were removed by vacuum distillation at 140^{o}C. The residue was then purified through column chromatography on silica using hexane as the mobile phase. The hexane solvent was removed using a rotary evaporator. The residual product was analyzed by ^{1}H-NMR and GC.

7. Sourcing of methyl octanoate

Coconut oil fatty acid distillate (100 g) was added to a round bottom flask equipped with a reflux condenser. The flask was immersed in an oil bath heated to 120^{o}C, placed on top of a magnetic stirrer, and subjected to vacuum distillation ($4x10^{-3}$ mbar) using rotary vane pump (Edwards RV3) as shown in Figure 29. The fraction from distillation at 120^{o}C was collected and analyzed by ^{1}H-NMR and GC. The collected fraction of 16.5 g was heated in sealed tubes at 80^{o}C for 90 minutes with 200 mL MeOH/H_2SO_4 (98:2). Then, allowed to cool down to room temperature, water 20 mL and hexane 80 mL were added to the reaction mixture and extraction was conducted. The hexane layer was collected and dried in vacuum to yield a mixture of the fatty acid esters (16.2 g). The fatty acid esters were purified by vacuum distillation

at $4x10^{-3}$ mbar, collecting the fractions at 60 to 65°C (5.3 g). A combined portion of fatty acid esters obtained in the previous step (13.0 g) was subjected to a repeat of the procedures getting a fraction rich in methyl octanoate (5.2 g) and a fraction rich in methyl decanoate (3.6 g).

8. Interesterification of coconut oil with MCFA esters from coconut oil fatty acid distillate

The interesterification reaction was carried out with coconut oil (1.0 g), methyl octanoate-rich MCFA ester mixture (1.16 g), and Lipozyme TL IM (0.12 g) as a catalyst. The reaction was stirred at 45ºC for 34.4 h. After the end of the reaction, the enzyme catalyst was removed by filtration using filter paper and washed with hexane. Next, hexane was removed by evaporation, and the fatty acid esters were removed by vacuum distillation at 140ºC. The residue was then purified through column chromatography on silica using hexane as the mobile phase. Finally, the hexane solvent was removed using a rotary evaporator. The residual product was analyzed by ^{1}H-NMR and GC techniques.

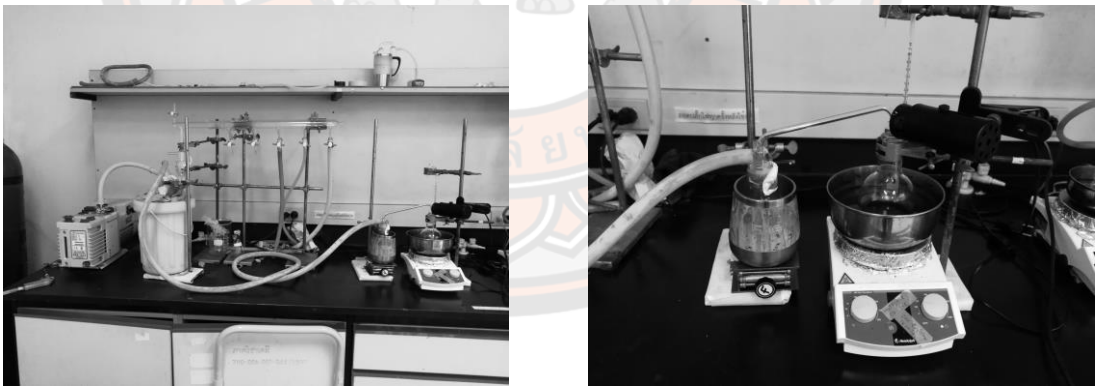


Figure 29 Set up for vacuum distillation

3.1.5.3 Preparation of modified coconut or low lauric coconut oil for biological testing

Coconut oil (Thai Pure Coconut Co., Ltd.) (100.0 g), methyl octanoate (72.0 g), methyl decanoate (48.0 g), and enzyme Lipozyme TL IM (11.0 g) were mixed. The reaction was stirred continuously at 45^{o}C for 96 h. The enzyme catalyst was removed by filtration and washed with hexane. A rotary evaporator removed the hexane from the reaction mixture and the fatty acids in the reaction mixture were removed by vacuum distillation using an oil pump. The residue was then purified through column chromatography on silica using hexane as the mobile phase. The hexane solvent was removed using a rotary evaporator. The desired product was analyzed by ^{1}H-NMR and GC spectroscopy.

3.2 Part II Enzymatic hydrolysis of capsaicin and dihydrocapsaicin and utilization of the fatty acid product for triglyceride modification

3.2.1 Material and Chemicals

Capsaicinoid powder containing primarily capsaicin and dihydrocapsaicin was obtained from Uthai research group (URG), Department of Chemistry, Naresuan University, Phitsanulok, Thailand. Coconut oil (Naturel brand/Lam Soon public company, Thailand) was purchased at a local supermarket. Lipozyme® TL IM and NOVOZYM® 435 were obtained from Strem Chemicals. Ethanol, ethyl acetate, acetone, sulfuric acid, hydrochloric acid, methanol, and phenolphthalein were obtained from Carlo Erba. Hexane was purchased from RCl Lab scan limited. Sodium hydroxide and sodium carbonate were obtained from UNIVAR. Sodium sulfate anhydrous and tri-Sodium orthophosphate dodecahydrate were obtained from LOBA CHEMIE. Chloroform-D was from Cambridge isotope laboratories, Inc. Silica was from Silicycle.

3.2.2 Apparatus

GC analysis was performed on a Shimadzu gas chromatography system (GC-2014) equipped with a flame ionization detector, and a capillary fused silica column (Rtx-Wax, length: 30 m, inner diameter: 0.25 mm, film thickness: 0.25 mm). The initial temperature was set to 120^{o}C and was held for 7 minutes. This was followed

up by a linear gradient up to 240^{o}C over the next 10 minutes, with the final temperature being held for further 50 minutes.

^{1}H-NMR analysis was recorded using $CDCl_3$ as a solvent on a Bruker Advance NMR spectrometer operating at 400 MHz. Chemical shifts (δ) are given in ppm relative to tetramethylsilane (TMS).

MS analysis was obtained on an Agilent 6540 ultra-high definition (UHD) accurate mass Q-TOF LC/MS system and Mass Hunter workstation data mining.

3.2.3 NMR analysis

The scope of modification of coconut oil acidolysis reaction was evaluated using ^{1}H-NMR spectroscopy. The critical signal for this evaluation is the doublet at 0.95 ppm. This signal of the integral was used as the observed variable to determine the scope of coconut oil modification. The integral of the proton at 4.1 to s 4.2 ppm representing protons of the sn-1,3 position on the glycerol backbone was set to 2 to standardize the integrals.

3.2.4 GC analysis

The Modified oil sample (0.03 g) was heated in sealed tubes at 80°C for 90 minutes with 3 ml MeOH/H_2SO_4 (98:2). The reaction mixture was let to cool down, and 1 ml of water was added to the mixture. Then the methyl esters were extracted with 5 ml of hexane and quantified using GC analysis.

The GC analysis was performed on a Shimadzu gas chromatography system (GC-2014) equipped with a flame ionization detector and a capillary fused silica column (Rtx-Wax, length: 30 m, inner diameter: 0.25 mm, film thickness: 0.25 µm). The temperature program was as follows: injector and detector temperatures were 250°C; the initial column temperature was set to 120^{o}C and was held for 7 minutes. Followed by a programmed rise to 240^{o}C at 10 minutes and then maintained temperature held for 50 minutes. The injection volume was 1.0 µl. The percent values reported below for individual fatty acids represent the percentages of their corresponding GC peak areas relative to the total peak areas of all reported fatty acids.

3.2.5 Experimental

3.2.5.1 Sourcing of fatty acid from capsaicinoid hydrolysis

1. Initial experiment for the preparation of fatty acid from the hydrolysis of capsaicinoids

Purified capcaicinoids (0.5 g) were dispersed in phosphate buffer (75 mL, pH 7.0, 80 mM), and lipase B from *Candida antarctica* immobilized on acrylic resin (0.75 g) was added to the reaction mixture in the Erlenmeyer flask. The reaction was carried out under a nitrogen atmosphere on a hotplate at 45°C for 7 days and left without agitation. After the reaction, the reaction mixture was removed from the Erlenmeyer flask using a pump with the inlet covered with a cloth. The pH of the solution was adjusted to 10 and it was extracted with ethyl acetate (3x20 mL). The combined ethyl acetate fractions were dried over sodium sulfate, filtered by filter paper, and evaporated by a rotary evaporator. The capsaicinoids present in the residue were analyzed by ^{1}H-NMR. The pH of the reaction mixture was then adjusted to pH 5 and it was extracted with ethyl acetate (3×20 mL). The combined ethyl acetate extracts were dried over sodium sulfate, filtered by filter paper, and evaporated by a rotary evaporator. The fatty acids present in the residue were analyzed by ^{1}H-NMR.

2. Enzyme selection for the preparation of fatty acids from the hydrolysis of capsaicinoids

The reaction was performed in 2 Erlenmeyer flasks as follows. Capsaicinoids (0.5 g) were dispersed in phosphate buffer (75 mL, pH 7.0, 80 mM). Enzyme catalysts, either Lipozyme TL IM (0.75 g) or NOVOZYM 435 (0.75 g), were added to each reaction mixture in the Erlenmeyer flask. The reaction mixture was carried out under a nitrogen atmosphere on a hotplate at 45°C for 7 days and left without agitation. After the reaction, the reaction mixture was removed from the Erlenmeyer flask using a pump with the inlet covered with a cloth. The pH of the solution was adjusted to 10 and it was extracted with ethyl acetate (3x20 mL). The combined ethyl acetate fractions were dried over sodium sulfate, filtered by filter paper, and evaporated by a rotary evaporator. The capsaicinoids present in the residue was analyzed by ^{1}H-NMR. The pH of the reaction mixture was then adjusted to pH 5 and it was extracted with ethyl acetate (3×20 mL). The combined ethyl acetate extracts were dried over

sodium sulfate, filtered by filter paper, and evaporated by a rotary evaporator. The fatty acids present in the residual product were analyzed by ^{1}H-NMR.

3. Effect of reaction time for the preparation of fatty acids from the hydrolysis of capsaicinoids

The reaction was performed in 2 Erlenmeyer flasks as follows. Capsaicinoids (0.5 g) were dispersed in phosphate buffer (75 mL, pH 7.0, 80 mM), and NOVOZYM 435 (0.75 g) was added to the reaction mixture in the Erlenmeyer flask. The reaction was carried out under a nitrogen atmosphere on a hotplate at 45°C and left without agitation. The two reactions were carried out at for different length of time; 3 days and 7 days. After the reaction, the reaction mixture was removed from the Erlenmeyer flask using a pump with the inlet covered with a cloth. The pH of the mixture was adjusted to 10, then extracted with ethyl acetate (3x20 mL). The combined ethyl acetate fractions were dried over sodium sulfate, filtered by filter paper, and evaporated by a rotary evaporator. The capsaicinoids present in the residue were analyzed by ^{1}H-NMR. Then, the pH of the mixture was adjusted to 5 and extracted with ethyl acetate (3×20 mL). The combined ethyl acetate extracts were dried over sodium sulfate, filtered by filter paper, and evaporated by a rotary evaporator. The fatty acids present in the residual product were analyzed by ^{1}H-NMR.

4. Effect of temperature for the preparation of fatty acids from the hydrolysis of capsaicinoids

The reaction was conducted in 3 Erlenmeyer flasks as follows. Capsaicinoids (0.5 g) were dispersed in phosphate buffer (75 mL, pH 7.0, 80 mM), and NOVOZYM 435 (0.75 g) was added to the reaction mixture in the Erlenmeyer flask. The reaction mixture was kept under a nitrogen atmosphere on a hotplate at different temperatures of 45, 50, and 55ºC and left without agitation. The reactions were carried out for 3 days. After the reaction, the solution was removed from the Erlenmeyer flask using a pump with the inlet covered with a cloth. The pH of the mixture was adjusted to 10 and then it was extracted with ethyl acetate (3x20 mL). The combined ethyl acetate fractions were dried over sodium sulfate, filtered by filter paper, and evaporated by a rotary evaporator. The capsaicinoids present in the residue were analyzed by ^{1}H-NMR. Afterward, the pH of the mixture was adjusted to 5 and the mixture was extracted with ethyl acetate (3×20 mL). The combined ethyl acetate extracts were dried over

sodium sulfate, filtered by filter paper, and evaporated by a rotary evaporator. The fatty acids present in the residual product were analyzed by ^{1}H-NMR.

5. Preparation of fatty acid from the hydrolysis of capsaicinoids

Capsaicinoids (0.5 g) were dispersed in phosphate buffer (75 mL, pH 7.0, 80 mM), and NOVOZYM 435 (0.75 g) was added to the reaction mixture in the Erlenmeyer flask. The reaction was carried out under a nitrogen atmosphere placed on a hotplate for 3 days at 50°C and left without agitation. After the reaction, the solution was removed from the Erlenmeyer flask using a pump with the inlet covered with a cloth. The pH of the mixture was adjusted to 10 and then it was extracted with ethyl acetate (3x20 mL). The combined ethyl acetate fractions were dried over sodium sulfate, filtered by filter paper, and evaporated by a rotary evaporator. The capsaicinoids present in the residue was analyzed by ^{1}H-NMR. After that, the pH of the mixture was adjusted to 5 and the mixture was extracted with ethyl acetate (3×20 mL). The combined ethyl acetate extracts were dried over sodium sulfate, filtered by filter paper, and evaporated by a rotary evaporator. The fatty acids present in the residual product were analyzed by ^{1}H-NMR. Afterward, these fatty acids were then collected for further application. After removing the solution, the lipase enzyme remained in the Erlenmeyer flask. This lipase enzyme was reused to set up repeated reaction runs in the following manner. Capsaicin (100 mg) and phosphate buffer (75 mL, pH 7.0, 80 mM) were added to the Erlenmeyer flask. The reaction was carried out and worked up as described above. The experiments were carried out continuously with reaction removal, workup, and renewal every 3 days. The fatty acids obtained from the continuous experiment was combined and analyzed by GC, MS, and ^{1}H-NMR.

3.2.5.2 Coconut oil acidolysis with the fatty acids from capsaicinoid hydrolysis

1. Initial experiment for coconut oil acidolysis with the fatty acids from capsaicinoids

Coconut oil (0.20 g), fatty acids (0.12 g), and Lipozyme TL IM enzyme (0.032 g, 10%) were added to a vial. The reaction mixture was agitated at 45°C for 72 h. After the reaction, the reaction mixture was filtered with filter paper. Ethanol (5 mL), acetone (5 mL), and a small amount of phenolphthalein indicator were added to

the reaction mixture. The solution was titrated with a 0.5 M NaOH solution until the color changed to pink. The solution was extracted with hexane (10 mL). The solution was separated into two layers; the hexane layer was collected and extracted with Na_2CO_3 (2x10 mL) followed by H_2O (10 mL). The hexane solution was dried over Na_2SO_4, filtered by filter paper, and evaporated by a rotary evaporator. The residue product was analyzed by ^{1}H-NMR and GC.

2. Adjusted conditions for coconut oil acidolysis with the fatty acids from capsaicinoids

Coconut oil (0.20 g), fatty acids (0.12 g), and Lipozyme TL IM enzyme (0.032 g, 10%) were added to a vial. The reaction mixture was agitated at 60^{o}C for 72 h. After the reaction, the reaction mixture was filtered with filter paper. Ethanol (5 mL), acetone (5 mL), and a small amount of phenolphthalein indicator were added to the solution. The solution was titrated with 0.5 M NaOH until the color changed to pink. The solution was extracted with hexane (10 mL). The solution was separated into two layers; the hexane layer was collected and extracted with Na_2CO_3 (2x10 mL) followed by H_2O (10 mL). The hexane solution was dried over Na_2SO_4, filtered by filter paper, and evaporated by a rotary evaporator. The residue was analyzed by ^{1}H-NMR and GC.

3. Investigation of the effect of enzyme loading

Coconut oil (0.20 g), fatty acids (0.12 g), and Lipozyme TL IM enzyme at different loading (0.0032 g, 0.016 g, 0.024 g, 0.032 g) were added to a vial. The reaction mixture was agitated at 60^{o}C for 72h. After the reaction, the reaction mixture was filtered with filter paper. Ethanol (5 mL), acetone (5 mL), and a small amount of phenolphthalein indicator were added to the solution. The solution was titrated with 0.5 M NaOH until the color changed to pink. The solution was extracted with hexane (10 mL). The solution was separated into two layers; the hexane layer was collected and extracted with Na_2CO_3 (2x10 mL) followed by H_2O (10 mL). The hexane solution was dried over Na_2SO_4, filtered by filter paper, and evaporated by a rotary evaporator. The residue was analyzed by ^{1}H-NMR and GC.

4. Optimization by Response Surface Methodology (RSM)

Design Expert 10.0.8 software was used to optimize the modified coconut oil from interesterification with fatty acid and Lipozyme TL IM lipase. The independent

variables investigated were three factors: 1) Reaction time (h) (X1), 2) Ratio of coconut oil and fatty acids (w/w) (X2), and 3) Reaction temperature (^{o}C) (X3). Three levels of each of the three independent variables were chosen for study, which was -1, 0, and +1. Table 8 shows the experiment design pattern of the three independent variables in coded and actual units.

The experiments of optimization by response surface methodology were carried out as follows. Coconut oil (0.1 g) mixed with fatty acids from capsaicinoids and Lipozyme TL IM enzyme at the desired ratio. The reaction mixture was agitated at the desired temperature for the desired amount of time. After the end of the reaction, the mixture was filtered with filter paper. Ethanol (5 mL), acetone (5 mL), and a small amount of phenolphthalein indicator were added to the solution. The solution was titrated with 0.5 M NaOH until its color changed to pink. The solution was extracted with hexane (10 mL). The solution was separated into two layers; the hexane layer was collected and extracted with 5% Na_2CO_3 (2x10 mL) and H_2O (10 mL). The hexane solution was dried over sodium sulfate, filtered, and evaporated. The residue was analyzed by ^{1}H-NMR.

Table 8 Experiment variables in coded and actual unit

Independent Variable	**symbol**	**Levels**		
		-1	**0**	**+1**
Reaction time (h)	X_1	24	48	72
Reagent ratio (w/w)	X_2	0.6	0.9	1.2
Temperature (^{o}C)	X_3	50	60	70

5. Increased fatty acid loading for coconut oil acidolysis with the fatty acids from capsaicinoids

Coconut oil (0.10 g), fatty acids (0.12 g), and Lipozyme TL IM enzyme loading (0.022 g, 10%) were added to a vial. The reaction mixture was agitated at 60^{o}C for 72 h. After the reaction, the reaction mixture was filtered with filter paper. Ethanol

(5 mL), acetone (5 mL), and a small amount of phenolphthalein indicator were added to the solution. The solution was titrated with 0.5 M NaOH until the color changed to pink. The solution was extracted with hexane (10 mL). The solution was separated into two layers; the hexane layer was collected and extracted with Na_2CO_3 (2x10 mL) followed by H_2O (10 mL). The hexane solution was dried over Na_2SO_4, filtered by filter paper, and evaporated by a rotary evaporator. The residue product was analyzed by ^{1}H-NMR and GC.

6. Reaction scale-up of coconut oil acidolysis with the fatty acids hydrolyzed from capsaicinoids

Coconut oil (0.20 g), fatty acids (0.24 g), and Lipozyme TL IM enzyme at different loading (0.044 g, 10%) were added to a vial. The reaction mixture was agitated at 60^{o}C for 72 h. After the reaction, the reaction mixture was filtered with filter paper. Ethanol (5 mL), acetone (5 mL), and a small amount of phenolphthalein indicator were added to the solution. The solution was titrated with 0.5 M NaOH until the color changed to pink. The solution was extracted with hexane (10 mL). The solution was separated into two layers; the hexane layer was collected and extracted with Na_2CO_3 (2x10 mL) followed by H_2O (10 mL). The hexane solution was dried over Na_2SO_4, filtered by filter paper, and evaporated by a rotary evaporator. The residue product was analyzed by ^{1}H-NMR and GC.

3.3 Part III Degradation of poly(ethylene terephthalate) (PET) by photooxidative and enzymatic catalysis

3.3.1 Material and Chemicals

The liquid preparations with Humicola insolens cutinase (HiC, product Novozym© 51032) were purchased from Strem Chemicals. PET bottle film used was from post-consumer non-carbonated water bottles (brand Singha drinking water©). PET granules and tris-buffer were purchased from Sigma-Aldrich. Sodium hydroxide was obtained from UNIVAR. Methanol was obtained from Carlo Erba. Iridium complex solution was from Filip Kielar Group.

3.3.2 Preparation of PET films

PET bottle film used from post-consumer non-carbonated water bottles was taken. It is washed using sterile distilled water to remove any impurities. It was cut into squares of approx. 0.5 cm, and it presented 0.1 mm thickness. The films were again washed, dried, and stored in a clean desiccator.

3.3.3 Preparation of Iridium complex

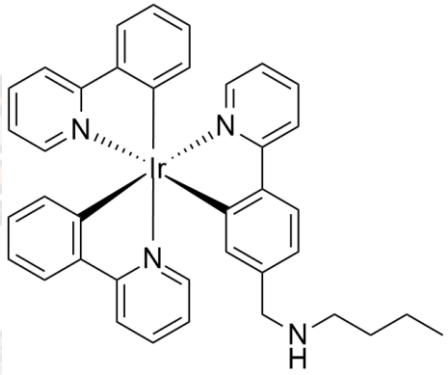


Figure 30 Structure of iridium complex

Iridium complex (modified from Filip Kielar Group) (Conc. 1000 ppm) 1 mg dissolves with 10 ml of water or organic solvent.

3.3.4 Experimental

3.3.4.1 Preliminary analysis by weight loss (calculating the percentage of weight loss of PET films)

The test for weight loss is calculated according to the equation: WL = 100x (W1 – W2)/W1), where W1 and W2 are the weights of the samples before and after treatment, respectively.

3.3.4.2 Degradation of poly (ethylene terephthalate) (PET) by enzymatic hydrolysis

1. Initial experiments

PET film (1.00 g), Tris buffer 12.5 mL (397mM pH 8.95), and enzyme (65 µl, 0.065 g/g) were added in a vial. The reaction was stirred at 62-65^{o}C and 70^{o}C for 1 week. The enzyme catalyst was removed by filtration using filter paper. PET film was

washed with distilled water and dried in a drying oven set at 100°C. The PET film after the end of the process was weighed for the calculation of percentage of weight loss according to the equation above.

2. Effect of temperature

PET film (1.00 g), Tris buffer 12.5 mL (397mM pH 8.95), and enzyme (65 µl, 0.065 g/g) were added in a vial. The reaction was stirred at 62-65 and 70°C for 1 week. The enzyme catalyst was removed by filtration using filter paper. PET film was washed with distilled water and dried in a drying oven set at 100°C. The PET film after the end of the process was weighed for the calculation of percentage of weight loss according to the equation above.

3. Effect of enzyme loading

PET film (1.00 g), Tris buffer 12.5 mL (397mM pH 8.95), and enzyme (65, 130, and 195µl)were added in a vial. The reaction was stirred at 62-70°C for 1 week. The enzyme catalyst was removed by filtration using filter paper. PET film was washed with distilled water and dried in a drying oven set at 100°C. The PET film after the end of the process was weighed for the calculation of percentage of weight loss according to the equation above.

4. Effect of reaction time

PET film (1.00 g), Tris buffer 12.5 mL (397mM pH 8.95), and enzyme **(**65 µl, 0.065 g/g) were placed in a vial. The reaction was stirred at 62-70°C for 1, 2, 3, 4, 6, and 8 weeks. The enzyme catalyst was removed by filtration using filter paper. PET film was washed with distilled water and dried in a drying oven set at 100°C. The PET film after the end of the process was weighed for the calculation of percentage of weight loss according to the equation above.

5. Effect of continuous enzyme loading

PET film (1.00 g), Tris buffer (12.5 mL, 397mM, pH 8.95), and enzyme (65 µl) were placed in a vial. The reaction was stirred at 62-70°C for one week. Afterward, the enzyme catalyst and Tris buffer were removed by filtration using filter paper. PET film was washed with distilled water, dried in a drying oven set at 100°C, and measured for weight loss. After that, PET film, enzyme catalyst, and Tris buffer were added to the vial for the continuous response. The experiments were carried out continuously every week for 8weeks.

3.3.4.3 PET degradation by photooxidative and enzymatic catalysis

PET film (1.00 g) and iridium complex dissolved in water (20 mL) were placed in a vial. The reaction was stirred using a magnetic stirrer at room temperature and irradiated for 24, 48, and 72 h. After reactions, the iridium complex was removed by filtration using filter paper. The PET film was washed with water. In the next step, PET film was mixed with tris buffer (12.5 mL,397 mM pH 8.95) and enzyme (65 µl, 0.065 g/g) in a vial. The reaction was stirred at 62-70°C for one week. Then, the enzyme catalyst was removed by filtration using filter paper. Finally, PET film was washed with distilled water and dried in a drying oven set at 100°C. The PET film after the end of the process was weighed for the calculation of percentage of weight loss according to the equation above.

PET film (1.00 g) and iridium complex dissolved in methanol (20 mL) were placed in a vial. The reaction was stirred using a magnetic stirrer at room temperature and irradiated for 24, 48, and 72 h. After reactions, the iridium complex was removed b filtration using filter paper. The PET film was washed with water. In the next step, PET film was mixed with tris buffer (12.5 mL (397 mM pH 8.95) and enzyme (65 µl, 0.065 g/g) in a vial. The reaction was stirred at 62-70°C for one week. Then, the enzyme catalyst was removed by filtration using filter paper. Finally, PET film was washed with distilled water and dried in a drying oven set at 100°C. The PET film after the end of the process was weighed for the calculation of percentage of weight loss according to the equation above.

3.3.4.3 PET surface activation by alkali pretreatment

PET films cut from post-consumer water bottles and PET granules from Sigma Aldrich were soaked in 10 M NaOH at room temperature for 24 hours. Then, the PET materials were removed by filtration with filter paper and washed using distilled water. The PET materials were dried in an incubator at 90–100°C for 24 hours and stored in a clean desiccator.

After the pretreatment of both types of PET films. PET sample (1.00 g), Tris buffer 12.5 mL (397 mM, pH 8.95), and Novozym 51032 enzyme (65 µl) were

added in a vial. The reaction was stirred at 62–70°C for 7 days. The enzyme catalyst was removed by filtration using filter paper. PET film was washed with distilled water and dried in a drying oven set at 100°C for 24 h. PET samples were tested for weight loss calculated according to the equation: WL = 100*(W1 – W2)/W1), where W1 and W2 are the weights of the samples before and after treatment, respectively.

CHAPTER IV

RESULT AND DISCUSSION:

This project investigates enzyme lipase applications for catalyzing transformations of natural and synthetic materials, including capsaicin hydrolysis, coconut oil modification, and PET decomposition. The reactions will use lipase enzymes as catalysts and will be evaluated using the ^{1}H-NMR and GC methods.

4.1 Part I Enzymatic modification of coconut oil by acidolysis or interesterification with MCFAs or their alkyl esters

It is known that medium-chain fatty acids (MCFAs) can be readily digested and absorbed. MCFAs can be directly transported to the liver and rapidly metabolized as a source of energy, making them less likely to accumulate as body fat. Moreover, fats and oils rich in MCFAs can benefit health, with a major emphasis on obesity control and improved cognitive function. One of the conventional ways of consuming MCFAs is in the form of medium-chain triglycerides (MCT) oils. MCT oils are synthetic fats containing only MCFAs, usually made by the esterification of glycerol with purified MCFAs. Thus, acidolysis or interesterification (Figure 31) are one of the possible ways for modifying the fatty acid composition of natural fats and oils to achieve composition, which is more alike to MCT oils. Coconut oil is one of the plant oils with higher content of MCFAs. Therefore, we decided to prepare more MCT like products by the modification of coconut oil using acidolysis and interesterification.

4.1.1 Coconut oil acidolysis reaction

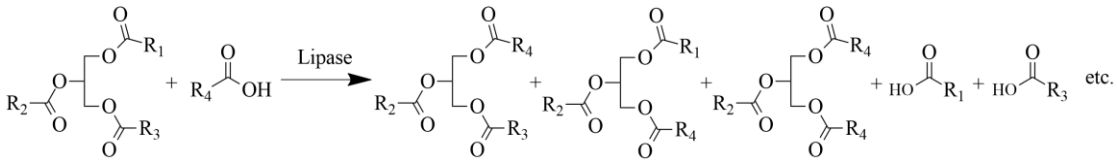


Figure 31 Triglyceride acidolysis reactions

1. Initial experiments: coconut oil acidolysis with fatty acid from commercial

Acidolysis involves the reaction between a fatty acid and oil or fat where the fatty acid and the triglyceride source are selected based on the desired product. We investigated acidolysis as one of the possible reactions for modification of the composition of coconut oil with MCFAs octanoic acid and decanoic acid. The initial acidolysis reactions were carried out at 45^{o}C for 24 h with a 1:3 mixture of coconut oil and octanoic acid (C8:0) in hexane as the solvent. The lipase enzyme loading for the initial experiments was 5% w/w relative to the weight of the reagents. The initial acidolysis reactions were carried out with lipase B from *Candida antarctica* immobilized on acrylic resin. The outcome of the experiment was evaluated using ^{1}H-NMR. The NMR spectrum of the product (Figure 32) was compared with the NMR spectrum of the original coconut oil (Appendix A Figure 94). The results indicated that the reaction did not result in any significant change in the fatty acid composition of coconut oil. Therefore, the procedure was modified by extending the reaction time to 72 h. The ^{1}H-NMR spectrum for this reaction is shown in Figure 33 and the fatty acid composition of the product has changed significantly relative to the starting coconut oil.

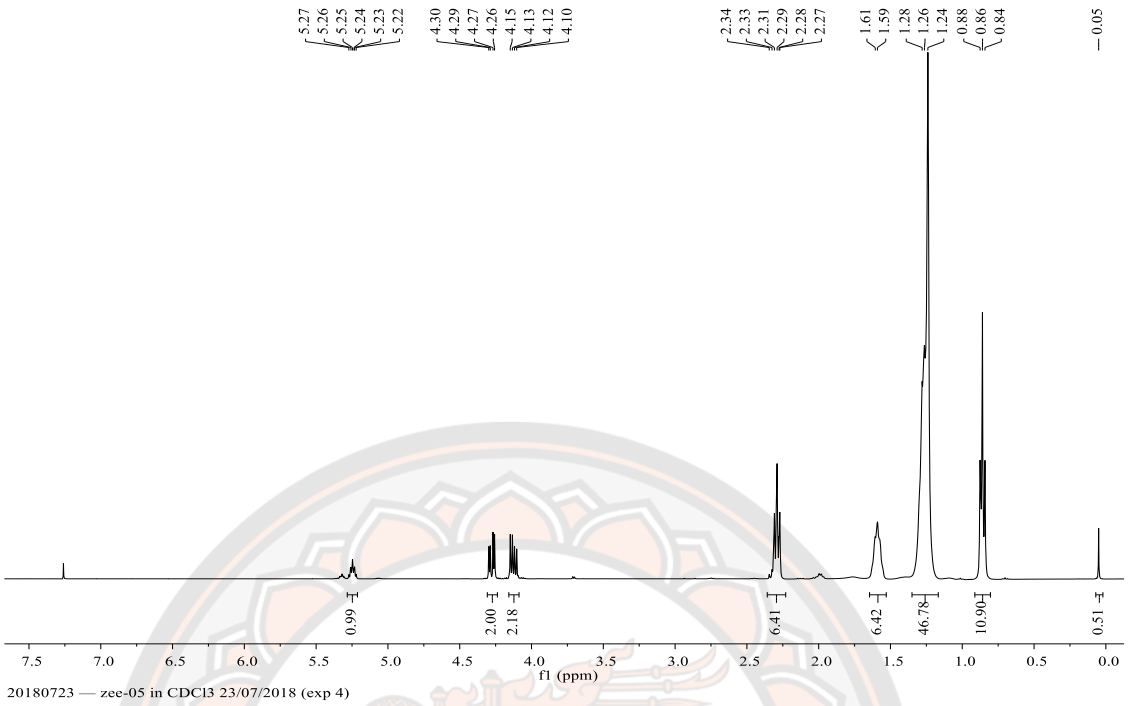


Figure 32 ^{1}H-NMR spectrum of oil obtained by enzyme lipase B from *Candida antarctica* immobilized on acrylic resin catalyzed acidolysis of coconut oil with octanoic acid at 24 h (initial reaction)

The NMR spectrum shown in Figure 33 shows that the integral of the proton signal at 1.1–1.3 ppm decreased from 51.8 in the original coconut oil to 41.99, which is significantly lower than in the original coconut oil. It demonstrates that the chain length of the fatty acids in the modified oil product has changed and is shorter than the original coconut oil, which is consistent with the exchange of fatty acids originally present in coconut oil with octanoic acid through acidolysis.

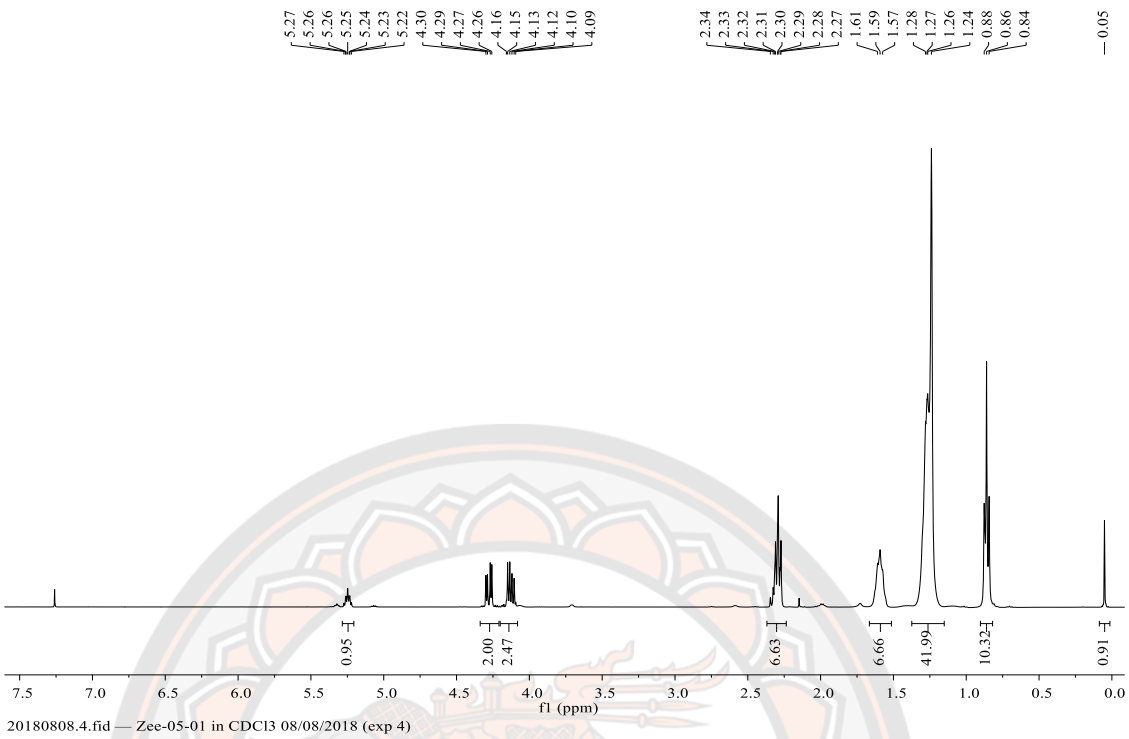


Figure 33 ^{1}H-NMR spectrum of oil obtained by enzyme lipase B from *Candida antarctica* immobilized on acrylic resin catalyzed acidolysis of coconut oil with octanoic acid at 72h (initial reaction)

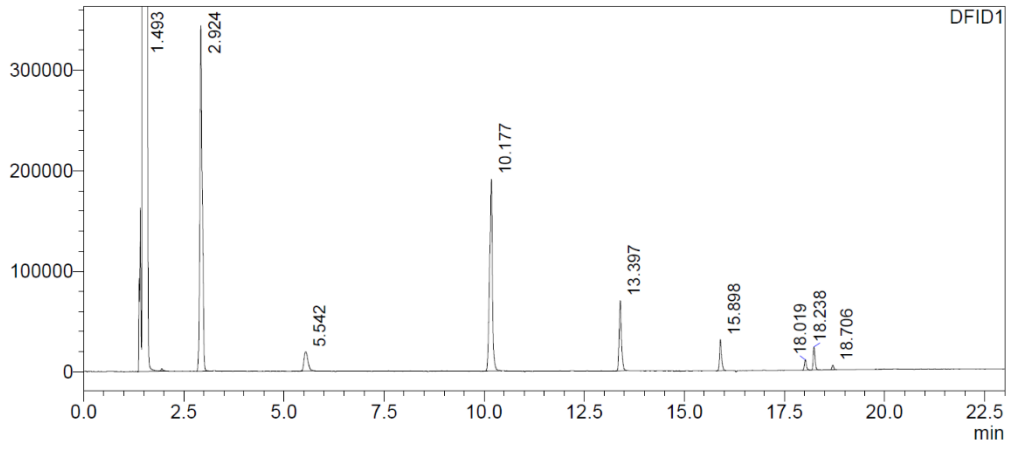


Figure 34 GC spectrum of oil obtained by enzyme lipase B from *Candida antarctica* immobilized on acrylic resin catalyzed acidolysis of coconut oil with octanoic acid at 72h (initial reaction)

Table 9 Fatty acid composition of modified coconut oil obtained by enzyme lipase B from *Candida antarctica* immobilized on acrylic resin catalyzed acidolysis of coconut oil with octanoic acid at 72h (initial reaction) determined by gas chromatography

Fatty acid symbol	Fatty acid trivial name	Retention time (min)	Specifications (%)
C8:0	Caprylic acid	2.924	47.73
C10:0	Capric acid	5.542	4.42
C12:0	Lauric acid	10.177	30.81
C14:0	Myristic acid	13.397	9.20
C16:0	Palmitic acid	15.898	3.71
C18:2	Linoleic acid	18.019	1.22
C18:1	Oleic acid	18.238	2.45
C18:0	Stearic acid	18.706	0.46

Furthermore, the results from GC analysis also confirmed the transformation in the composition of coconut oil as shown in Figure 34 and the percentage of content of individual fatty acids can be seen in Table 9. The content of octanoic acid (C8:0) increased from 8.20% in the original coconut oil to 47.73% while the content of lauric acid decreased from 48.5% to 30.81%. These results confirm that the acidolysis reaction can be used for the modification of chain length of the fatty acids in coconut oil.

2. Select suitable enzyme for coconut oil acidolysis

Having established the possibility to perform the acidolysis reaction in the initial experiments, we decided to investigate other lipase enzymes to select a suitable

one. The lipase enzymes we tested were B from *Candida antarctica* acrylic resin, lipase from *Pseudomonus cepacia* immobilized on immobead 150, lipase from *Rhizopus oryzae* immobilized on immobead 150, lipase from *Candida rugosa* immobilized on immobead 150, and Lipozyme TL IM. The reactions were carried out using the conditions described above. The transformation of coconut oil achieved in these reactions was evaluated using ^{1}H-NMR (Appendix A Figure 107 - 111) and is summarized in Figure 35

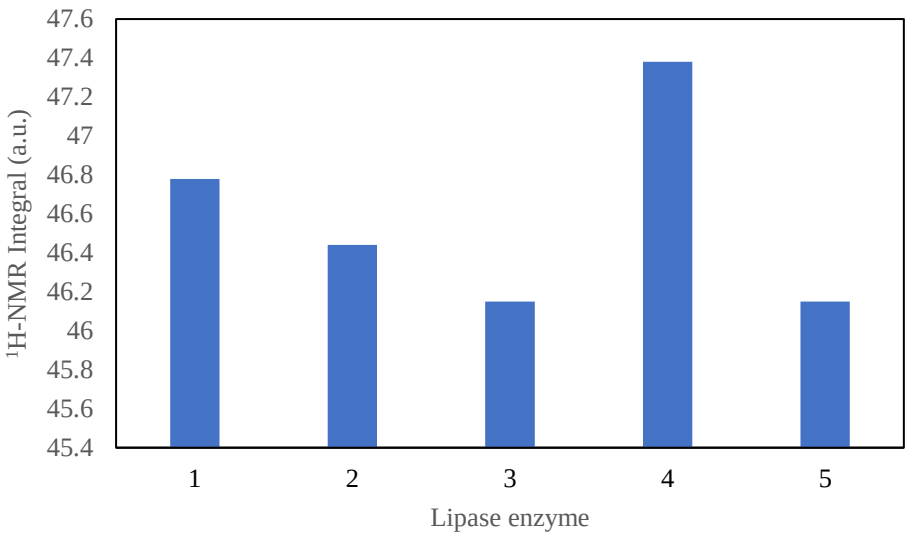


Figure 35 The integral of ^{1}H-NMR signal at position 1.1-1.3 ppm observed in modified coconut oil obtained by acidolysis using 1) lipase B from *Candida antarctica* acrylic resin, 2) lipase from *Pseudomonus cepacia* immobilized on immobead 150, 3) lipase from *Rhizopus oryzae* immobilized on immobead 150 , 4) lipase from *Candida rugosa* immobilized on immobead 150, and 5) Lipozyme TL IM

The ^{1}H-NMR integrals for the signal at position 1.1-1.3 ppm observed in the products of the modification reactions were 46.78, 46.44, 46.15, 47.38, and 46.15 for 1) lipase B from *Candida antarctica* acrylic resin, 2) lipase from *Pseudomonus cepacia* immobilized on immobead 150, 3) lipase from *Rhizopus oryzae* immobilized

on immobead 150, 4) lipase from *Candida rugosa* immobilized on immobead 150, and 5) Lipozyme TL IM, respectively. The best results were obtained with lipase from *Rhizopus oryzae* immobilized on immobead 150 and Lipozyme TL IM. However, the differences in the performance of these enzymes were rather small. Given these results, the follow-up experiments focused on Lipozyme TL IM and lipase B from *Candida antarctica* immobilized on acrylic resin due to their good performance and comparatively low cost.

3. Investigation of the effect of temperatures on coconut oil acidolysis

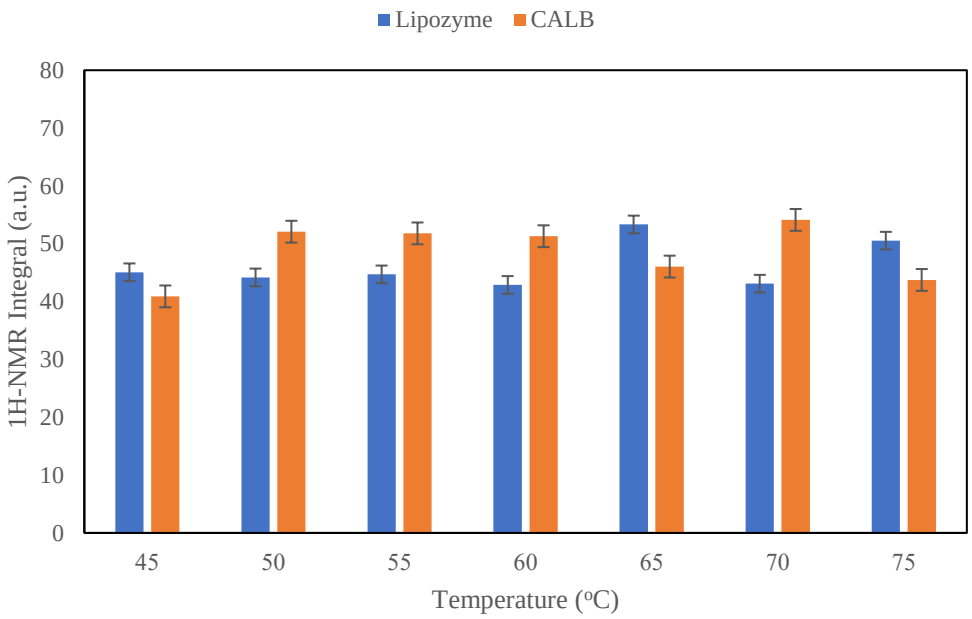


Figure 36 The integral signal of proton at position 1.1-1.3 ppm observed in modified coconut oil obtained by acidolysis using lipase Lipozyme TL IM and lipase B from *Candida antarctica* acrylic resin versus reaction temperature

To get further insight into the performance of the process, the effect of temperature on the acidolysis reaction catalyzed by the two selected enzymes has been investigated between 45˚C and 75˚C. The results are shown in Figure 36.

Both enzymes respond differently to changes in temperature. The performance of Lipozyme TM IM at temperatures of 50°C, 55°C, and 60°C differs only a little from the performance at 45°C. Subsequently a large drop in performance was observed at 65°C and 75°C. For lipase B from *Candida antarctica* immobilized on acrylic resin, the performance at 50°C, 55°C, and 60°C is worse relative to the performance at 45°C. A slight improvement in performance is observed at 65°C. However, the performance deteriorates further at 70°C. The results indicate that the performance of the enzymes does not improve at higher temperatures and likely gets compromised by temperature-promoted denaturization of the enzymes.

4. Investigation of the potential for enzyme recycling on coconut oil acidolysis

Enzyme catalysts are expensive, therefore utilizing them efficiently is essential. The immobilized enzyme catalysts used in this study can be separated from the reaction mixture and recycled for future use. Therefore, we performed an experiment in which the enzyme catalysts were removed from the reaction mixture by filtration and then reused in another round of acidolysis of coconut oil. The reactions were carried out using conditions described above with the enzymes used in five repeats. The results of this experiment can be seen in Figure 37. The results indicated that both enzymes remain active after recycling. In the case of Lipozyme TL IM there is a gradual decrease in the activity with repeated runs of the reaction. The experiment results with lipase B from *Candida antarctica* immobilized on acrylic resin show that the enzyme can maintain its activity throughout the experiment to a greater extent than Lipozyme TL IM.

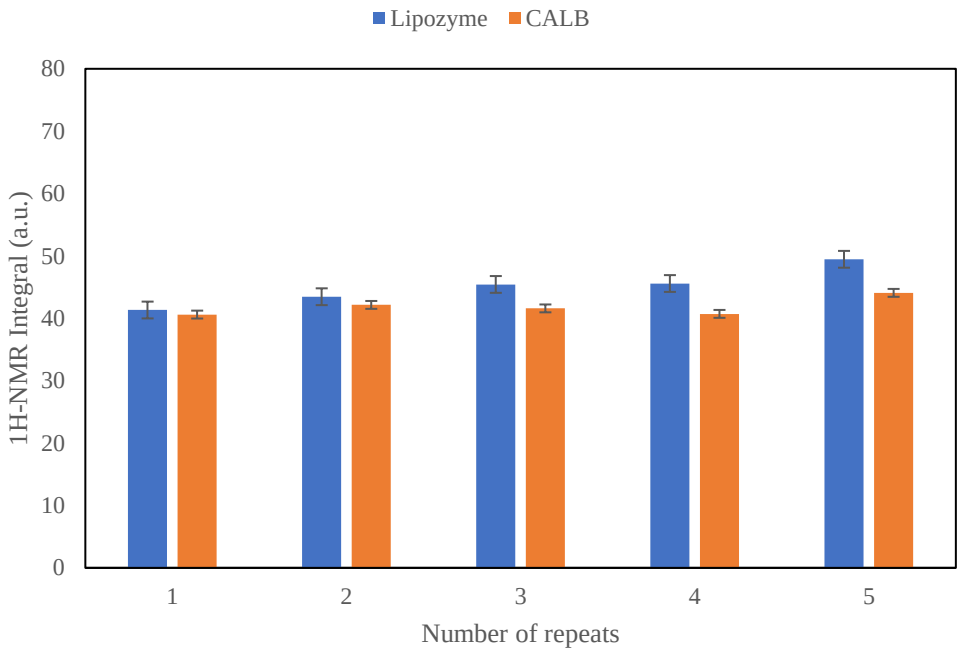


Figure 37 The integral signal of proton at position 1.1-1.3 ppm observed in modified coconut oil obtained by acidolysis using lipase Lipozyme TL IM and lipase B from *Candida antarctica* acrylic resin versus reaction number of repeating

5. Investigation of the potential of enzyme loading on coconut oil acidolysis

Further experiments focused on investigating the potential for enhancing the effectiveness of the utilization of the enzyme catalyst were carried out by investigating the effects of enzyme loading on the outcomes of the reaction. The reaction was tested with both enzymes and the enzyme loading was 10%, 5%, and 2.5% (w/w) while other reaction conditions were kept as described above. The progress of these experiments was analyzed by ^{1}H-NMR technique and the results are shown in Appendix Figure 140-145.

Furthermore, the results are summarized in Figure 38 and show that the reaction, for both lipase enzymes, experiences only minor changes in performance in response to the decrease in enzyme loading. This indicates the possibility of decreasing the amount of enzyme catalyst used in the reaction.

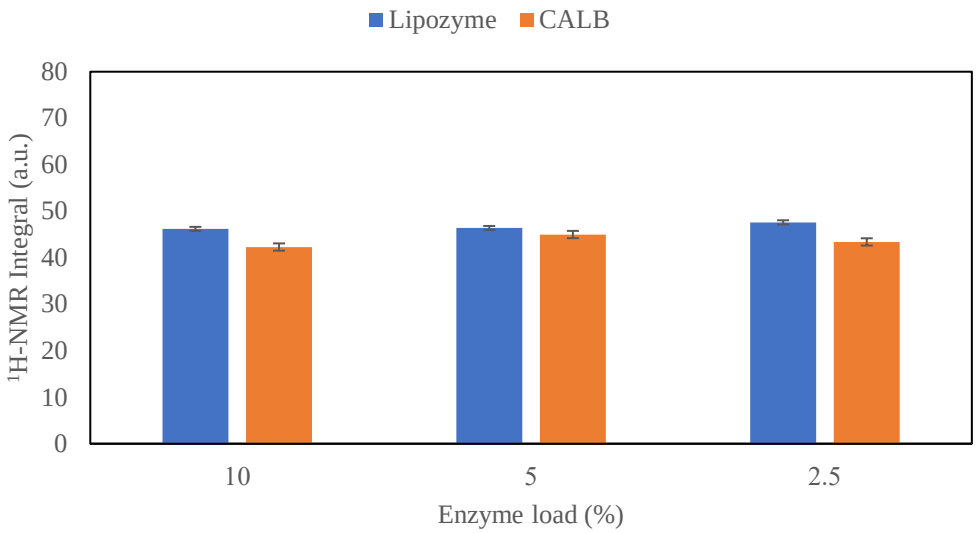


Figure 38 The integral signal of proton at position 1.1-1.3 ppm observed in modified coconut oil obtained by acidolysis using lipase Lipozyme TL IM and lipase B from *Candida antarctica* acrylic resin versus enzyme loading

6. Reaction scale-up of coconut oil acidolysis

In addition to the above investigation of the effects of various factors on the performance of the acidolysis reaction, we also simultaneously conducted the preparation of the modified coconut oil for the purpose of biological testing in mice. The required amount of the product for this work was on the order of 100s of grams and therefore we needed to scale up the reaction. Based on the similar performance of the two enzymes in the investigations described above we chose Lipozyme TL IM for this work due to its lower cost. The acidolysis reaction was scaled up to runs using 40 g of coconut oil. The reaction temperature for the scaled-up reactions was kept at 45°C. However, the reaction time was extended from 72 h to 168 h. Furthermore, the ratio of coconut oil to octanoic acid was changed from 1:3 to 1:5. The results were analyzed by the ^{1}H-NMR and a representative spectrum is shown in Figure 39.

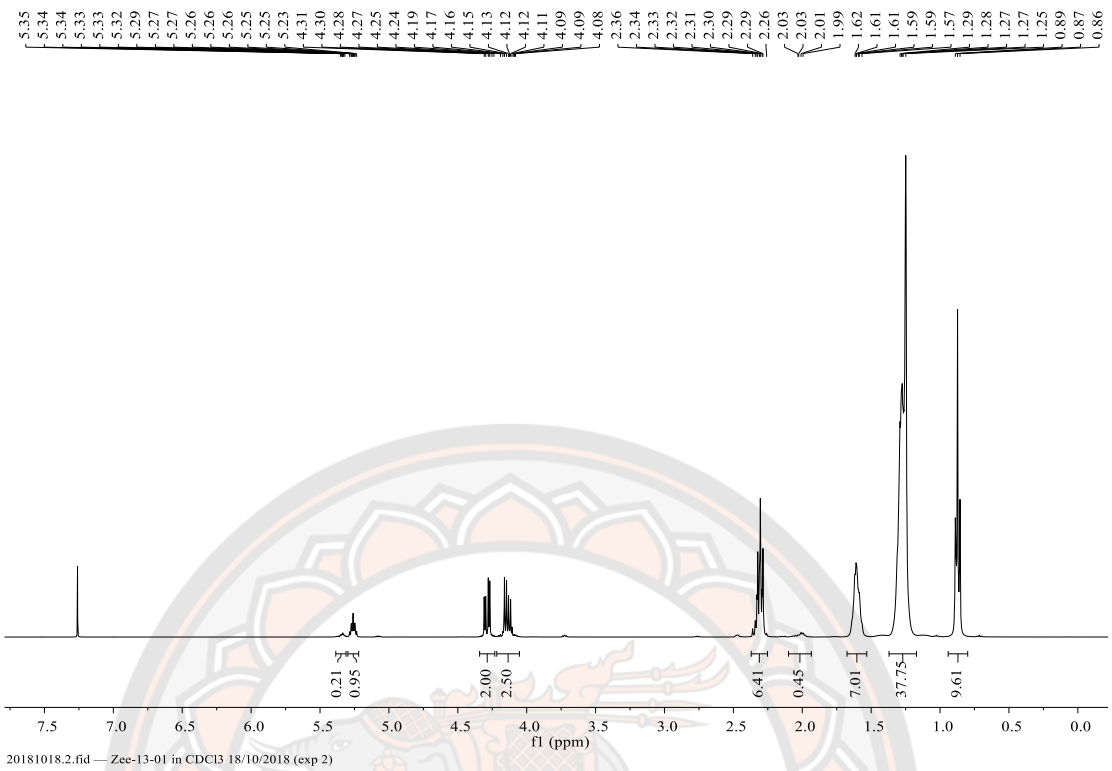


Figure 39 ^{1}H-NMR spectrum of upscaling modified coconut oil prepared using enzymatic acidolysis for biological testing

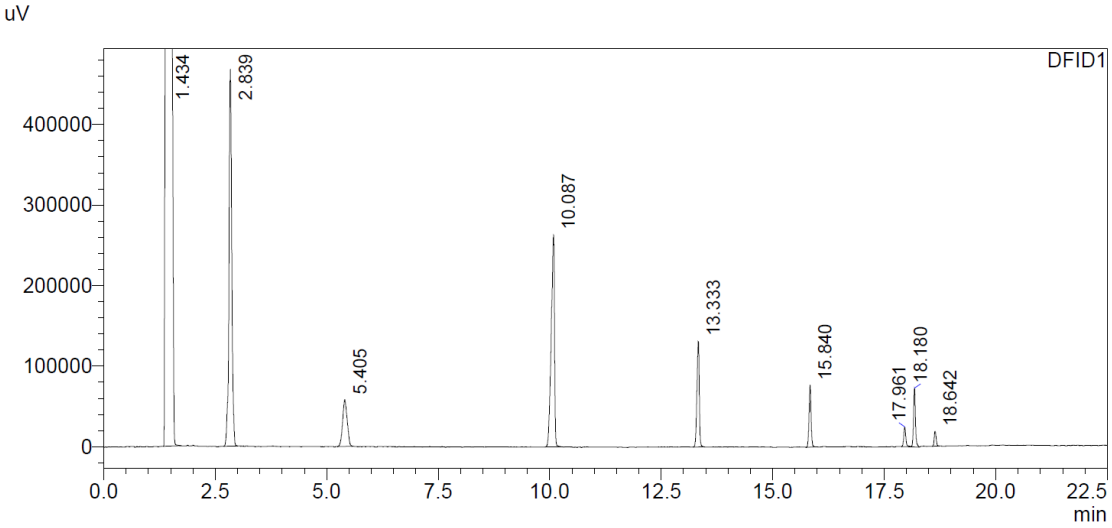


Figure 40 GC spectrum of upscaling modified coconut oil prepared using enzymatic acidolysis for biological testing

The products were also analyzed by GC and the results are shown in Figure 40 and Table 10. The observed ^{1}H-NMR integral value of the peak of interest at 1.1-1.3 ppm for the scaled-up reaction was 37.75, which indicates that a higher degree of incorporation of octanoic acid than in the previous experiments has been achieved. The GC analysis shows that the content of octanoic acid in the obtained product was 41.3%. (Figure 40, Table 10). These results demonstrate that the performance of the acidolysis reaction can be maintained in scaled-up reactions with minimum performance degradation.

Table 10 Fatty acid composition of modified coconut oil obtained after modification at 40 g scale using enzymatic acidolysis determined by gas chromatography

Fatty acid symbol	Fatty acid trivial name	Retention time (min)	Specifications (%)
C8:0	Caprylic acid	2.839	41.3
C10:0	Capric acid	5.405	8.5
C12:0	Lauric acid	10.087	27.8
C14:0	Myristic acid	13.333	9.9
C16:0	Palmitic acid	15.840	5.1
C18:2	Linoleic acid	17.961	1.6
C18:1	Oleic acid	18.180	4.5
C18:0	Stearic acid	18.642	1.2

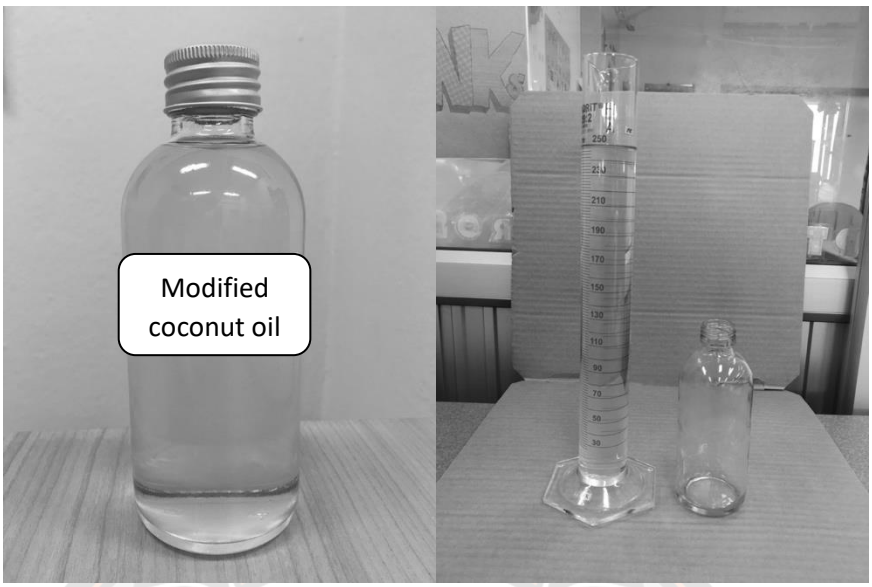


Figure 41 Modified coconut oil prepared using enzymatic acidolysis for biological testing

Ultimately, 250 ml of the modified coconut oil prepared using enzyme catalyzed acidolysis has been obtained (Figure 41). This has been then used in biological testing.

7. Coconut oil acidolysis from commercial (octanoic acid (C8:0) and decanoic acid (C10:0))

All the above work on enzymatic acidolysis of coconut oil has been carried out with octanoic acid (C8:0) as the sole MCFA reagent. However, based on the knowledge that most MCTs contain a mixture of octanoic acid (C8:0) and decanoic acid (C10:0), we decided to carry out the acidolysis reaction with a mixture of these fatty acids. The initial acidolysis reactions were carried out at 45^{o}C for 72 hours with a 1:3 mixture of coconut oil and fatty acids (octanoic acid and decanoic acid), hexane as the solvent, and a 5% weight loading of the Lipozyme TL IM enzyme. The conversion of the triglycerides taking place in these reactions was evaluated using ^{1}H-NMR and GC analyses. The ^{1}H NMR spectrum for this reaction can be seen in Figure 42, and a corresponding GC spectrum can also be found in Figure 43. Reference NMR and GC

spectra for coconut oil and commercial MCT oil (octanoic and decanoic acids) can be found in Appendix Figure 100-109 and Table 31-35.

The NMR spectrum shown in Figure 42 shows that the integral of the proton signal at 1.1–1.3 ppm decreases from 51.8 in the original coconut oil to 46.15, which is significantly lower than in the original coconut oil. It demonstrates that the chain length of the fatty acids in the modified oil product has changed and is shorter than the original coconut oil.

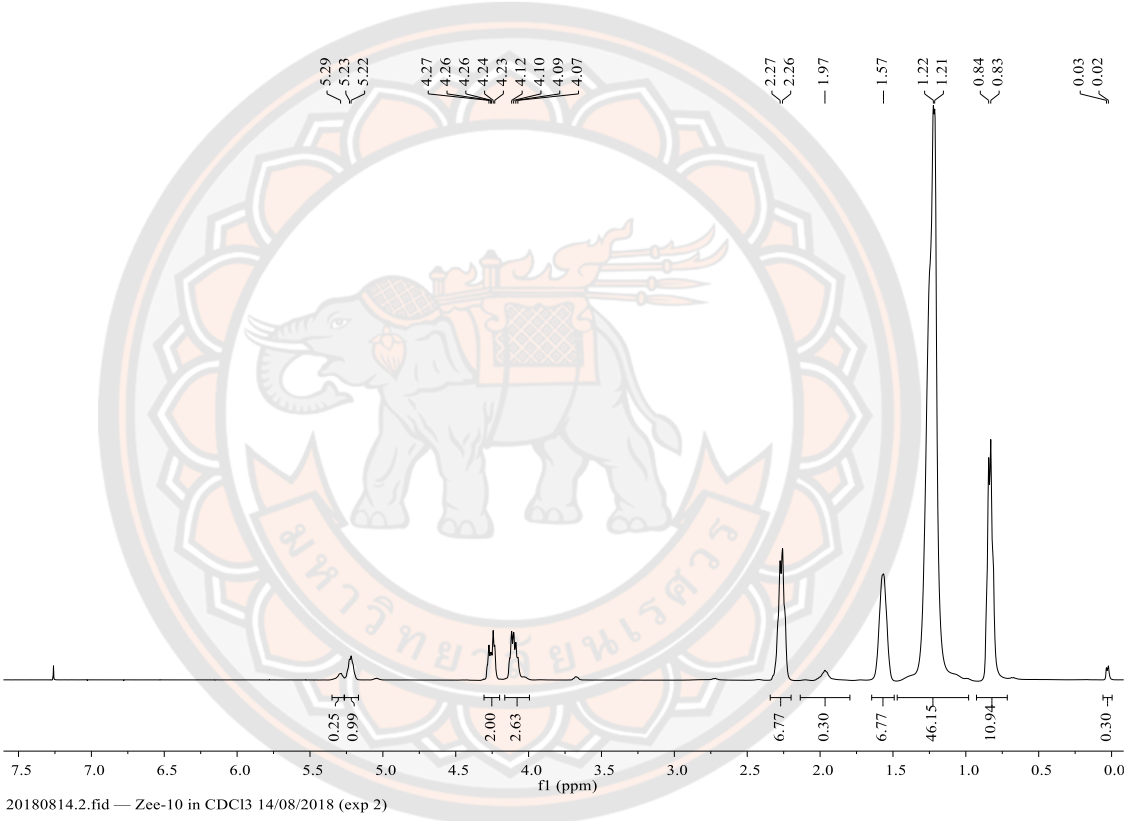


Figure 42 ^{1}H-NMR spectrum of oil obtained by enzyme Lipozyme TL IM catalyzed acidolysis of coconut oil with octanoic acid (C8:0) and decanoic (C10:0)

Then, GC analysis was used to confirm the results from the ^{1}H-NMR spectra and obtain detailed information on the fatty acid composition of the modified oil. The corresponding GC spectrum for this representative reaction can be seen in

Figure 43, and the relative contents of individual fatty acids in the product can be seen in Table 11. The GC analysis confirmed the peak with a retention time of 2.9 and 5.5 minutes, which corresponds to octanoic acid and decanoic acid (Figure 43, Table 11). The results from the GC analysis indicate that the content of octanoic acid (C8) and decanoic acid (C10) increases significantly in the product at 35% compared to the starting coconut oil. While the content of lauric acid is 35.2%, which is significantly lower than in the original coconut oil, which is 48.5%, The results from the initial experiment have shown that products from these initial reactions can indeed proceed.

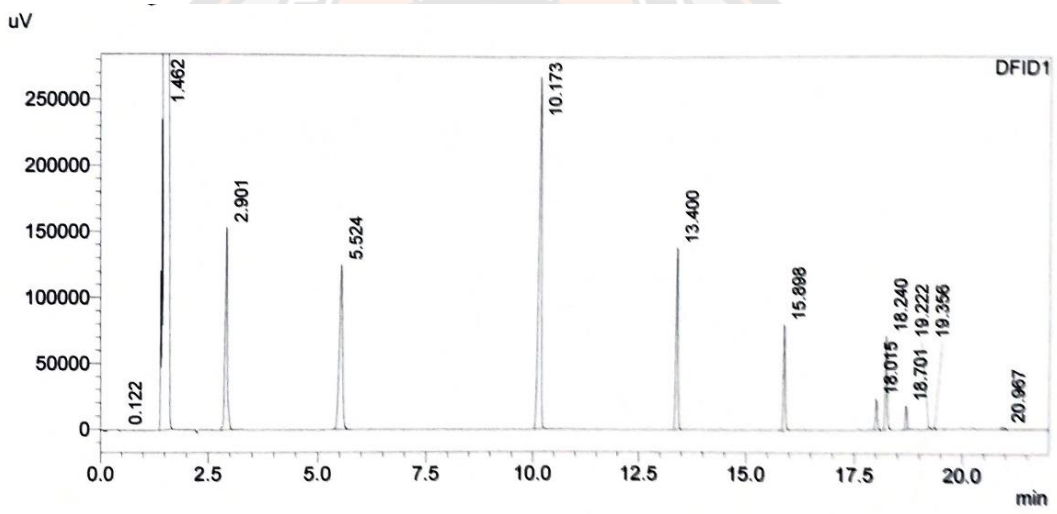


Figure 43 GC spectrum of modified coconut oil prepared by the acidolysis reaction using octanoic acid and decanoic (ratio 1:0.6) and catalyzed by the enzyme Lipozyme TL IM

Table 11 Fatty acid composition of modified coconut oil produced by acidolysis at the ratio of coconut oil with fatty acid (1:0.6)

Fatty acid symbol	Fatty acid trivial name	Retention time (min)	Specifications (%)
C8:0	Caprylic acid	2.901	16.22
C10:0	Capric acid	5.524	18.86
C12:0	Lauric acid	10.173	35.24
C14:0	Myristic acid	13.400	13.14
C16:0	Palmitic acid	15.898	6.96
C18:2	Linoleic acid	18.015	2.09
C18:1	Oleic acid	18.240	6.03
C18:0	Stearic acid	18.701	1.46

4.1.2 Coconut oil interesterification

While our previous experiments have shown the possibility to modify the composition of coconut oil with MCFAs and thus resulting in a more MCT like product the acidolysis reaction had its drawbacks. Firstly, the purification of the reaction is based on a rather tedious extraction procedure. Secondly, the product prepared for the biological testing led to dubious results, which were attributed to presence of impurities (e.g.diglycerides) likely resulting from the extraction purification procedure. Therefore, we decided to investigate interesterification as an alternative coconut oil modification procedure. As can be seen in Figure 44, interesterification uses fatty acid esters rather than fatty acids as the starting materials.

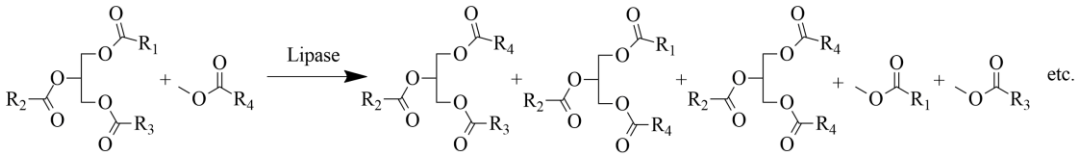


Figure 44 Triglyceride interesterification reaction

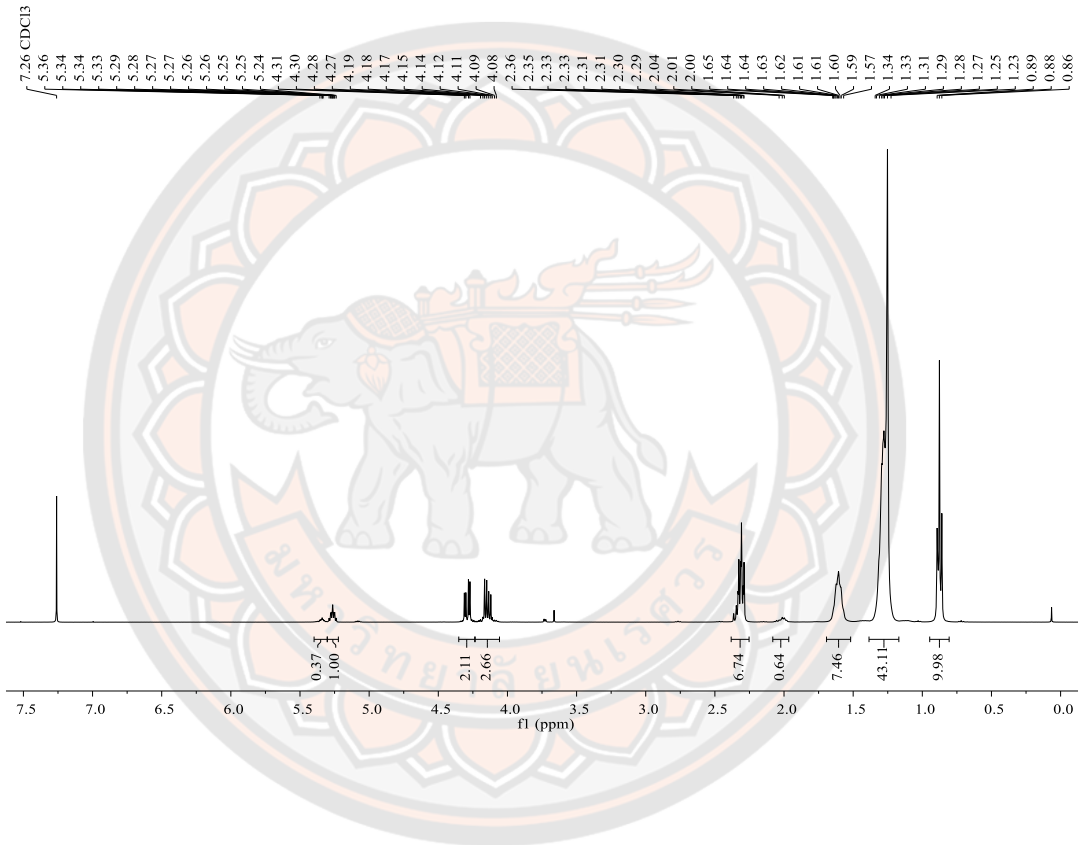


Figure 45 ^{1}H-NMR spectrum of oil obtained by enzyme lipase B from *Candida antarctica* immobilized on acrylic resin catalyzed interesterification of coconut oil with methyl octanoate

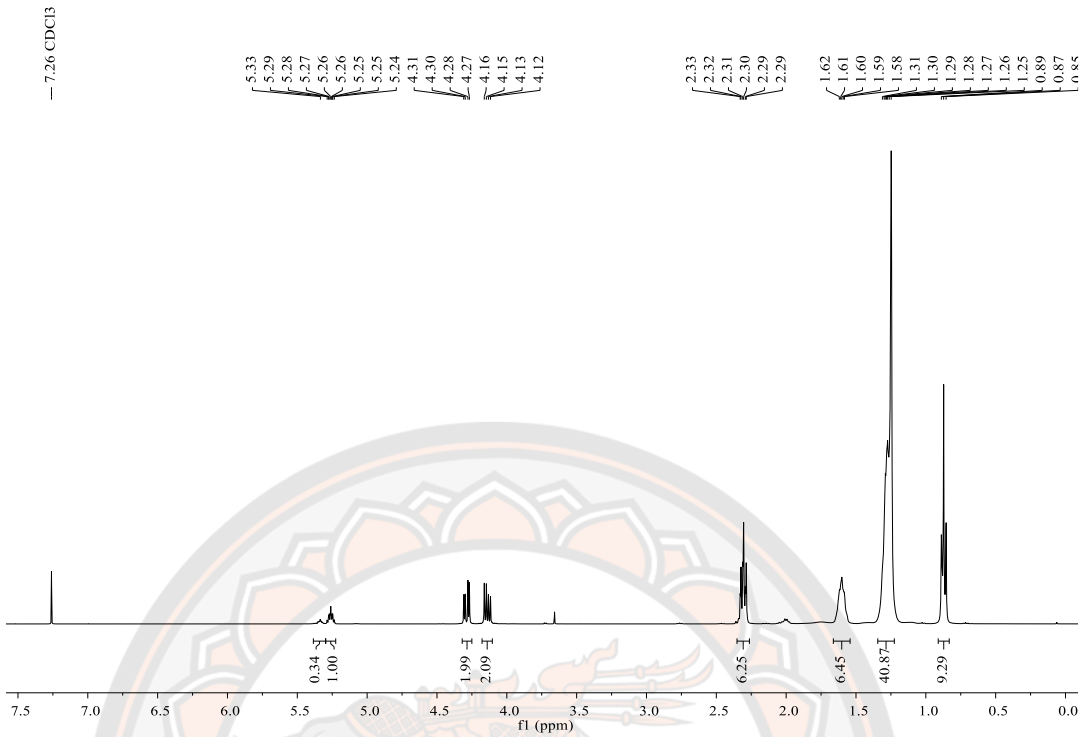


Figure 46 ^{1}H-NMR spectrum of oil obtained by enzyme Lipozyme TL IM catalyzed interesterification of coconut oil with methyl octanoate

The initial experiments of coconut oil interesterification were conducted with lipase B from *Candida antarctica* immobilized on acrylic resin and Lipozyme TL IM as they have been shown effective in the previous experiments on coconut oil acidolysis. In addition, these lipase enzymes have previously been shown to be effective in catalyzing the interesterification of fats and oils and thus changing their fatty acid composition or rearranging the fatty acids in these lipid molecules. The conditions of initial experiments of coconut oil interesterification were chosen by modification of our previous work with methyl octanoate as the acyl donor, coconut oil to methyl octanoate ratio of 1:3, temperature of 45°C, 10% wt. loading of the enzyme, and 72 h reaction duration as in the case of coconut oil acidolysis, the transformation of the triglycerides taking place in these reactions was evaluated using ^{1}H-NMR and GC analyses. The ^{1}H-NMR spectra for the initial coconut oil interesterification reactions with methyl

octatanoate catalyzed by lipase B from *Candida antarctica* and Lipozyme TL IM can be seen Figure 45 and Figure 46, respectively.

GC analysis was then used to confirm the results from the ^{1}H-NMR spectra and to obtain more detailed information on the fatty acid composition of the modified oil. The GC spectrum for the reaction catalyze by lipase B from *Candida antarctica* immobilized on acrylic resin be seen in Figure 47. The percentage contents of individual fatty acids in the product can be seen in Table 12.

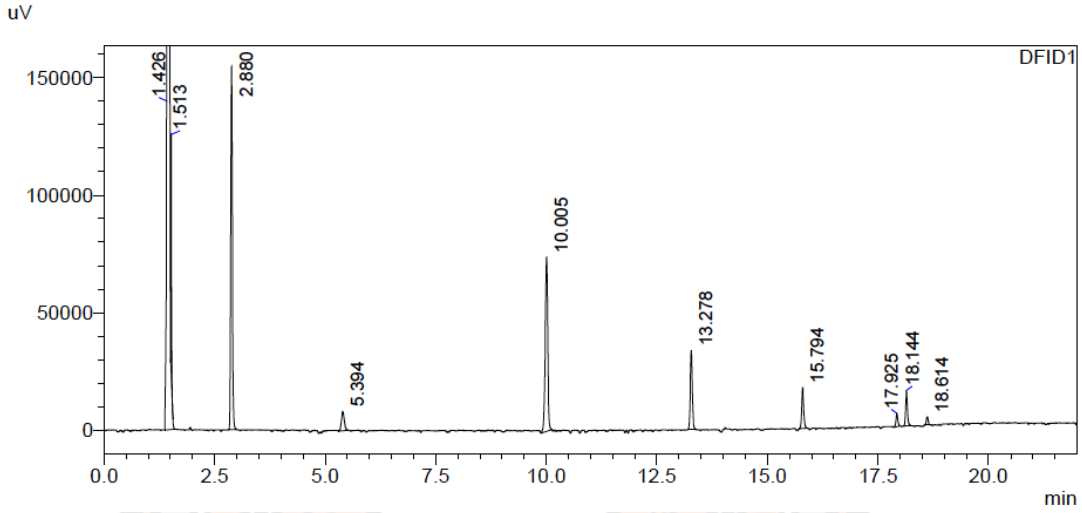


Figure 47 A representative GC spectrum of modified coconut oil prepared by the interesterification reaction using methyl octanoate and catalyzed by lipase B from *Candida antarctica* immobilized on acrylic resin

The results from ^{1}H-NMR (Figure 45 and 46) and GC (Figure 47) show that the initial interesterification reactions have indeed resulted in the desired modification of coconut oil. Firstly, ^{1}H-NMR (Figure 45 and 46) analysis shows that the integral of the proton signal at 1.1-1.3 ppm decreases from 51.8 in the original coconut oil (Appendix Figure 94) to 43.1 and 40.9 for reactions catalyzed by lipase B from *Candida antarctica* and Lipozyme TL IM, respectively. Thus, the integral values of the signal of interest in the products are significantly lower than in the original coconut oil, which

indicates that the chain length of the acyl residues of the triglycerides in the modified oil produced by interesterification is shorter than in the original coconut oil. This is consistent with the exchange of longer-chain fatty acids in coconut oil with octanoic acid from methyl octanoate. The GC analysis was used to gain further understanding by focusing on the peak with a retention time of 2.9 minutes, which corresponds to octanoic acid (Figure 47, Table 12). The results from GC analysis indicate that the content of octanoic acid (C8) increases significantly from 8.2% in the starting coconut oil to 41.8% in the product (Table 12). While the content of lauric acid in the product is 30.3%, which is significantly lower than in the original coconut oil, which is 48.5% (Appendix A Figure 94, Table 30).

Table 12 Percent content of individual fatty acids in original coconut oil, and modified oil produced by interesterification

Fatty acid	**Retention time (min)**	**Coconut oil**	**Modified oil (Interesterification)**
C8	2.78	8.2	41.8
C10	5.51	6.3	3.8
C12	10.17	48.5	30.3
C14	13.40	17.8	11.3
C16:0	15.90	8.6	5.6
C18:2	18.01	2.4	1.8
C18:1	18.24	6.6	4.5
C18:0	18.70	1.6	1.0

Both enzymes utilized in the initial interesterification modification of coconut oil have led to similar outcomes. Therefore, we investigated the performance of both enzymes in reactions exploring the effects of temperature and the possibility of enzyme recycling.

1. Investigation of the effect of temperature

The first parameter to be investigated was the reaction temperature. The effect of reaction temperature was studied in the range from 45°C to 75°C. The reaction was carried out with both enzymes while maintaining other reaction parameters identical to the initial tests. The experimental results can be seen in Figure 48, which plots graphs of the ^{1}H-NMR integrals versus temperature. Figure 48 shows that between 45°C and 65°C, the performance of both enzymes decreased slightly as the temperature increased. At 70°C, the performance dropped rapidly, and at 75°C, the enzymes lost most of their activity.

Furthermore, the graph can be used to compare the activity of the enzymes. The activity of Lipozyme TL IM is better than that for lipase B from *Candida antarctica* immobilized on acrylic resin as Lipozyme TL IM gives lower integral values at 1.1-1.3 ppm than CAL B at every temperature, which corresponds to a more extensive integration of octanoic acid into the product.

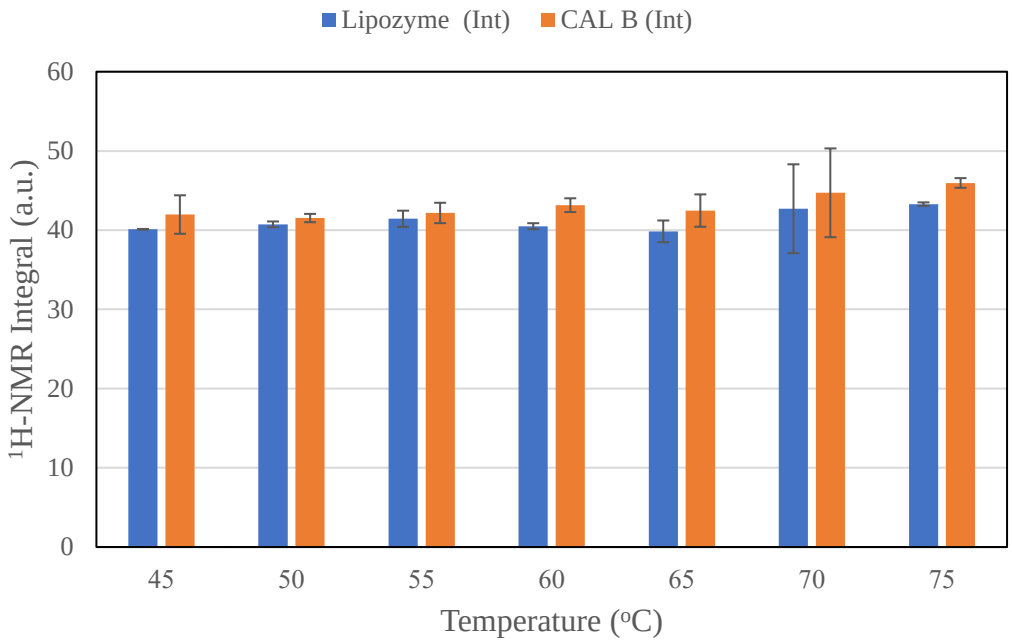


Figure 48 A plot of the integral of the peak of interest (1.1-1.3 ppm) in the ^{1}H-NMR spectrum of a product of modified coconut oil produced by enzyme catalyzed interesterification using lipase B from *Candida antarctica* immobilized on acrylic resin and Lipozyme TL IM as a function of reaction temperature

2. Investigation of the potential for enzyme recycling

The second parameter to be investigated was the possibility of recycling and reusing the enzyme catalyst. Therefore, the reactions were carried out using the initial conditions, and the enzyme was recovered and used in further reaction repeats. The reactions were carried out with both enzymes and repeated five times. The results of these experiments are shown in Figure 49.

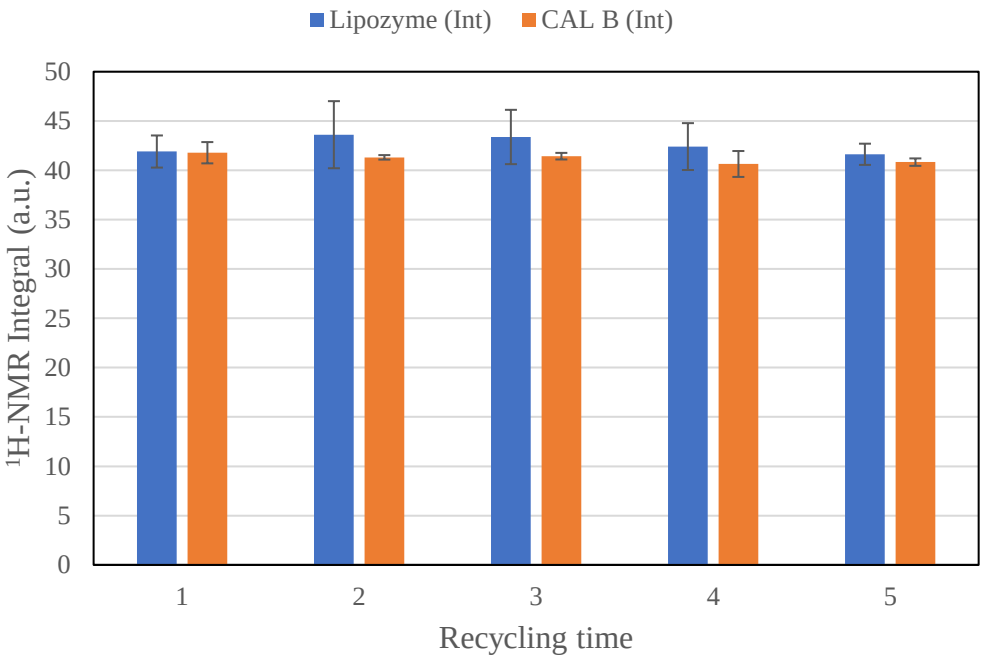


Figure 49 A plot of the integral of the peak of interest (1.1-1.3 ppm) in the ^{1}H-NMR spectrum of a product of modified coconut oil produced by enzyme-catalyzed interesterification using lipase B from *Candida antarctica* immobilized on acrylic resin and Lipozyme TL IM versus the number of uses of the enzyme catalyst in a recycling experiment

The results indicate that the activity of both enzymes in the reaction repeats was maintained throughout this experiment, as no deteriorating trend can be observed. The results from these experiments show that the performance of both enzymes is very similar. Therefore, we decided to conduct further experiments only with Lipozyme TL IM because of its lower price**.**

To obtain further insights into the effects of key parameters on the performance of the interesterification reaction in terms of the extent of incorporation of octanoic acid. Design of Experiments (DOE) and Response Surface Methodology (RSM) were used to determine the best conditions for running the process.

3. Optimization by Response Surface Methodology (RSM)

The previous results have shown that it is possible to perform interesterification of coconut oil with methyl octanoate and to select the appropriate enzyme. We have therefore carried out further experiments with the aim of finding optimal conditions that give the product with the highest incorporation of methyl octanoate. Therefore, in this work, the response surface methodology (RSM) approach was used to optimize the process parameters and identify the optimal conditions. The experiments were carried out to investigate the combined effects of three factors, including reaction time, the ratio of methyl octanoate and coconut oil, and the loading of the enzyme catalyst. The effect of reaction temperature, an important parameter affecting the performance of enzymatic catalysis, was not chosen for this investigation, given the limited effects observed in the experiments described above. Furthermore, it was expected that increasing the reaction temperature above 45^{o}C would negatively affect the possibility of recycling the enzyme catalyst. Lipozyme TM IL was selected as the enzyme for this work due to its lower price and comparable performance relative to Lipase B from *Candida antarctica* immobilized on acrylic resin. The reactions were carried out as described above. Additionally, the product was purified using filtration on a silica gel plug and analyzed using ^{1}H-NMR. The RSM analysis was carried out using the Box-Behnken approach with 3 center points and 1 block. The three investigated levels of reaction time (X1) were 12 hours (-1), 24 hours (0), and 36 hours (+1). The three investigated levels of the methyl octanoate to coconut oil ratio (X2) were 0.6 (-1), 0.9 (0), and 1.2 (+1). Finally, the three investigated levels of enzyme loading (X3) were 1% (-1), 5% (0), and 10% (+1). Integral of the peak between 1.1 and 1.3 ppm in the ^{1}H-NMR spectra of the product was used as the independent variable (Y). Regression analysis was performed to fit the response variable as a function of the independent variables. The integral values, the independent variable (Y), range between 36.20±1.24 and 40.55±0.66. The results of the experiments carried out are shown in Table 14. All these values are lower than the integral value observed for unmodified coconut oil is 51.37 indicating the incorporation of octanoic acid into the products at all conditions.

The experimental data were fit to a modified quadratic model. The regression equation as a function of reaction time (X_1), reagent ratio (X_2), and enzyme loading (X_3) is shown in Equation 1.

Equation 1:

$$y = 36.29 - 0.63 * X_1 - 0.84 * X_2 - 1.08 * X_3 - 0.70 * X_1 * X_2 + 1.28 * X_1^2 + 0.11 * X_2^2 + 1.13 * X_3^2$$

Table 13 Independent variables of products made from modified coconut oil

Independent variable and interactions	**Coefficient**	**SE Coefficient**	**t-value**	**p-value**
Constant	36.29	0.13	279.2	<0.0001
X_1	-0.63	0.082	-7.7	0.0001
X_2	-0.84	0.082	-10.2	<0.0001
X_3	-1.08	0.082	-13.2	<0.0001
X_1^2	1.28	0.12	10.7	<0.0001
X_2^2	1.11	0.12	9.2	<0.0001
X_3^2	1.13	0.12	9.4	<0.0001
X_1X_2	-0.70	0.12	-5.8	0.0005

Table 14 Level values for the Box-Behnken design of the response surface methodology experiments

Run	Independent Variables			Responses
	X_1	X_2	X_3	Integral
1	0	0	0	36.28 (±1.55)
2	-1	-1	0	39.53 (±1.04)
3	0	1	-1	38.85 (±0.99)
4	-1	0	1	38.47 (±1.21)
5	-1	0	-1	40.29 (±1.58)
6	1	0	-1	39.08 (±0.76)
7	0	0	0	36.39 (±1.74)
8	1	0	1	36.95 (±0.61)
9	1	1	0	36.43 (±0.71)
10	0	-1	-1	40.55 (±0.66)
11	0	1	1	36.81 (±0.86)
12	0	-1	1	37.90 (±0.93)
13	0	0	0	36.20 (±1.24)
14	-1	1	0	38.98 (±0.90)
15	1	-1	0	40.54 (±0.48)

The experimental data were analyzed at the 95% confidence level, and the modified quadratic model resulted in an R^2 value of 98.9%. Table 11 shows the results of the regression, including standard error of coefficients, t-values, and p-values. All three factors as exhibited significant ($p<0.05$) linear and quadratic effects on the observed integral values. As for the interaction factors, the only used factor X1*X2 was significant. A 3D plot of the ^{1}H-NMR integral as a function of reaction time and methyl octanoate to coconut oil ratio can be seen in Figure 50.

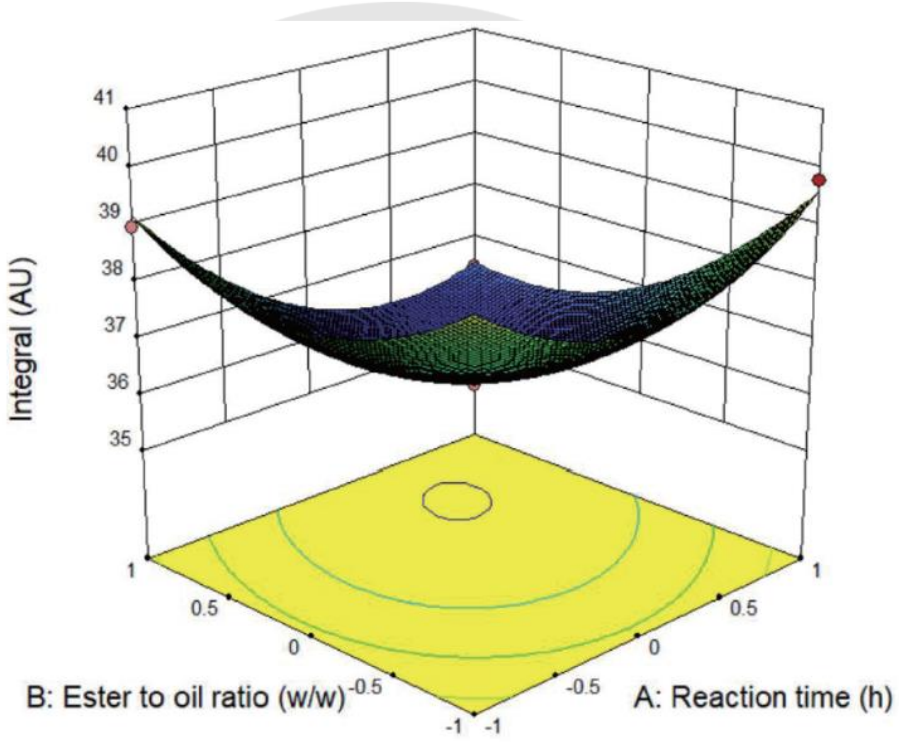


Figure 50 Three-dimensional plot of integral values versus time and oil to ester ratio

Based on the RSM analysis, the optimum conditions for synthesis that caused the maximum increase in the incorporation of octanoic acid were a reaction time of 34.4 h, a methyl octanoate to coconut oil ratio of 1.16, and enzyme loading of 5.6%. According to the analysis, under these optimized conditions, the expected integral value is 36.17± 0.23. The reaction was carried out under these conditions at the scale of 1 g of oil and the observed integral value was 36.14± 0.78. A representative ^{1}H-NNMR

spectrum can be found in Figure 51. GC analysis of the resulting oil has indicated that this corresponds to a 40.9± 2.5% content of octanoic acid in the product. The representative gas chromatogram and contents of individual fatty acids can be found in Figure 52 and Table 15, respectively.

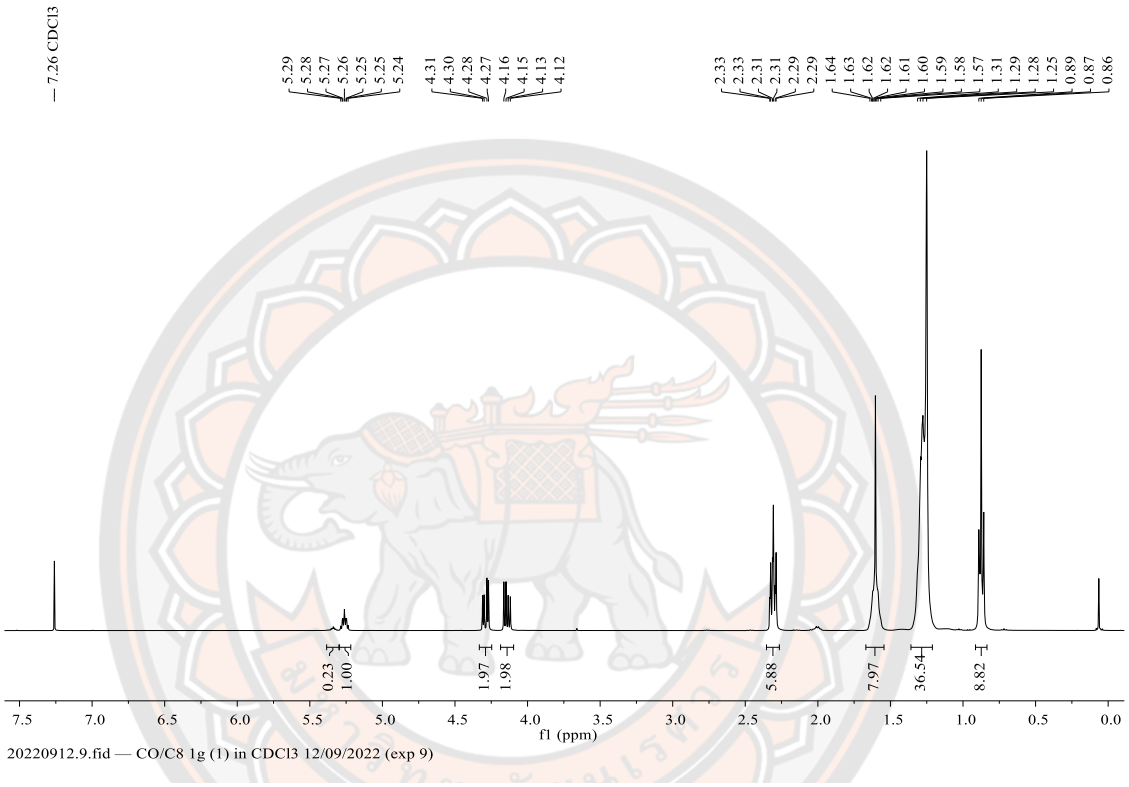


Figure 51 ^{1}H-NMR spectrum of modified coconut oil obtained after modification at 1 g scale using optimized reaction conditions

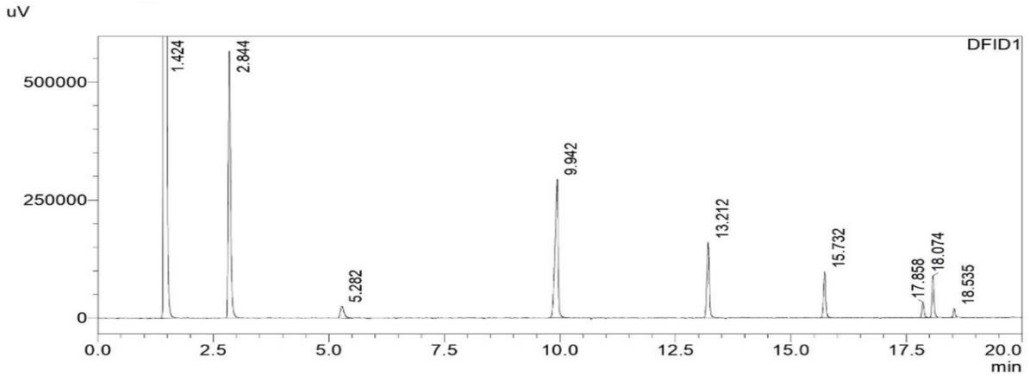


Figure 52 Gas chromatogram of modified coconut oil obtained after modification at 1 g scale using optimized reaction conditions

Table 15 Fatty acid composition of modified coconut oil obtained after modification at 1 g scale using optimized reaction conditions determined by gas chromatography

Fatty acid symbol	Fatty acid trivial name	Retention time (min)	Specifications (%)
C8:0	Caprylic acid	2.844	41.70
C10:0	Capric acid	5.282	2.96
C12:0	Lauric acid	9.942	28.12
C14:0	Myristic acid	13.212	11.95
C16:0	Palmitic acid	15.732	6.58
C18:2	Linoleic acid	17.858	2.23
C18:1	Oleic acid	18.074	5.36
C18:0	Stearic acid	18.535	1.10

The reaction was then carried out using the optimized conditions at a scale of 20 g of coconut oil. Figures 53, 54 and Table 16 show the ^{1}H-NMR spectrum, GC chromatogram, and fatty acid content for the product from the reaction with 20 g of coconut oil, respectively. The observed integral value for this scaled-up reaction was 36.53, and the GC-based content of octanoic acid in the obtained product was 38.0%. These results indicate that the performance of the optimized reaction can be maintained with minimal performance deterioration in scaled-up reactions.

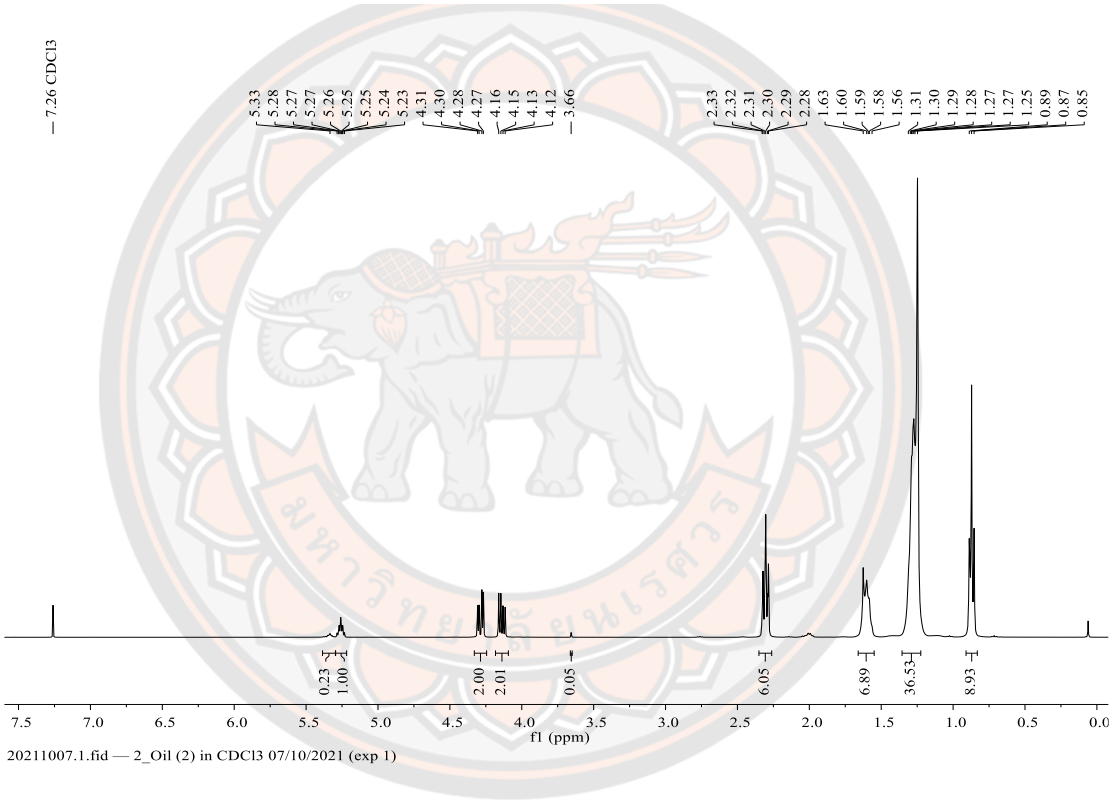


Figure 53 ^{1}H-NMR spectrum of modified coconut oil obtained after modification at 20 g scale using optimized reaction conditions

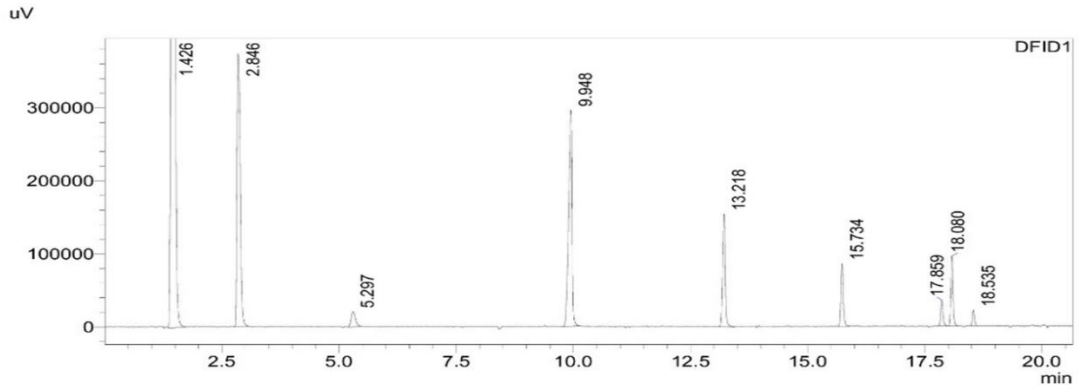


Figure 54 Gas chromatogram of modified coconut oil obtained after modification at 20 g scale using optimized reaction conditions

Table 16 Fatty acid composition of modified coconut oil obtained after modification at 20 g scale using optimized reaction conditions determined by gas chromatography

Fatty acid symbol	Fatty acid trivial name	Retention time (min)	Specifications (%)
C8:0	Caprylic acid	2.846	38.04
C10:0	Capric acid	5.297	2.81
C12:0	Lauric acid	9.948	30.93
C14:0	Myristic acid	13.218	11.94
C16:0	Palmitic acid	15.734	6.27
C18:2	Linoleic acid	17.859	2.52
C18:1	Oleic acid	18.080	6.18
C18:0	Stearic acid	18.535	1.31

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4. Sourcing of methyl octanoate

Finally, we were interested in exploring the source of the MCFAs (octanoic and decanoic acids) and their methyl esters to be used in the coconut oil transformation reactions. The experiments described above were carried out with these reagents from commercial sources. We expected that coconut oil fatty acid distillate, a side product obtained during coconut oil deodorization, to be an interesting potential source for the MCFAs and their methyl esters used in the transesterification reaction. The methyl esters of octanoic and decanoic acids employed in this study for the interesterification of coconut oil were obtained using a three-step process.

Firstly, coconut oil fatty acid distillate (100 g) was distilled under heating at 120^{o}C and subjected to vacuum distillation ($4x10^{-3}$ mbar) to obtain a mixture of fatty acids enriched in the shorter ones. Following this process, the fatty acid distillate yielded 16.5 g of a mixture containing significant amounts of only octanoic (C8:0), decanoic (C10:0), dodecanoic (C12:0), and tetradecanoic acids (C14:0) (Figure 55, 56 and, Table 17). After that, the fatty acid mixture (16.5 g) was esterified with MeOH/H_2SO_4 to yield a mixture of the corresponding mixture of methyl esters (Figure 57). This fatty acid methyl ester mixture was then subjected to vacuum distillation under heating at 60-65^{o}C. The first fraction (5.3 g) contained 71.5 % of methyl octanoate, 23.5 % of methyl decanoate, and 5% of methyl dodecanoate (Figure 58, 59 and Table 18). The second fraction (3.6 g) then contained 8.0 % of methyl octanoate, 85.5 % of methyl decanoate, and 6.5 % of methyl dodecanoate (Figure 60, 61 and Table 19).

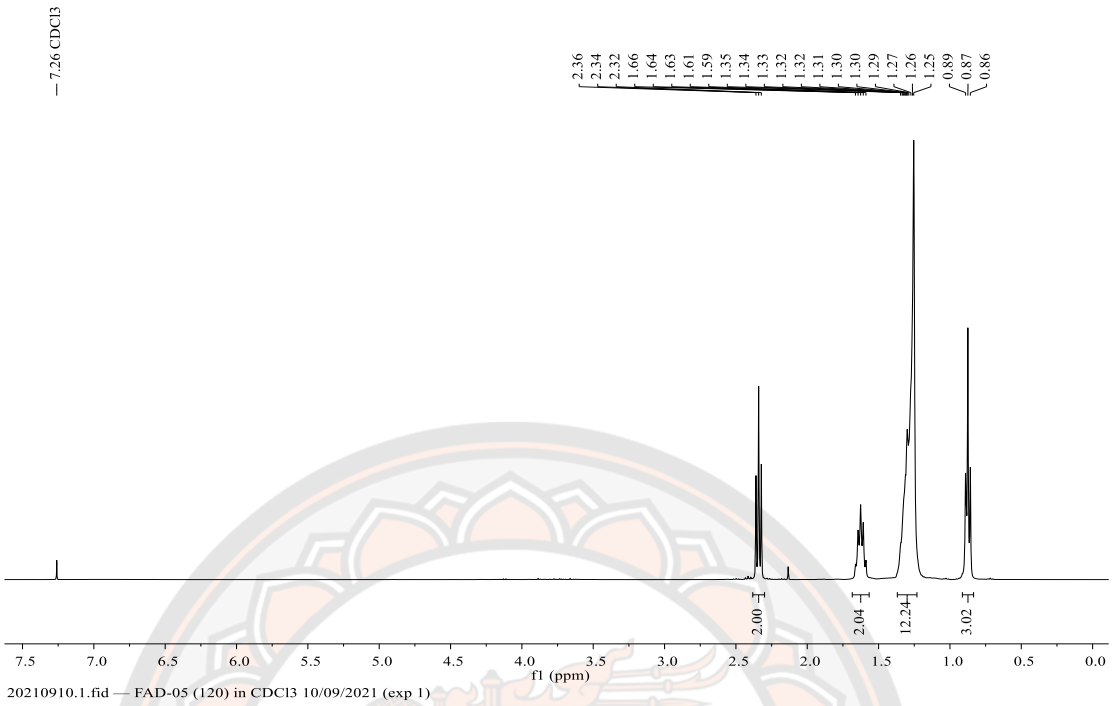


Figure 55 ^{1}H-NMR spectrum of fatty acids obtained from distillation at 120^{o}C

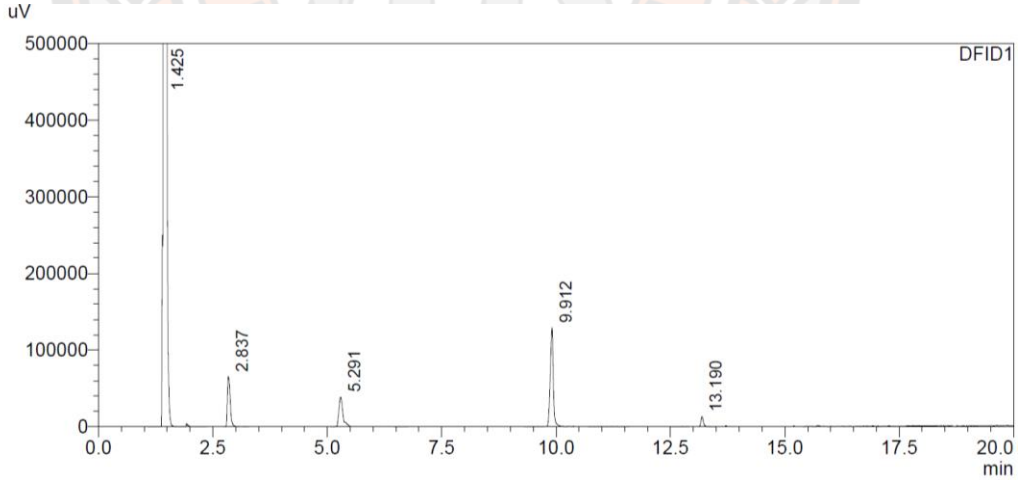


Figure 56 GC spectrum of fatty acids obtained from distillation at 120^{o}C

Table 17 Percent content of fatty acids obtained from distillation at 120°C

Fatty acid symbol	Fatty acid trivial name	Retention time (min)	Specifications (%)
C8:0	Caprylic acid	2.837	23.00
C10:0	Capric acid	5.291	20.33
C12:0	Lauric acid	9.912	52.28
C14:0	Myristic acid	13.190	4.39

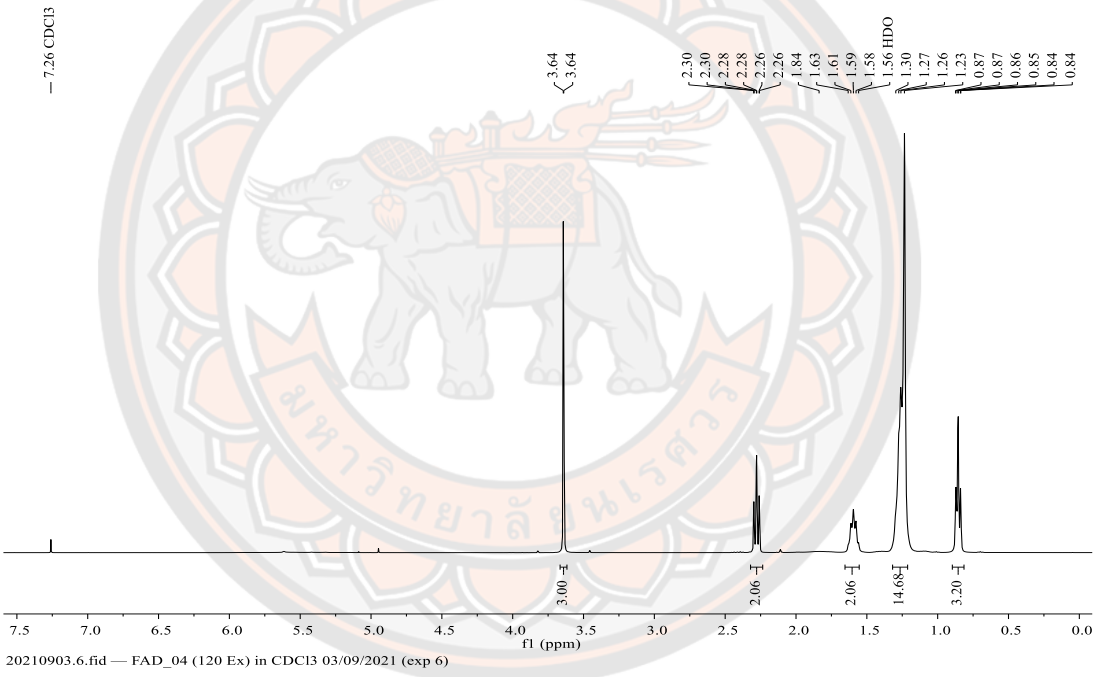


Figure 57 ^{1}H-NMR spectrum of the mixture of fatty acid methyl esters from coconut oil fatty acid distillate, which was distilled at 120°C and then esterified with MeOH/H_2SO_4

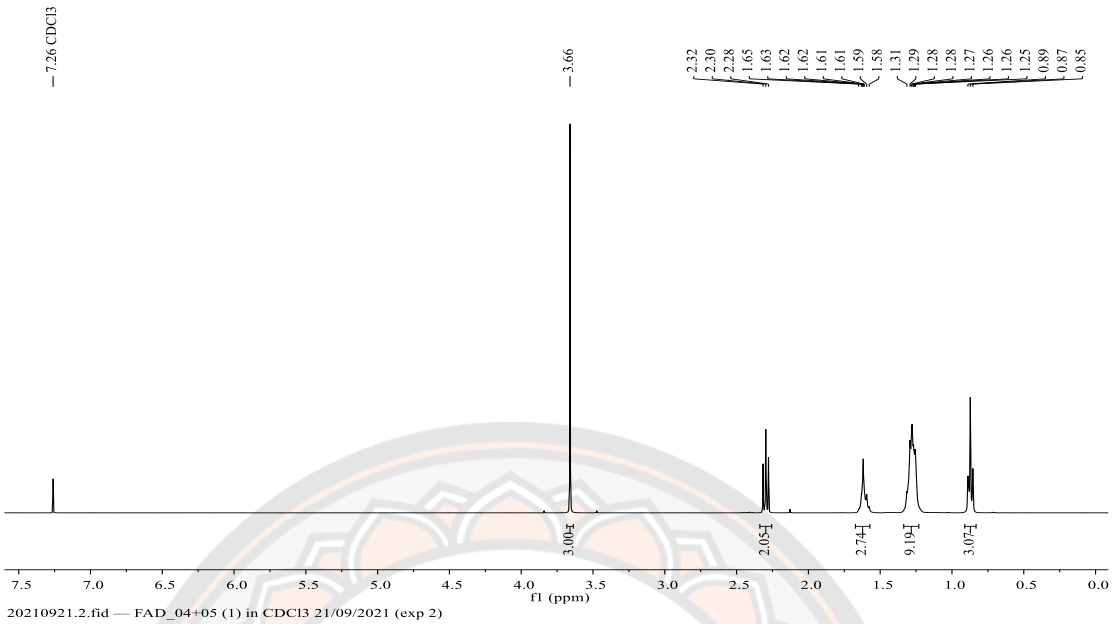


Figure 58 ^{1}H-NMR spectrum of the mixture of fatty acid methyl esters obtained from coconut oil fatty acid distillate using distillation (60ºC <T<65ºC) as the first fraction

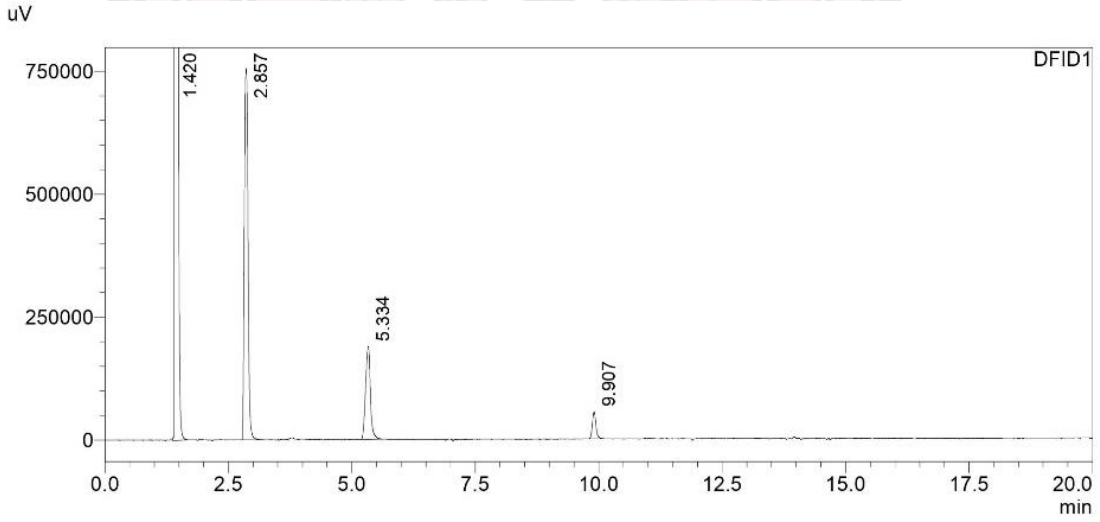


Figure 59 GC spectrum of a mixture of fatty acid methyl esters obtained from coconut oil fatty acid distillate using distillation (60ºC <T<65ºC) as the first fraction

Table 18 Percent content of mixture fatty acid methyl esters first fractions obtained after vacuum distillation of coconut oil fatty acid distillate (60ºC <T<65ºC)

Fatty acid symbol	Fatty acid trivial name	Retention time (min)	Specifications (%)
C8:0	Caprylic acid	2.857	71.52
C10:0	Capric acid	5.334	23.52
C12:0	Lauric acid	9.907	4.96

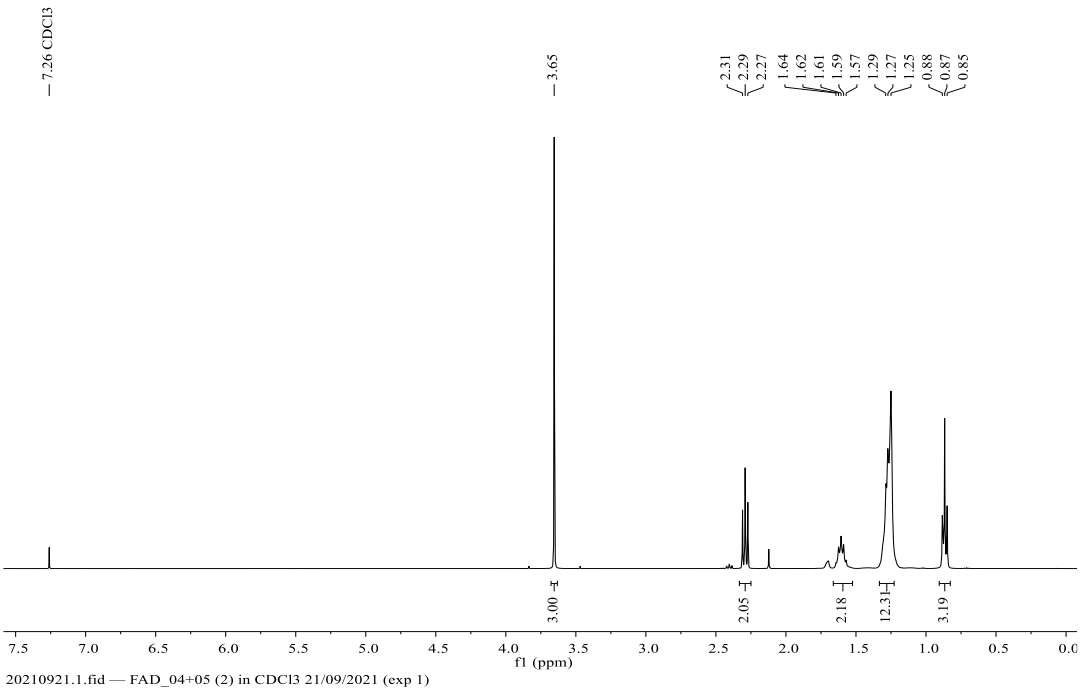


Figure 60 ^{1}H-NMR spectrum of the mixture of fatty acid methyl esters obtained from coconut oil fatty acid distillate using distillation (60ºC <T<65ºC) as the second fraction

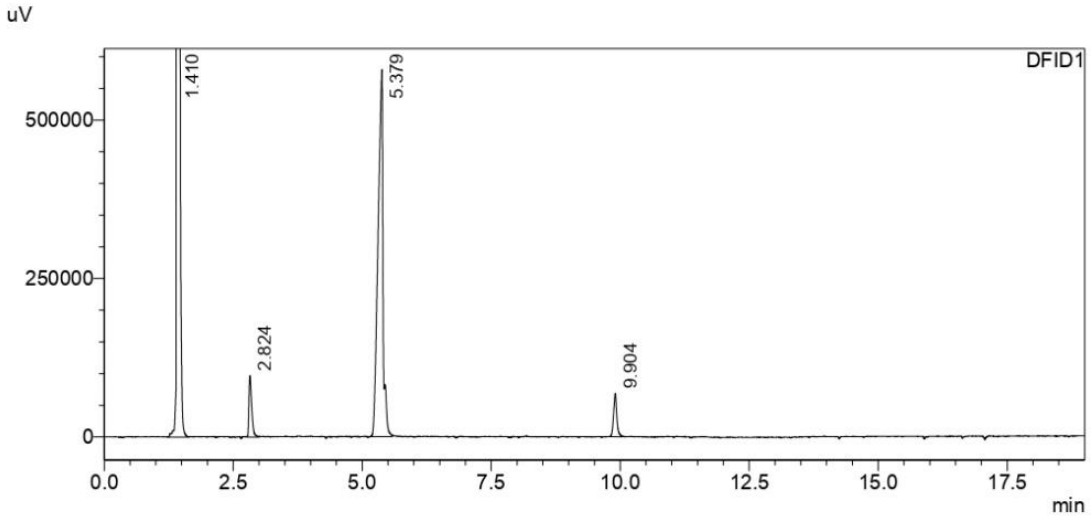


Figure 61 GC spectrum of a mixture of fatty acid methyl esters obtained from coconut oil fatty acid distillate using distillation (60ºC <T<65ºC) as the second fraction

Table 19 Percent content of a mixture of fatty acid methyl esters second fractions obtained after vacuum distillation of coconut oil fatty acid distillate (60ºC <T<65ºC)

Fatty acid symbol	Fatty acid trivial name	Retention time (min)	Specifications (%)
C8:0	Caprylic acid	2.824	8.03
C10:0	Capric acid	5.379	85.49
C12:0	Lauric acid	9.904	6.48

The distillations in this work have been carried out with simple vacuum distillation apparatuses indicating the possibility to obtain highly MCFA enriched materials from fatty acid distillate in relatively simple manner.

5. Interesterification of coconut oil with MCFA esters from coconut oil fatty acid distillate

The first fraction of methyl esters from coconut oil fatty acid distillate (5.3g) containing 71.50% of methyl octanoate was then used to carry out the interesterification reaction under the optimized conditions. The result is shown in Figure 62, which shows the ^{1}H-NMR spectrum of the product. The integral value of the peak of interest at 1.1-1.3 ppm of the product was 37.13, and the content of octanoic acid in the product, based on GC analysis, was 24.90%, as shown in Figure 63 and Table 20. This is less than what was obtained with pure (commercial) octanoic acid. However, considering the fact that the material obtained from the fatty acid distillate contains significant amount of methyl decanoate, it is important to note that the combined content of octanoic and decanoic acids in the product, as determined by the GC, is 39.14%, which is comparable to the results obtained in performance enhancement studies conducted with pure octanoic acid.

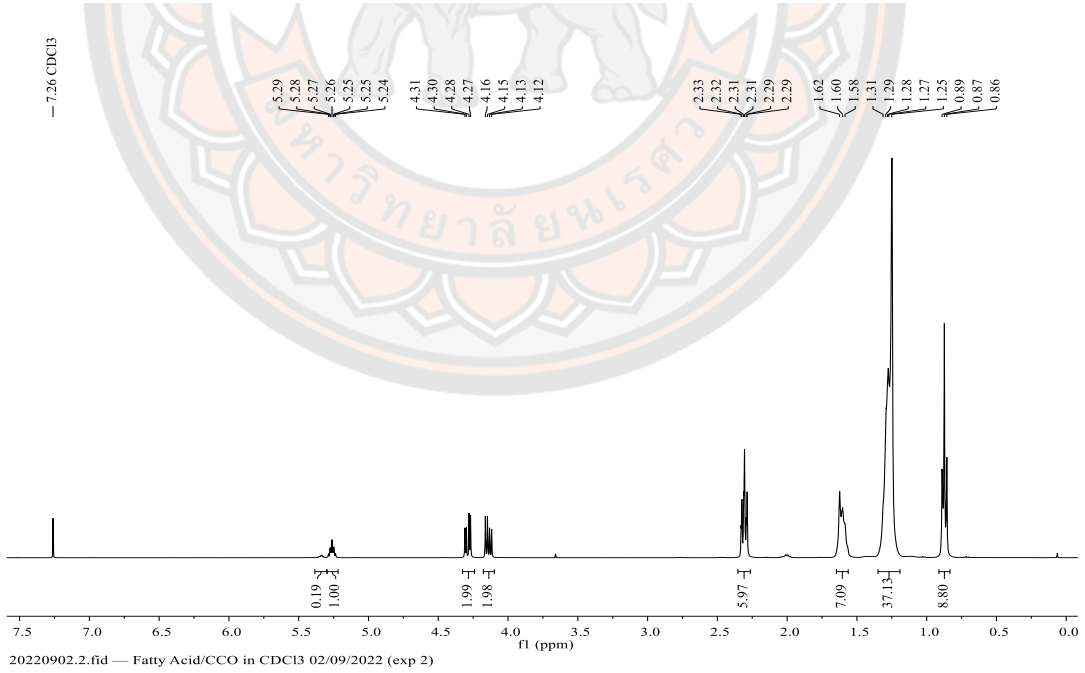


Figure 62 ^{1}H-NMR spectrum of modified coconut oil obtained after modification at 1 g scale using optimized reaction conditions and methyl esters sourced from coconut oil fatty acid distillate

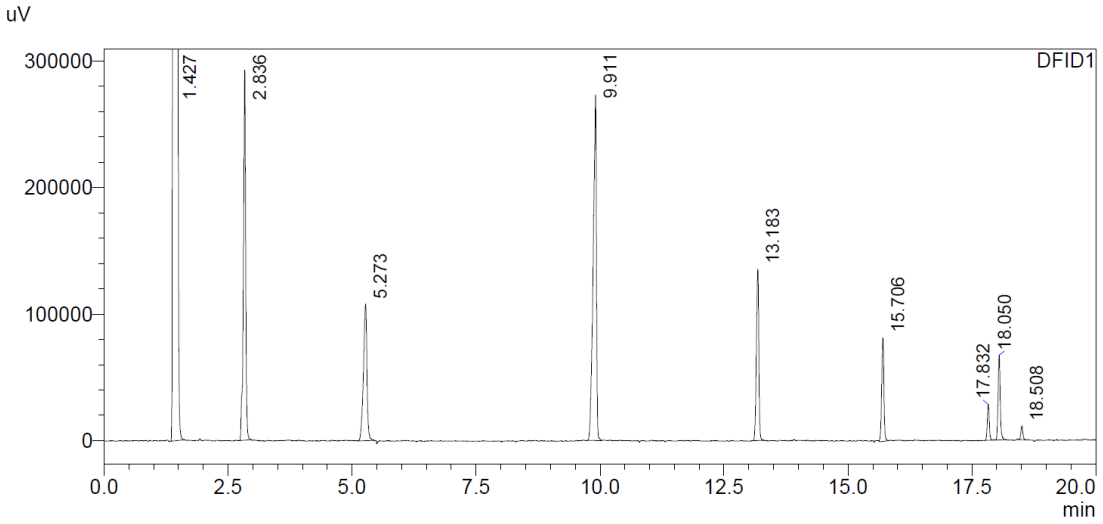


Figure 63 Gas chromatogram of modified coconut oil obtained after modification at 1 g scale using optimized reaction conditions and methyl esters sourced from coconut oil fatty acid distillate

Table 20 Fatty acid composition of modified coconut oil obtained after modification at 1 g scale using optimized reaction conditions and methyl esters sourced from fatty acid distillate determined by gas chromatography

Fatty acid symbol	Fatty acid trivial name	Retention time (min)	Specifications (%)
C8:0	Caprylic acid	2.836	24.93
C10:0	Capric acid	5.273	14.21
C12:0	Lauric acid	9.911	33.50
C14:0	Myristic acid	13.183	12.26
C16:0	Palmitic acid	15.706	6.82
C18:2	Linoleic acid	17.832	2.13
C18:1	Oleic acid	18.050	5.36
C18:0	Stearic acid	18.508	0.79

4.1.3 Preparation of modified coconut or low lauric coconut oil for biological testing

As was the case for acidolysis, modified coconut oil rich in medium-chain fatty acids (MCFAs) for biological testing in mice had to be prepared using interesterification concomitantly with the above experiments. This material had to be prepared in significant amounts, which required reaction scale up. The reaction was typically carried out with 100 g of virgin coconut oil (Thai Pure Coconut Co., Ltd.) mixed with 72 g of methyl octanoate, 48 g of methyl decanoate, and 11 g of lipase. The reaction was carried out at 45^{o}C for 96h. These reactions have used different conditions in comparison to the reactions discussed above as they were carried out before the completion of the optimization experiments. Furthermore, as the reaction scale was

larger we increased the reaction time ensure sufficient modification. After the end of the process, the modified coconut oil was obtained, and the composition was analyzed by NMR and GC techniques (Figures 64, 65, and Table 21).

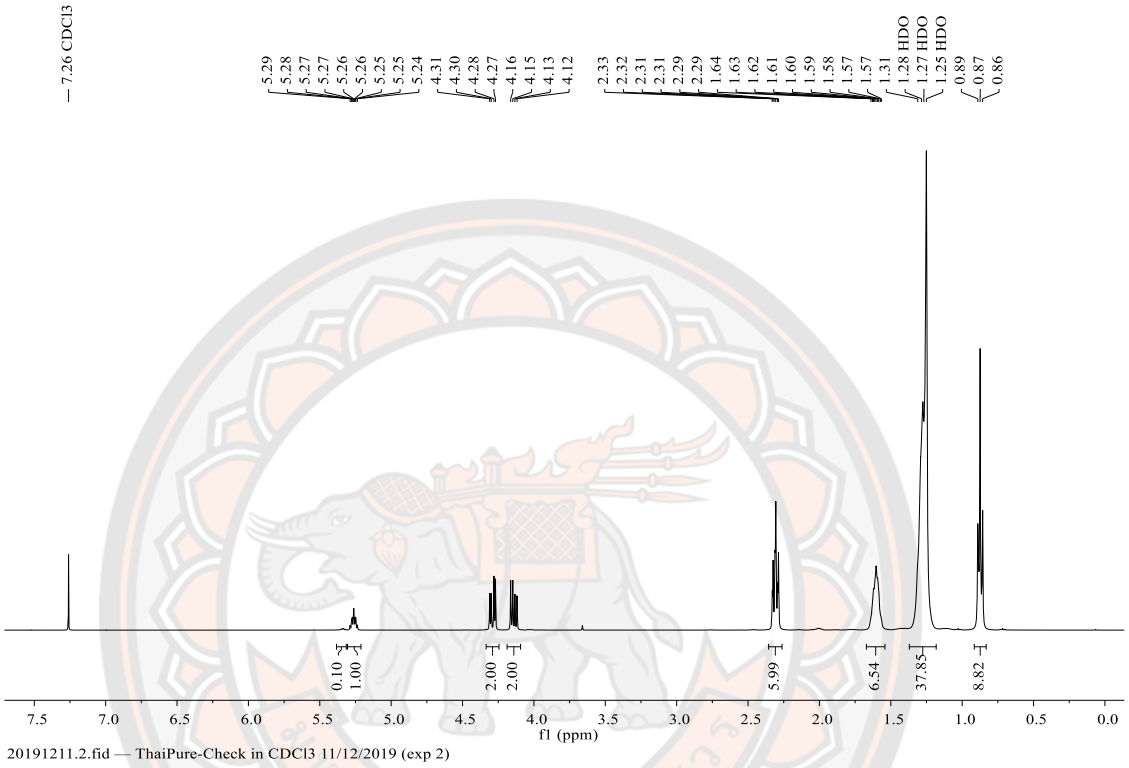


Figure 64 ^{1}H-NMR spectrum of modified coconut oils rich in medium-chain fatty acids (MCFAs) and low lauric acid

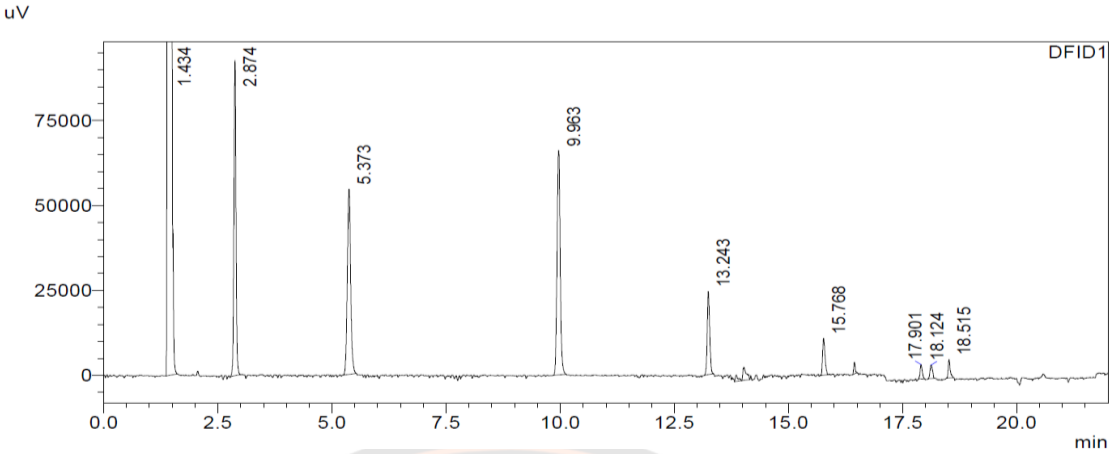


Figure 65 Gas Chromatogram of modified coconut oils rich in medium-chain fatty acids (MCFAs) and low lauric acid

Table 21 The percentage content of fatty acids in modified coconut oil rich in medium-chain fatty acids (MCFAs) and low lauric acid determined by gas chromatography

Fatty acid symbol	Fatty acid trivial name	Retention time (min)	Specifications (%)
C8:0	Caprylic acid	2.874	27.50
C10:0	Capric acid	5.373	26.00
C12:0	Lauric acid	9.963	29.80
C14:0	Myristic acid	13.243	8.70
C16:0	Palmitic acid	15.768	3.80
C18:2	Linoleic acid	17.901	1.30
C18:1	Oleic acid	18.124	1.40
C18:0	Stearic acid	18.515	1.50

Table 22 The percentage content of fatty acids in medium-chain triglyceride (MCT), virgin coconut oil (VCO), and modified virgin coconut oil or low lauric acid (LLA) determined by gas chromatography

Fatty acid symbol	Fatty acid trivial name	MCT	VCO (Thai pure)	LLA (Modified oil)
C8:0	Caprylic acid	56.70	6.70	27.50
C10:0	Capric acid	43.30	6.40	26.00
C12:0	Lauric acid	-	51.60	29.80
C14:0	Myristic acid	-	18.40	8.70
C16:0	Palmitic acid	-	8.00	3.80
C18:2	Linoleic acid	-	2.70	1.30
C18:1	Oleic acid	-	5.10	1.40
C18:0	Stearic acid	-	0.90	1.50

The information from Table 22 shows the percentage content of fatty acids in medium-chain triglyceride (MCT) oil, virgin coconut oil (VCO), and modified coconut oil or low lauric acid (LLA) oil. Further analytical information, ^{1}H-NMR spectra and GC chromatograms can be found in Appendix Figure 100-103 and Table 31-32. Results of animal tests using modified coconut oil (low lauric acid: LLA) are shown in Appendix B.

4.2 Part II Enzymatic hydrolysis of capsaicinoids and utilization of the fatty acid product for triglyceride modification

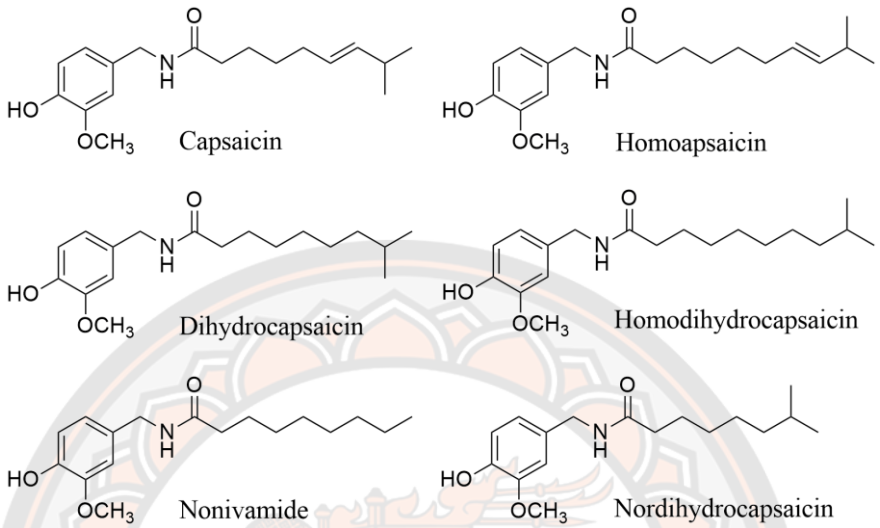


Figure 66 Chemical structure of the main capsaicinoids

It is well known that capsaicin is the major pungent compound found in chili peppers, and that it can activate TRPV1 cation channels of neuronal receptors employed in nociception. The evidence from the literature reporting the properties of capsaicin shows that it can be helpful in the treatment of pathologies and problems. While capsaicin has been shown to possess beneficial properties, it also has drawbacks, as it causes burning and irritation. Furthermore, previous studies reported that the biological effects of capsaicin (e.g., effects on energy metabolism) could be elicited by its biodegradation products. It is likely that one of the products of capsaicin biodegradation is *E*-8-methyl-6-nonenoic acid, which is a medium-chain fatty acid (MCFA). MCFAs are known to possess effects on energy metabolism and have other biological effects. One of the potential approaches to obtaining this medium-chain fatty acid (MCFA) is the enzymatic hydrolysis of capsaicin. Furthermore, naturally sourced materials containing capsaicin are likely to contain other capsaicinoids. In our case the starting material contained both capsaicin and dihydrocapcaicin. Thus the hydrolysis of this

material will produce 8-methylnonanoic acid in addition to the *E*-8-methyl-6-nonenoic acid.

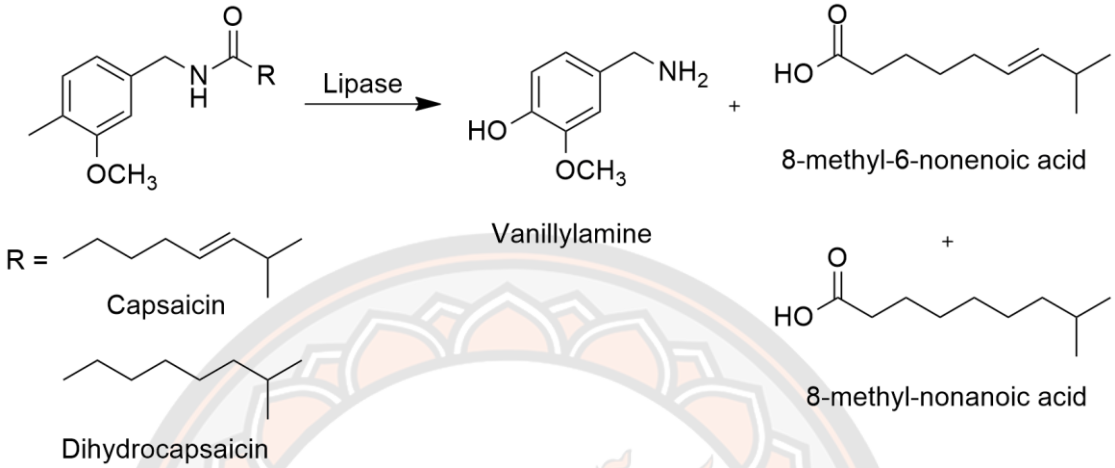


Figure 67 Enzymatic hydrolysis of a mixture of capsaicin and dihydrocapsaicin

4.2.1 Preparation of fatty acids from the hydrolysis of capsaicinoids

Initial experiments for the preparation of fatty acids from the hydrolysis of capsaicinoids were based on previous results obtained in our group (2017, Kanprakobkit, and Kielar). In these earlier experiments, enzymatic hydrolysis of capsaicinoids to medium-chain fatty acids was developed using lipase B from *Candida antarctica* immobilized on acrylic resin. We studied the effects of the enzymatic reaction, focusing on the effects of temperature, pH, and buffer concentration. The reaction was investigated by ^{1}H-NMR and HPLC. The optimal conditions identified during this work were 45^{o}C, pH 7.0, and 80-mM phosphate buffer concentration. The results obtained in terms of the content of vanillyl amine in the reaction mixture after 72 hours reached 77%. The study focused on the analysis of the side product vanillyl amine due to the possibility to detect it using HPLC. The mixture of the desired *E*-8-methyl-6-nonenoic acid and 8-methylnonanoic acid was isolated in 54.8% yield after column chromatography. In the final section of the earlier work, the experiments demonstrated the feasibility of the proposed methodology.

The previous results have provided a starting point for the continuation of this work described in the following section. This work has focused on further increasing the practicality of the hydrolysis reaction.

The first aspect of the reaction that we decided to address was the work up and purification of the hydrolysis reaction mixture. We hypothesized that performing extraction of the aqueous reaction mixture at different pH values could achieve simplification of the purification of the reaction mixture. We decided to first extract the reaction mixture at pH 10 to eliminate the vanillyl amine byproduct and any dissolved capsaicin, and to continue the process by changing the pH of the reaction mixture to 5 and conducting a second extraction to isolate the desired product. After extraction, the products obtained at both pH at 10 and 5 were analyzed using NMR spectroscopy. An example of the spectrum is shown in Figures 68 and 69 for pH 10 and 5, respectively. Satisfyingly, when the extraction workup was carried out using ethyl acetate as a solvent, the *E*-8-methyl-6-nonenoic acid and 8-methylnonanoic acid, were isolated without any additional contaminants.

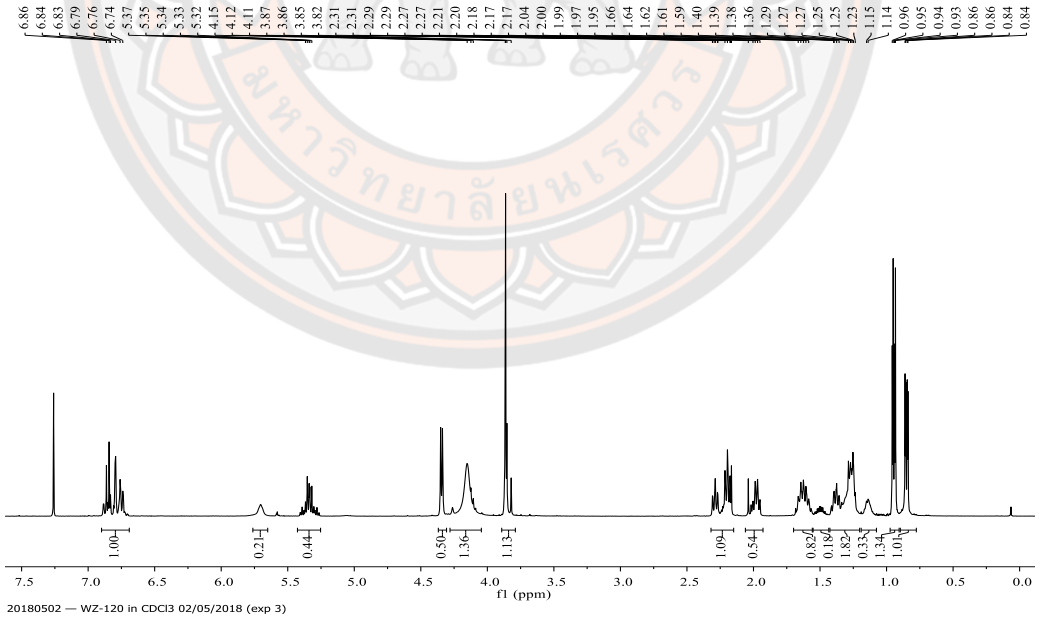


Figure 68 ^{1}H-NMR spectrum of EtOAc extract of capsaicin hydrolysis reaction mixture obtained with extraction at pH 10

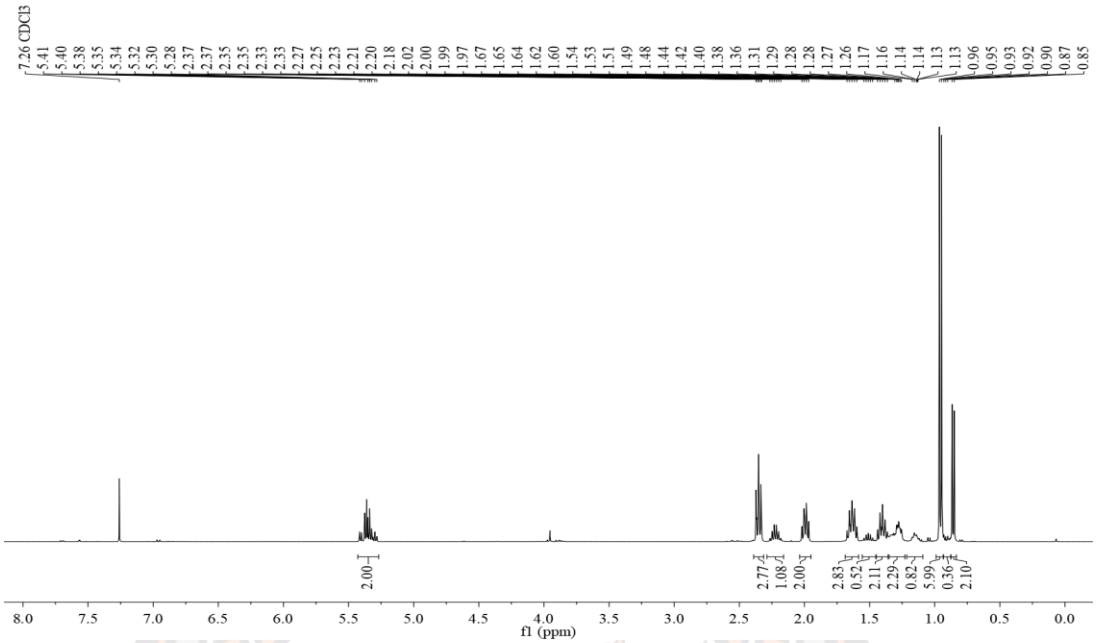


Figure 69 ^{1}H-NMR spectrum of EtOAc extract of capsaicin hydrolysis reaction mixture obtained with extraction at pH 5

We have continued our efforts of improving the practicality of the capsaicinoid hydrolysis reaction by addressing selection of the enzyme catalyst. The originally selected lipase B from *Candida antarctica* immobilized on acrylic (Sigma Aldrich) resin was selected after a screening of nearly 40 enzyme preparations. However, a similar enzyme Novozym® 435 (Strem) has not been part of this screening process. Therefore, we conducted an experiment to compare these two enzyme catalysts. The preliminary experimental conditions were adapted from the previous experiments. The reaction was carried out with phosphate buffer (pH 7.0, 80 mM) at 45^{o}C for 7 days. Both enzymes, lipase B from *Candida antarctica* immobilized on acrylic resin and Novozym® 435 were selected to be tested in the hydrolysis reaction. The desired product was obtained after extraction at pH 5. The product was weighed and analyzed to confirm the structure using the ^{1}H-NMR technique (Appendix A Figure 168). The results of productivity are shown in Figure 70. The amount of fatty acids obtained using lipase B from *Candida antarctica* immobilized on acrylic resind on average was 6.5 mg/day. On the other hand, the reaction catalyzed by Novozym® 435 produced on average 7.7 mg/day of fatty acids. The amount of product obtained using

Novozym® 435 as the catalyst was higher than when lipase B from *Candida antarctica* immobilized on acrylic resin. Furthermore, analysis of the ^{1}H-NMR spectra of products made by both enzymes indicated the product made using Novozym® 435 had a higher purity. Finally, Novozym® 435 can be purchased at a lower price. Based on these results, Novozym® 435 was selected for further study of other parameters.

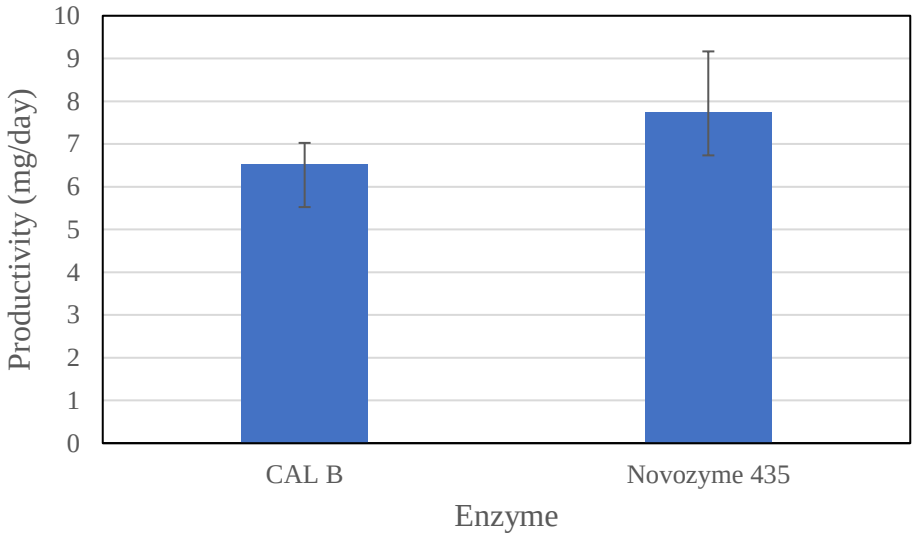


Figure 70 Productivity of enzymatic hydrolysis of capsaicin with the enzymes lipase B from Candia antarctia immobilized on acrylic beads and Novozym® 435

Having selected the enzyme to be used in further work, we turned to reaction time as a parameter to be investigated. The reactions were carried out using the initial conditions with the enzyme Novozym® 435 using different reaction times: 3 and 7 days. The product was obtained after extraction at pH 5, and the products were weighed and analyzed using ^{1}H-NMR. The results of this experiment are shown in Figure 71, which shows that the average daily productivity for the fatty acids was 12.1 mg and 7.7 mg for reactions carried out with 3 days and 7 days reaction turnover time, respectively. Therefore, further reactions were carried out with a reaction mixture turnover time of 3 days as this increases the productivity of the reaction and represents a more efficient use of the enzyme catalysts.

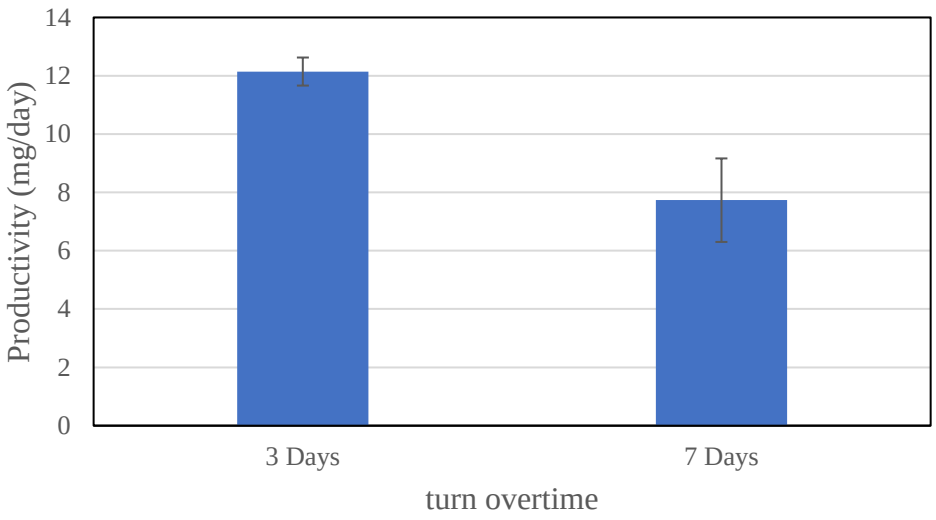


Figure 71 Productivity of enzymatic hydrolysis of capsaicin with reaction mixture turnover times of 3 days and 7 days

Finally, the effect of the temperature was investigated. The reaction was carried out using the initial conditions for 3 days of turnover time at 45°C, 50°C, and 55°C. The results are shown in Figure 72, which shows that the average daily productivity for the fatty acids was 12.1 mg/day at 45°C and increased to 16.8 mg/day at 50°C. Finally, fatty acid was decreased to 13.2 mg/day at 55°C. Thus, the results indicate that the optimal performance is attained around 50°C, which was utilized in further work. Thus, the results have shown that the best performance is achieved at 50°C, which was used in further work.

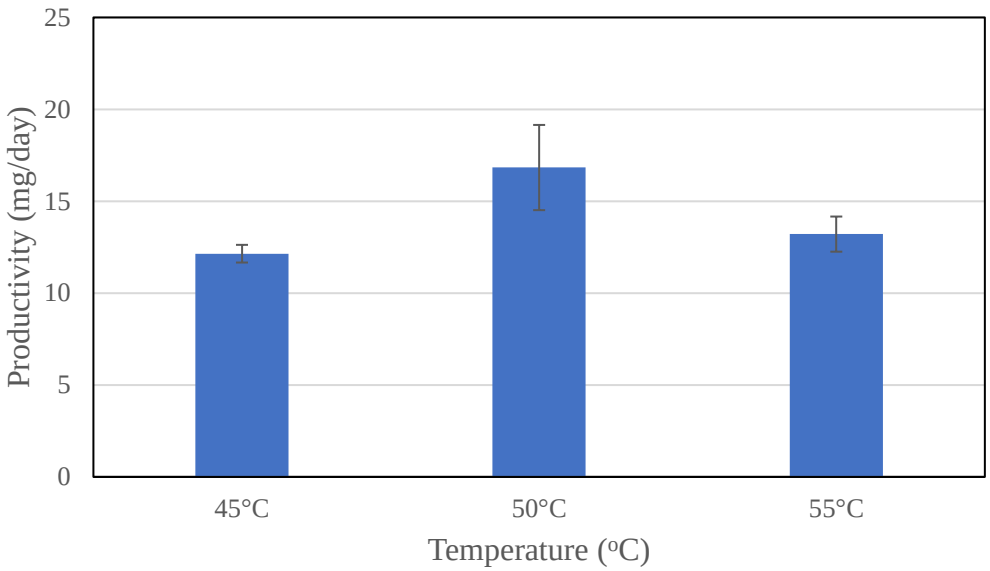


Figure 72 Productivity of enzymatic hydrolysis of capsaicin with reaction mixture turnover times of 3 days at 45°c, 50°C and 55°C

Finally, we obtained the optimal conditions for preparing fatty acids from the hydrolysis of capsaicinoids. The optimal hydrolysis reaction was conducted with Novozym 435 enzyme at 50°C with a 3 day reaction mixture turn over time. The reactions were carried out with more than 10 repeats. The fatty acids obtained from the individual reaction mixture turn overs were combined, and the structure was confirmed using ^{1}H-NMR, GC, and MS, as shown in Figure 73-75.

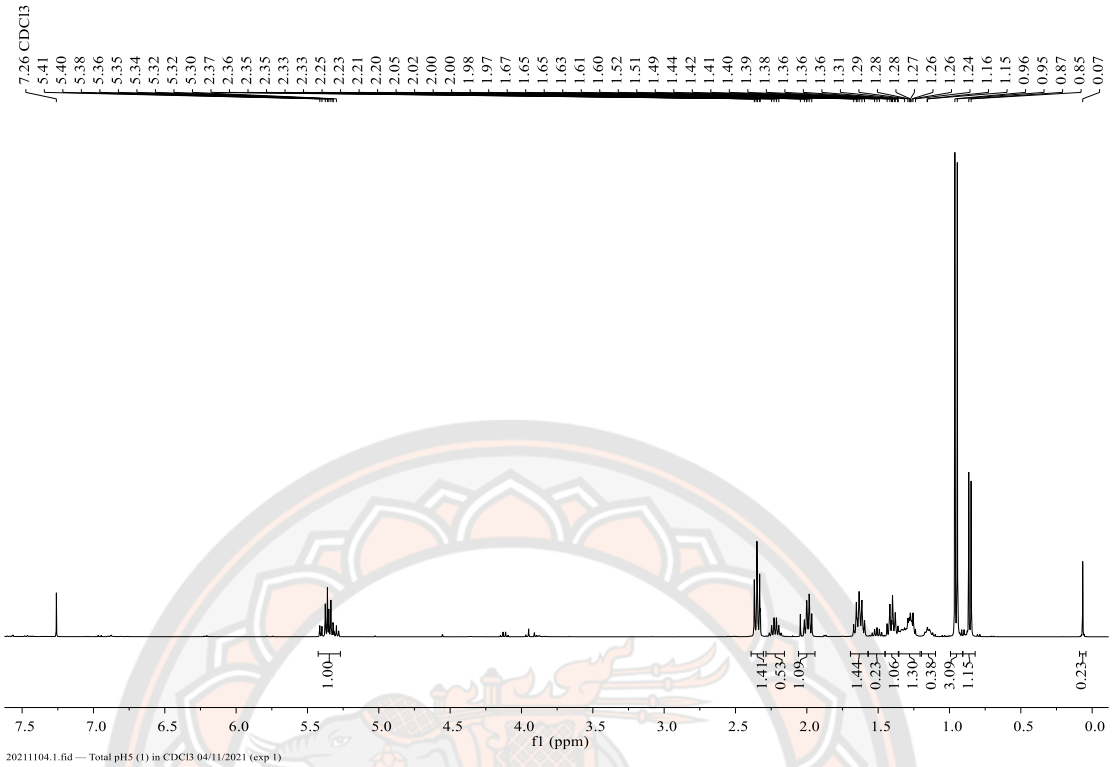


Figure 73 ^{1}H-NMR spectrum of fatty acid from the hydrolysis of capsaicin

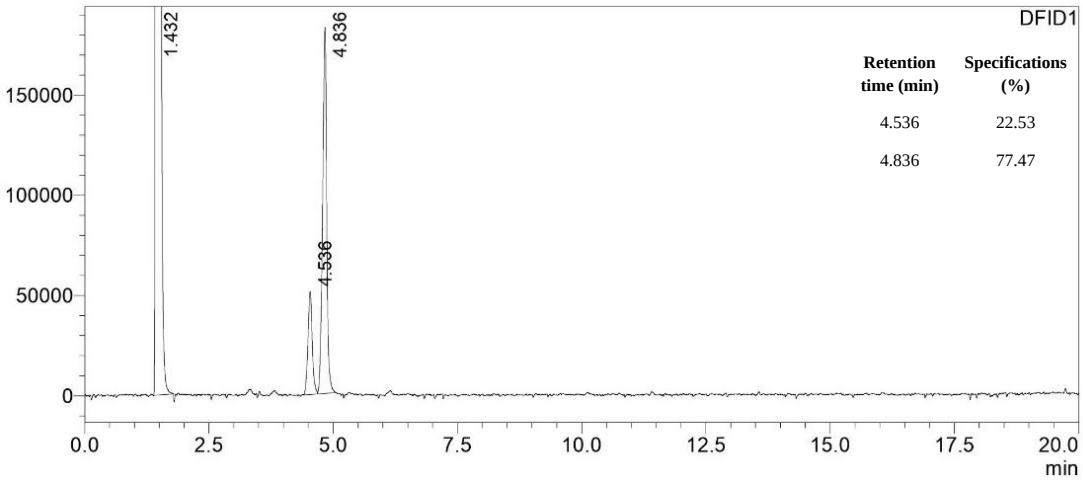


Figure 74 Gas chromatogram of fatty acid from the hydrolysis of capsaicin

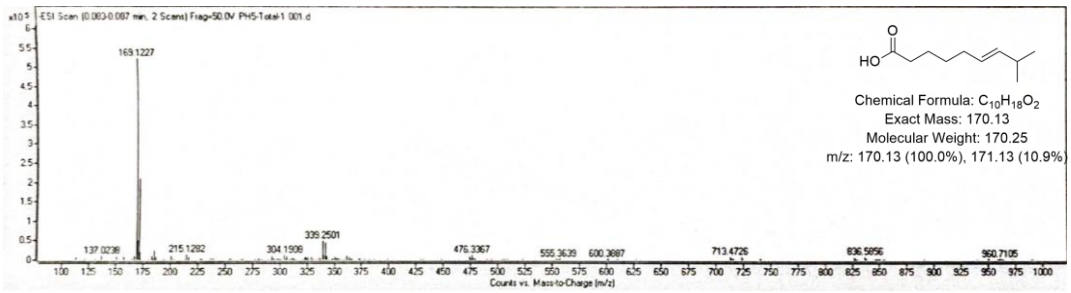


Figure 75 Mass chromatogram of fatty acid from the hydrolysis of capsaicin

4.2.2 Initial experiments for the modification of coconut oil using fatty acids obtained from the hydrolysis of capsaicinoids

Having established a working protocol for the enzymatic hydrolysis of capsaicionoids and a way of isolating the resulting fatty acids, we continued the work with modification of coconut oil using these fatty acids in an enzyme catalyzed acidolysis reaction. The initial conditions for the acidolysis reaction were based on our previous work with the reaction being carried out with coconut oil to fatty acid ratio of 1:3 at 45^{o}C for 72h. with 10% wt. loading of the enzyme catalyst (Lipozyme TL IM). The result of the acidolysis reaction was evaluated using ^{1}H-NMR, and GC was used to confirm the results. The ^{1}H-NMR was utilized to evaluate the alteration of the structure of coconut oil by exchange of originally present fatty acids with fatty acids from capsaicinoids. These changes in the structure of the oil lead to the appearance of clear changes in the ^{1}H-NMR spectrum. Firstly, the incorporation of the unsaturated fatty acid from capsaicin leads to the increase in the integral value of the signal at 5.1-5.2 ppm; the protons attached to the double bond give distinct signals. In addition, the terminal isopropyl group gives a distinct doublet for the methyl groups around 0.95 ppm. Finally, the integral of the signal for intermediate methylene groups of the acyl groups at 1.1-1.3 ppm is expected to decrease because of this transformation. The ^{1}H-NMR spectrum of the reaction mixtures from the initial experiments has indicated the incorporation of the fatty acids from capcaicinoids into the product (Figure 76). In addition, the ^{1}H-NMR spectrum demonstrates that the average length of the fatty acids in the modified product has been reduced as the integral of the signal at 1.1-1.3 ppm decreased from 51.87 (Appendix A Figure 94) in the original coconut oil to 36.76 in

the product. The corresponding GC spectrum for this representative reaction can be seen in Figure 77, and the relative contents of incorporation of fatty acids in the product can be seen in Table 23. Nonetheless, the incorporation was rather limited. Thus, the reaction was repeated at a temperature of 60ºC. The result is shown in Figure 78. It is found that incorporation of the fatty acids from capsaicinoids was significantly increased as a result of this change. The results from GC analysis indicate that the content of incorporation of fatty acid from capsaicinoids reached 31.17% (Figure 79, Table 24). While the content of lauric acid is 38.67%, which is significantly lower than in the original coconut oil, which is 48.5% (Appendix A Figure 95, Table 30). When comparing the amount of fatty acids from capsacinoids incorporated into the product at 45ºC and 60ºC, it was found that the incorporation of fatty acid obtained from 60ºC was higher than at 45ºC. Based on these results, The temperature at 60ºC was selected for the next study on other parameters.

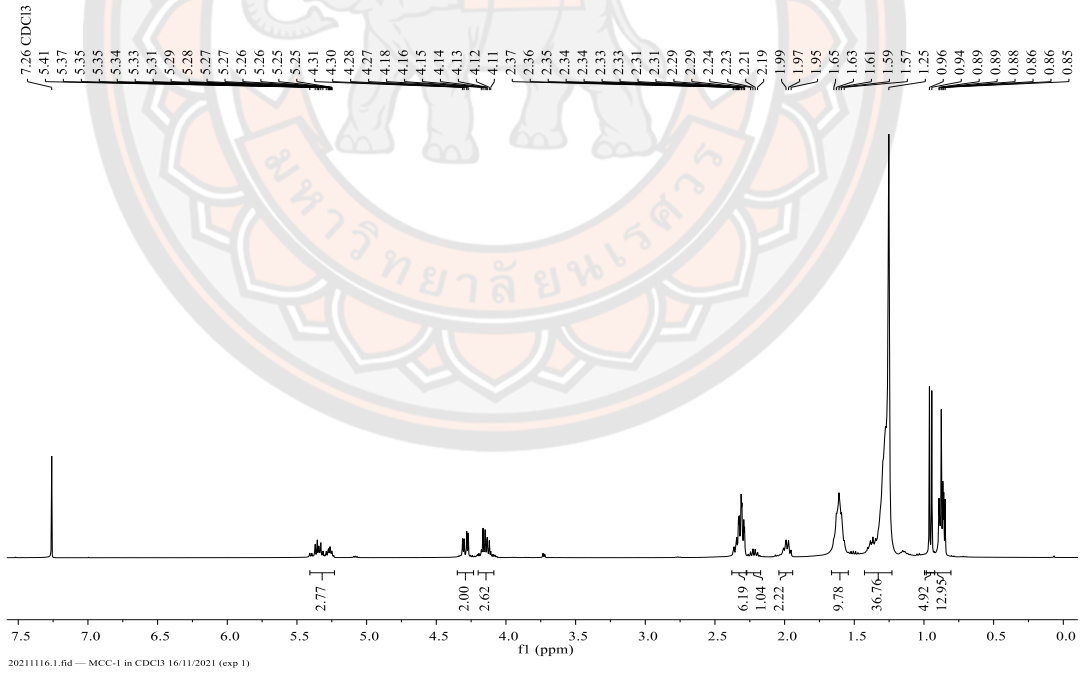


Figure 76 ^{1}H-NMR spectrum of product from initial modification of coconut oil with Lipozyme TL IM 10% and fatty acid from the hydrolysis of capsaicin at 45ºC

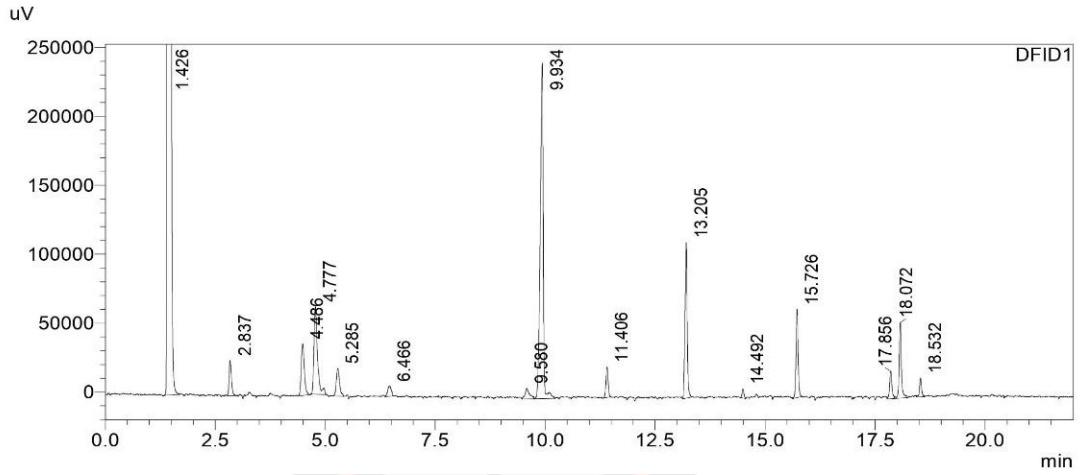


Figure 77 Gas chromatogram of product from initial modification of coconut oil with Lipozyme TL IM 10% and fatty acid from the hydrolysis of capsaicin at 45ºC

Table 23 Fatty acid composition of the product from the initial modification of coconut oil with Lipozyme TL IM 10% and fatty acid from the hydrolysis of capsaicin at 45°C determined by gas chromatography

Fatty acid symbol	Fatty acid trivial name	Retention time (min)	Specifications (%)
C8	Caprylic acid	2.837	3.14
	8-methylnonanoic acid	4.486	6.16
	E-8-methyl-6-nonenoic acid	4.777	11.86
C10	Capric acid	5.285	3.49
	-	6.466	1.40
	-	9.580	1.81
C12	Lauric acid	9.934	38.67
	-	11.406	2.57
C14	Myristic acid	13.205	13.11
	-	14.492	0.66
C16:0	Palmitic acid	15.726	6.90
C18:2	Linoleic acid	17.856	2.45
C18:1	Oleic acid	18.072	6.34
C18:0	Stearic acid	18.532	1.44

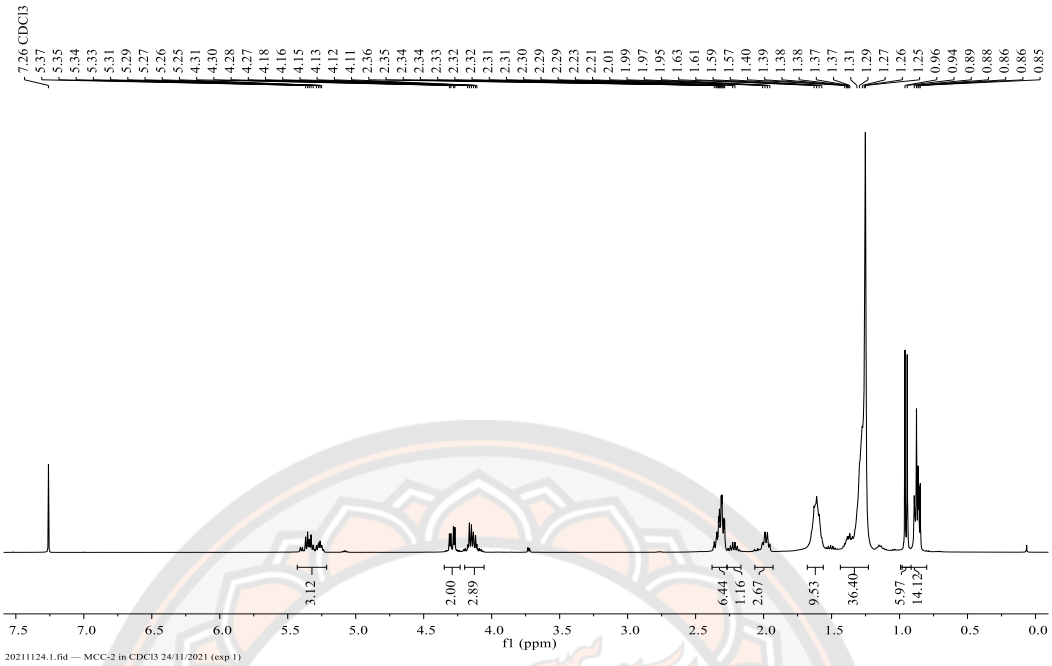


Figure 78 ^{1}H-NMR spectrum of product from initial modification of coconut oil with Lipozyme TL IM 10% and fatty acid from the hydrolysis of capsaicin at 60^{o}C

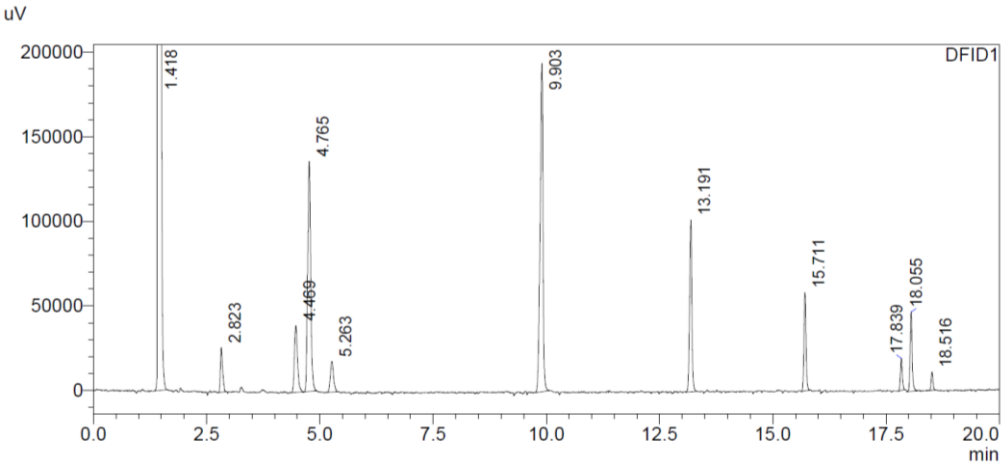


Figure 79 Gas chromatogram of product from initial modification of coconut oil with Lipozyme TL IM 10% and fatty acid from the hydrolysis of capsaicin at 60^{o}C

Table 24 Fatty acid composition of product from initial modification of coconut oil with Lipozyme TL IM 10% and fatty acid from the hydrolysis of capsaicin at 60°C determined by gas chromatography

Fatty acid symbol	Fatty acid trivial name	Retention time (min)	Specifications (%)
C8	Caprylic acid	2.823	3.50
	8-methylnonanoic acid	4.469	7.11
	E-8-methyl-6-nonenoic acid	4.765	24.06
C10	Capric acid	5.263	3.37
C12	Lauric acid	9.903	32.80
C14	Myristic acid	13.191	13.37
C16:0	Palmitic acid	15.711	6.95
C18:2	Linoleic acid	17.839	2.07
C18:1	Oleic acid	18.055	5.62
C18:0	Stearic acid	18.516	1.15

4.2.3 Investigation of the effect of enzyme loading

A further experiment aimed at improving the efficiency of the enzyme catalyst was carried out, focusing on the enzyme loading in the reaction. The reaction was carried out at 60°C for 72 hours with coconut oil to fatty acid ratio of 1:3. with the enzyme Lipozyme TL IM loading at 1.0%, 5.0%, 7.5%, and 10%. The results of the experiments can be found in Appendix A Figure 168-175 and Table 36-39. The results from the ^{1}H-NMR spectra show that the average integral values for the ^{1}H-NMR peak between 1.1 and 1.3 ppm were 51.08, 39.75, 36.59, and 36.40 for enzyme loading of

1.0%, 5.0%, 7.5%, and 10%, respectively. The GC analysis further confirmed the results of the incorporation of fatty acids from capsaicinoids. The fatty acid incorporation contents were 9.0%, 30.4%, 34.9%, and 38.0%, respectively. These results demonstrate that extent of incorporation of fatty acids from capsaicinoids into the modified products drops with lower enzyme loading.

4.2.4 Optimization by Response Surface Methodology (RSM)

We have conducted further experiments in a more systematic manner to determine the optimal conditions for increasing the content of incorporated capsaicinoid fatty acids in the product. The response surface methodology (RSM) approach was used for the optimization of the process parameters and for identifying the optimal conditions. The experiments were carried out to investigate the effects of three parameters, including reaction time, temperature, and the ratio of fatty acids from capsaicin and coconut oil. The RSM analysis was carried out using the Box-Behnken approach with 3 center points and 1 block. The reactions were carried out as described above and analyzed using ^{1}H-NMR spectroscopy. The integral of the peak at 0.95 ppm is a means of analyzing the reaction and the degree of incorporation of the fatty acids from capsaicinoids into the product. The three investigated levels of temperature (X1) were 50^{o}C (-1), 60^{o}C (0), and 70^{o}C (1). The three investigated levels of reaction time (X2) were 24 h (-1), 48 h (0), and 72 h (1). Finally, the three investigated levels of the fatty acids to the coconut oil weight ratio were 0.6 (-1), 0.9 (0), and 1.2 (1). The results of the experiments carried out are shown in Table 25. The integral values, which are the independent variable (Y), range between 4.24±0.29 and 7.97±0.46.

Table 25 Level values for the Box-Behnken design of the response surface methodology experiments

Run	Independent Variables			Responses
	X_1	X_2	X_3	Integral
1	0	-1	1	6.77±0.33
2	0	-1	-1	5.56±0.94
3	0	0	0	6.80±0.26
4	-1	0	-1	5.70±0.21
5	0	0	0	6.65±0.39
6	0	1	-1	4.24±0.29
7	1	0	-1	6.64±0.47
8	0	1	1	7.97±0.46
9	1	0	1	7.19±0.14
10	-1	-1	0	6.72±0.43
11	-1	0	1	7.75±0.48
12	1	-1	0	6.20±0.11
13	-1	1	0	6.75±0.19
14	0	0	0	7.03±0.42
15	1	1	0	6.90±0.56

The experimental data were fit to an equation containing the linear and interaction terms (Equation 1) of temperature (X_1), reaction time (X_2), and reagent ratio (X_3). The experimental data were analyzed at the 95% confidence level and the R^2 value was 81.6% with an insignificant lack of fit. The values of the coefficients as well as t-values and p-values are shown in Table 26. It is evident that only the reagent ratio (X_3) variable and its interaction with reaction time ($X_2 * X_3$) give significant ($p<0.05$).

Equation 1:

$$y = 6.59 + 0.00125 * X_1 + 0.076 * X_2 + 0.94 * X_3 + 0.17 * X_1 * X_2 - 0.37 * X_1 * X_3 + 0.63 * X_2 * X_3$$

Table 26 Independent variables of products made from modified coconut oil (coefficients, standard error coefficients, t-values, and p-values for ^{1}H-NMR integral of peak at 1.2-1.3 ppm (Box Behnken design))

Independent variable and interactions	Coefficient	SE Coefficient	t-value	p-value
Constant	6.59	0.13	50.7	0.0128
X_1	0.00125	0.18	0.01	0.9947
X_2	0.076	0.18	0.4	0.6875
X_3	0.94	0.18	5.2	0.0009
X_1X_2	0.17	0.26	0.6	0.5350
$X_1 X_3$	-0.37	0.26	-1.4	0.1848
X_2X_3	0.63	0.26	2.4	0.0407

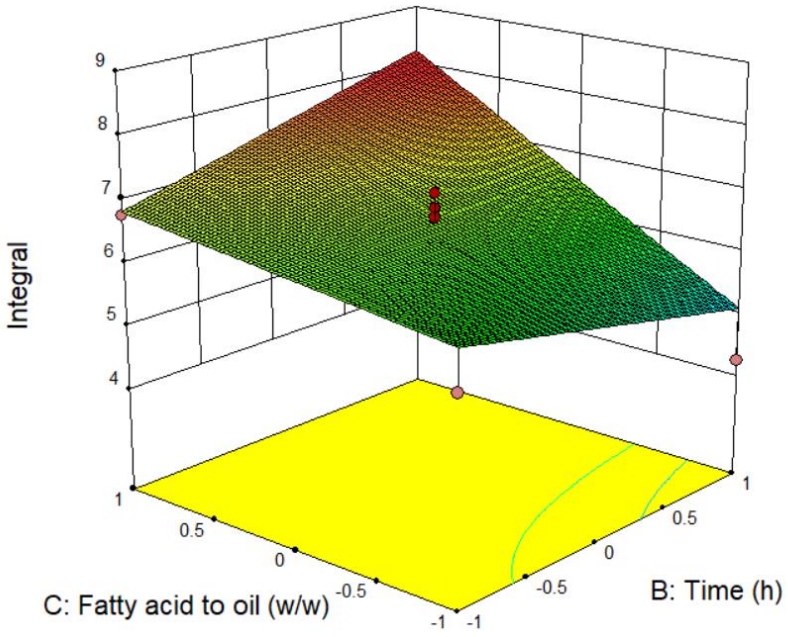


Figure 80 Three-dimensional plot of integral values versus time and oil to fatty acid ratio

The plot of the integral values versus reaction time and ester to oil ratio can be seen in Figure 80. The lack of significance of both temperature and reaction time as well as the R^2 value of 0.816 suggest limited value of the model. This could be due to the relatively large dispersion of the observed integral values. The key takeaway is that performance of the reaction could be improved by increasing the weight ratio of the fatty acids relative to coconut oil.

We tested the model by predicting the composition of the product made in a reaction of fatty acids with coconut oil with a reaction time of 72 h, a fatty acid from capsaicin to coconut oil ratio of 1.2, and a reaction temperature of 60°C. The predicted value of the integral was 8.24±0.52. The reaction was then carried out at a scale of 0.1 g of coconut oil, and 0.12 g of fatty acid with 10% enzyme loading at 60°C. The results from the ^{1}H-NMR spectrum shown in Figure 81 can be observed that the value of the integral of the signal of interest in the ^{1}H NMR reached a value of 7.10 and the GC-based content of unsaturated and saturated fatty acids from capsaicin in the obtained product were 38.0% and 16.1%, respectively. (Figure 82, Table 27). Repeating the

reaction five times using these conditions and analyzing the results by ^{1}H-NMR provided a value of the integral of the peak of interest of 7.10±0.39, indicating the degree of repeatability of the result. However, this shows significant discrepancy from the predicted value, which is in line with the difficulties observed in the RSM experiments.

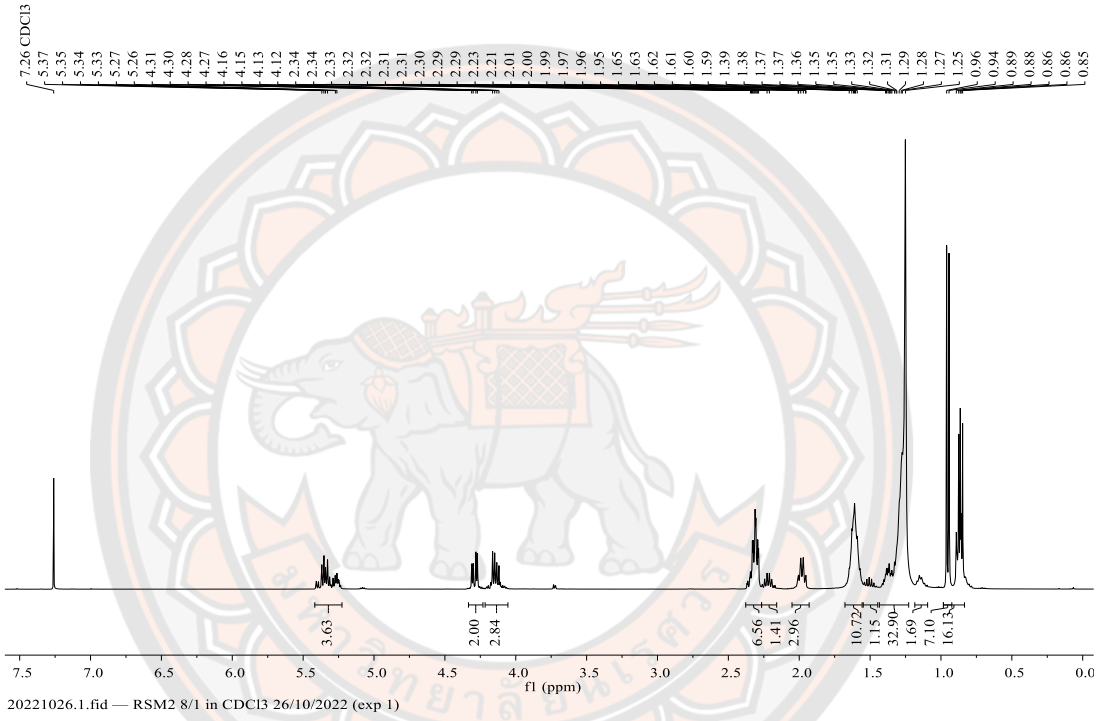


Figure 81 ^{1}H-NMR spectrum of product from modification of coconut oil under optimal conditions from RSM

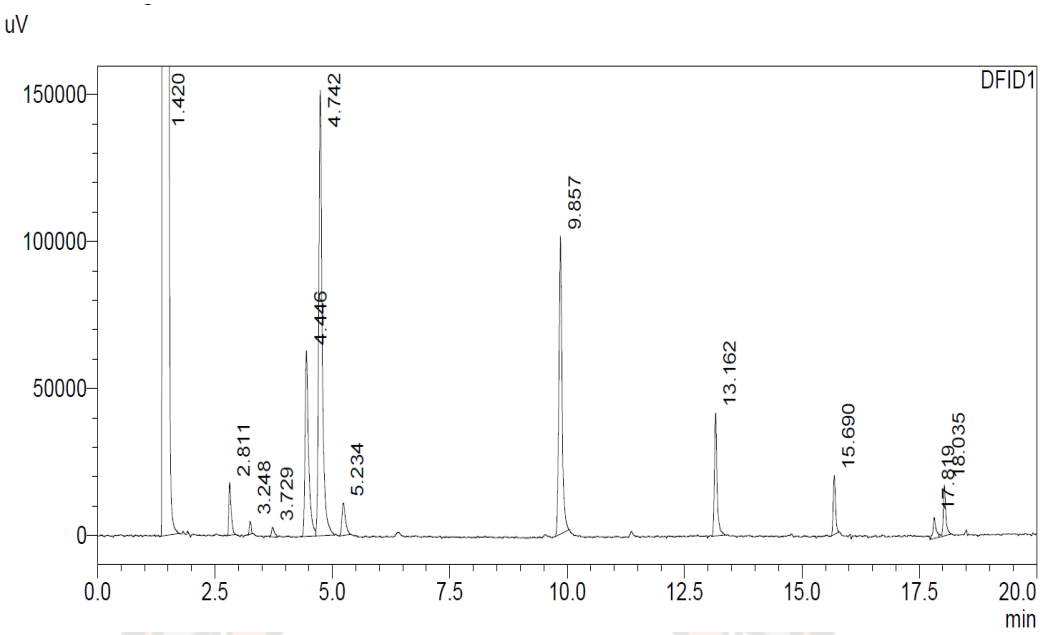


Figure 82 Gas chromatogram of product from modification of coconut oil under optimal conditions from RSM

Table 27 Fatty acid composition of product from modification of coconut oil under optimal conditions from RSM determined by gas chromatography

Fatty acid symbol	Fatty acid trivial name	Retention time (min)	Specifications (%)
C8	Caprylic acid	2.811	2.97
	-	3.248	0.56
	-	3.729	0.68
	8-methylnonanoic acid	4.446	16.06
	E-8-methyl-6-nonenoic acid	4.742	38.01
C10	Capric acid	5.234	2.76
C12	Lauric acid	9.857	23.07
C14	Myristic acid	13.162	7.85
C16:0	Palmitic acid	15.690	3.37
C18:2	Linoleic acid	17.819	1.52
C18:1	Oleic acid	18.035	3.15

Finally, the reaction was scaled up from the scale of 0.1 g used in the RSM experiments to the scale of 0.2 g of coconut oil and 0.24 g of fatty acid at 60^{o}C. The integral of the signal of interest at 0.95 ppm in the ^{1}H-NMR has a value of 5.65. The GC analysis indicates that the content of the unsaturated and saturated fatty acids from capsaicin in the product was 28.88 % and 9.12 %, respectively shown in Figure 84, Table 28. The reaction was carried out in triplicate giving a value for the integral of 6.10±0.47. This result further shows that some performance deterioration is observed upon scale up and further demonstrates the sensitive nature of this reaction, which will need to be addressed in future work.

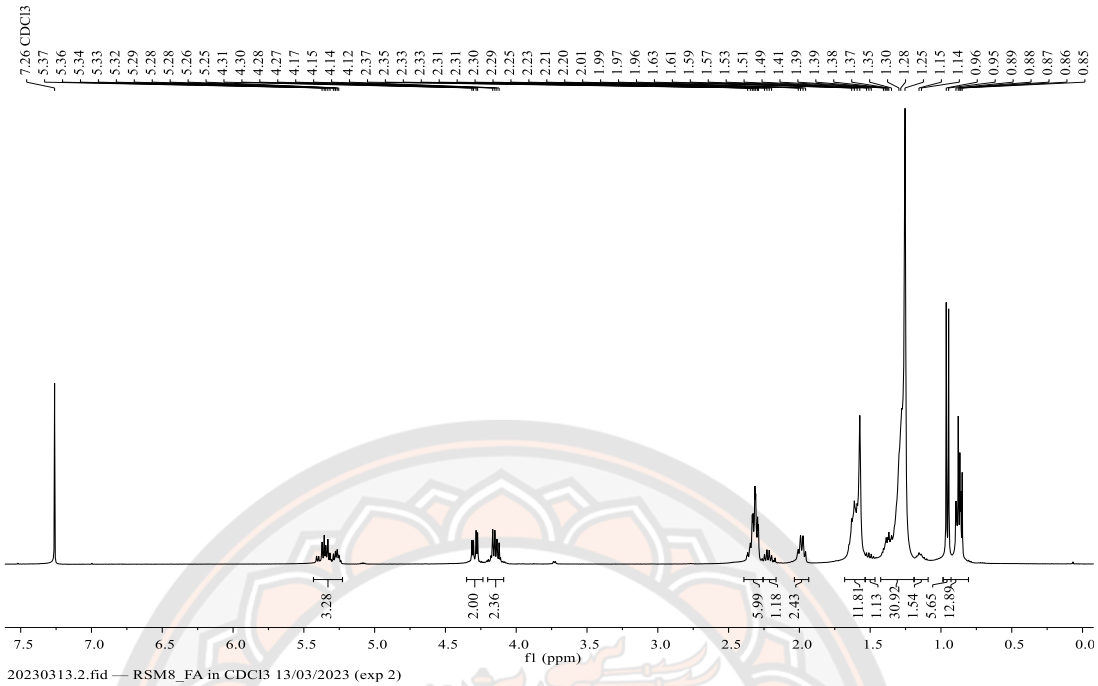


Figure 83 ^{1}H-NMR spectrum of product from modification of coconut oil under optimal conditions from RSM (up-scale)

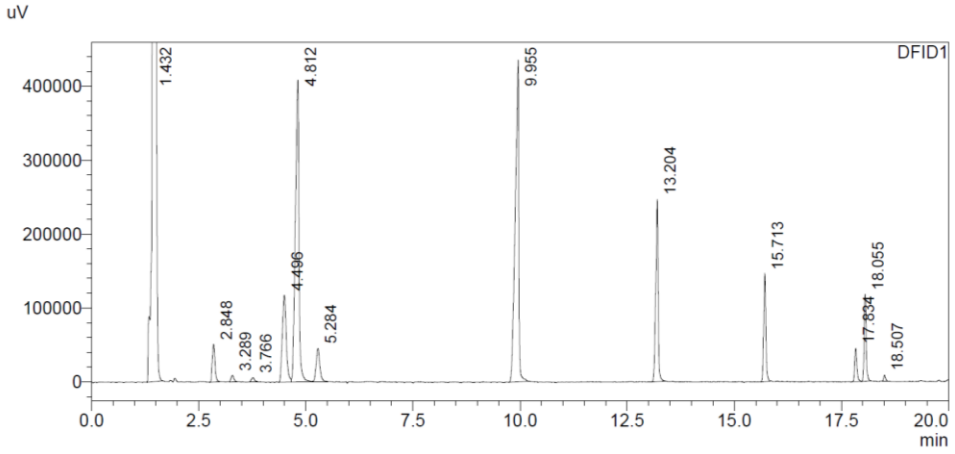


Figure 84 Gas chromatogram of product from modification of coconut oil under optimal conditions from RSM (up-scale)

Table 28 Fatty acid composition of product from modification of coconut oil under optimal conditions from RSM determined by gas chromatography (upscale)

Fatty acid symbol	Fatty acid trivial name	Retention time (min)	Specifications (%)
C8	Caprylic acid	2.848	2.68
	-	3.289	0.49
	-	3.766	0.37
	8-methylnonanoic acid	4.496	9.12
	E-8-methyl-6-nonenoic acid	4.812	28.88
C10	Capric acid	5.284	3.29
C12	Lauric acid	9.955	30.66
C14	Myristic acid	13.204	11.50
C16:0	Palmitic acid	15.713	6.11
C18:2	Linoleic acid	17.834	1.96
C18:1	Oleic acid	18.055	4.62
C18:0	Stearic acid	18.507	0.32

4.3 Part III Enzymatic degradation of poly (ethylene terephthalate) (PET)

Poly(ethylene terephthalate) (PET) is the copolyester of ethylene glycol (EG) and terephthalic acid (TPA). The characteristics of PET are high strength and resilience, resistance to abrasion, and stretching. PET is widely used in packaging plastic bottles, resulting in an increase in the amount of PET plastic made, utilized, and discarded is increasing day by day. Thus, dealing with PET plastic has become an important issue. One of the most promising alternatives is to use enzymes as catalysts for PET depolymerization to its monomers. However, previous reports indicate that some enzymes or microorganisms are capable of degrading PET to its component monomers, which could potentially be recycled. Despite these promising results, full recycling of post-consumer PET remains a challenge. Therefore, in this part, we aimed to combine photooxidative and enzymatic methods for PET degradation. In this study, the combined approach is that the photooxidative degradation step, carried out with the help of a photocatalyst, would be used to partially degrade the structural integrity of the PET material with the aim of making it more readily susceptible to enzymatic degradation. The photoactive iridium complexes developed in our group for other research projects were used as the photocatalyst in the photooxidative degradation step.

4.3.1 Initial Experiments of enzymatic hydrolysis of poly(ethylene terephthalate) (PET)

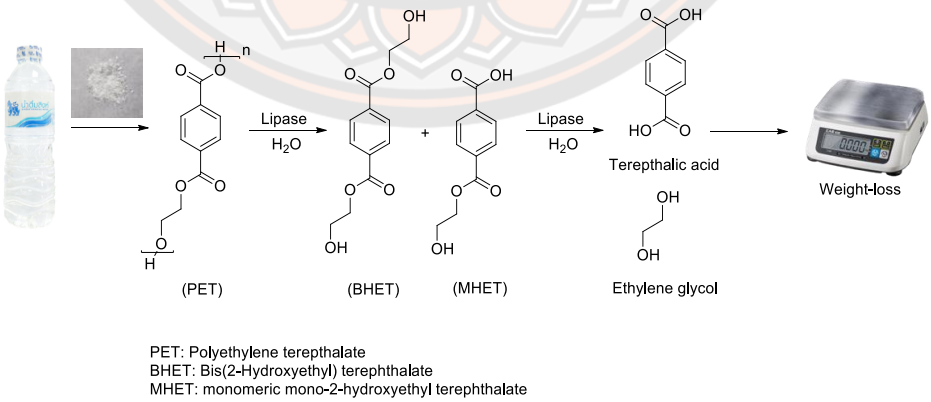


Figure 85 Enzymatic hydrolysis of poly(ethylene terephthalate) (PET)

1. Initial Experiments

We aimed to investigate the degradation or recycling of poly(ethylene terephthalate) (PET) from water bottles using enzymes as catalysts and to try to identify optimal conditions for this reaction. The initial experiments of degradation or recycling of poly(ethylene terephthalate) (PET) from water bottles using enzymes as catalysts. The enzyme used in the initial work have been lipase Humicola insolens cutinase (HiC, product Novozym© 51032), which is among the most efficient biocatalysts for PET depolymerization reported so far. For optimal conditions, PET hydrolysis reactions were carried out following a previously published report (de Castro, 2019). The conditions of the initial experiments were carried out with PET film with tris buffer (397 mM pH 8.95) and enzyme loading (0.065 g/g) at 62–65^{o}C for one week. The transformation of these reactions was calculated from the difference between the sample weights before and after according to the equation: WL=100x(W1 – W2)/W1), where W1 and W2 are the weights of the samples before and after treatment, respectively. The results demonstrated that the weight loss of the samples was 0.84% after hydrolysis by lipase HiC at 62-65^{o}C for 1 week. The lost weight showed that the decomposition of PET was observed to only a very limited degree. We have therefore conducted further experiments in an attempt to identify conditions resulting in a more extensive PET degradation.

2. Effect of temperature

The reaction temperature was the first effect to be studied. The literature report inspiring this work suggests that the optimal reaction temperature is 62.6°C. Reaction temperature is an important parameter. Therefore, we repeated the experiment at two different temperatures, 62-63^{o}C and 70^{o}C, to probe if the increase in temperature would lead to improve results. The reaction was carried out with the same enzyme as the previous experiment and reaction parameters, other than temperature, were kept identical to the initial tests. The observed weight losses of PET film were 0.80% and 0.90% at 62-63^{o}C and 70^{o}C respectively. Therefore, the results suggest minimal change in the performance as a result of increasing the temperature.

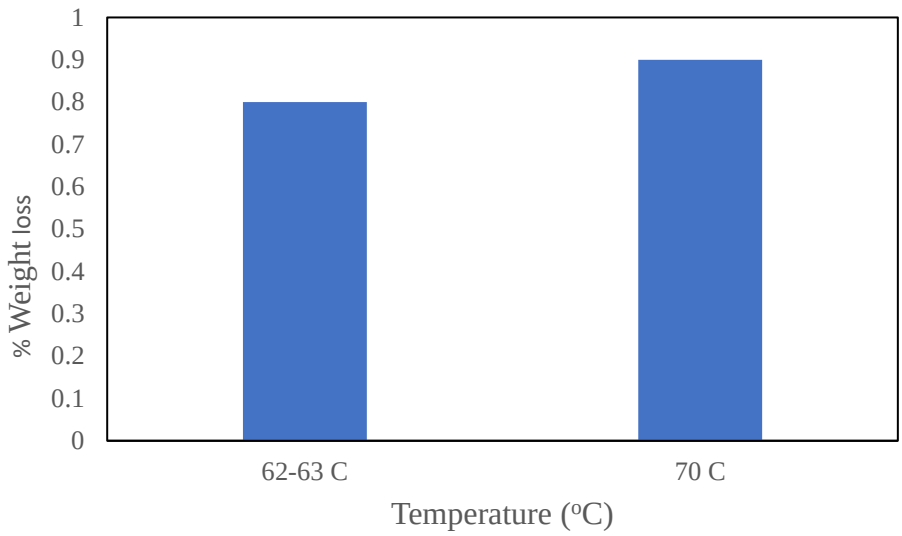


Figure 86 %Weight loss of PET film with reaction temperatures of 62-63ºC and 70ºC

3. Effect of enzyme loading

The second parameter to be studied was the concentration of the enzyme. The reactions were carried out with enzyme HiC loading of 65, 130, and 195 µl while other reaction parameters were identical to the initial tests. The results of these experiments obtained the weight loss of PET film were 0.84%, 0.74%, and 0.84%, respectively. However, it can be observed that increasing the concentration of enzyme did not significantly affect to degradation of PET film in terms of weight loss.

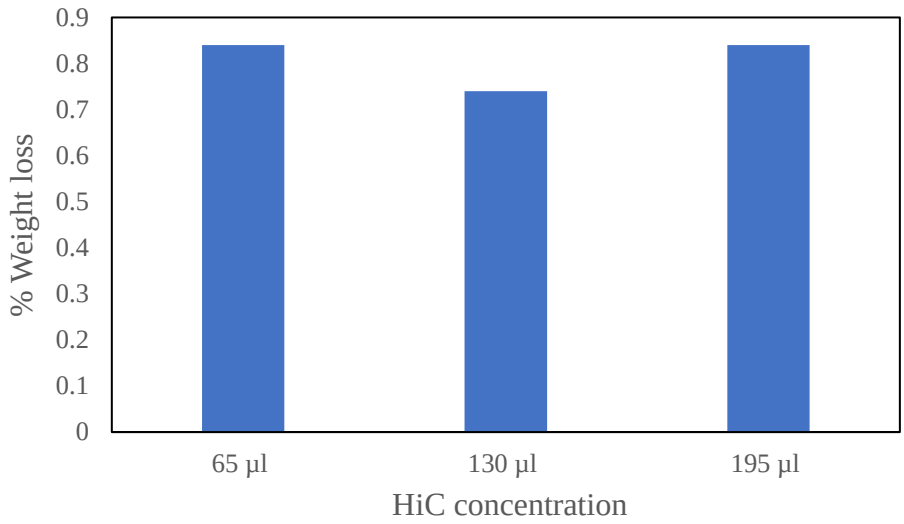


Figure 87 %Weight loss of PET film with enzyme HiC concentration at 65, 130, and 195 µl

4. Effect of reaction time

The third parameter to be studied was the effect of the reaction time. The reactions were carried out at different times; 1, 2, 3, 4, 6, and 8 weeks and maintained other reaction parameters identical to the initial tests. The results of these experiments obtained the weight loss of PET film were 0.84%, 0.70%, 0.60%, 0.60%, 0.95%, and 1.35%, respectively. The results showed that PET film degradation at 1–4 weeks, resulting in weight loss, differed slightly. However, the weight loss of PET film at 6 and 8 weeks indicated that weight loss increased over time.

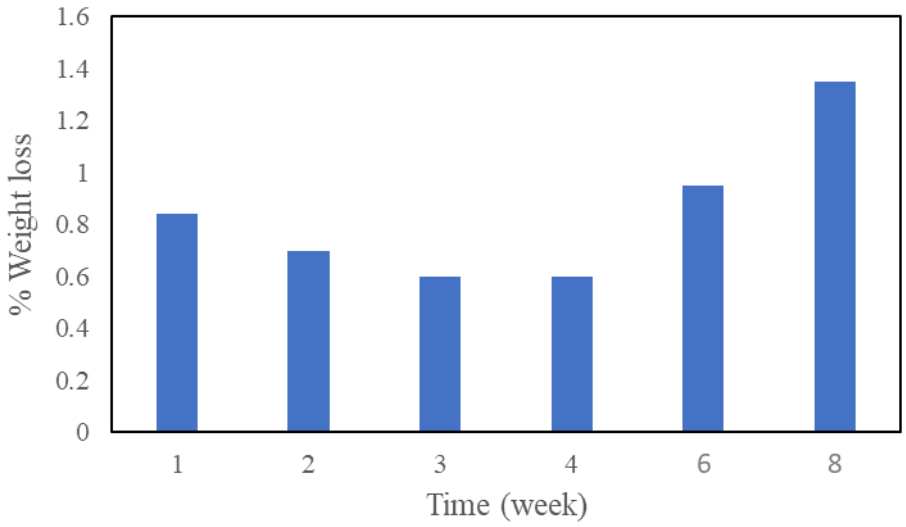


Figure 88 %Weight loss of PET film with reaction time of 1, 2, 3, 4, 6, and 8 weeks

5. Effect of continuous enzyme loading

Finally, the effect of continuous enzyme loading was investigated. The reaction was carried out the same as the initial condition. From week 2 onwards, the enzyme catalyst and Tris buffer were removed, and the PET film was dried and weighed. The results show that the weight loss of PET film decreased slightly from 1.00 g to 0.9960 g, 0.9923 g, and 0.9911 g after weeks 2, 3, and 4, respectively. However, the weight loss of PET film from continuous enzyme loading indicated that the lifetime of enzymes is limited and affects the hydrolysis of PET film.

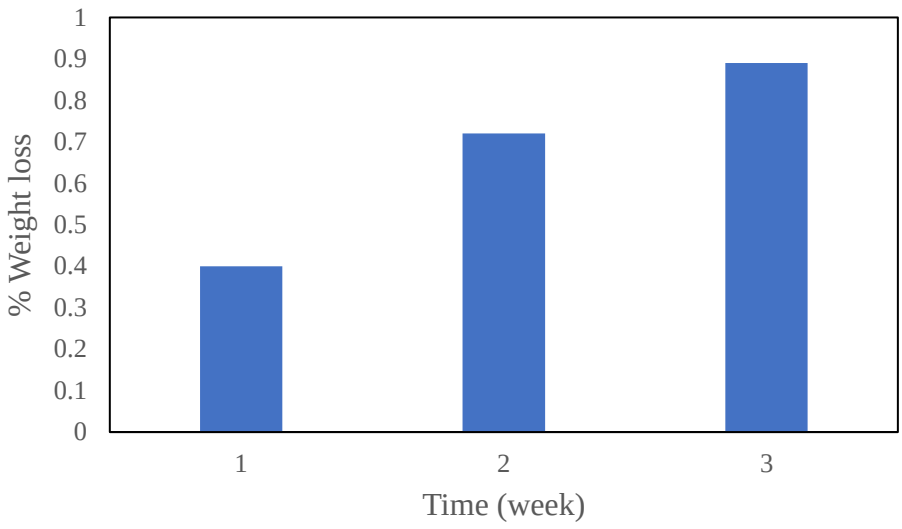


Figure 89 %Weight loss of PET film with reaction continuous enzyme loading every week

4.3.2 PET degradation by photooxidative and enzymatic catalysis

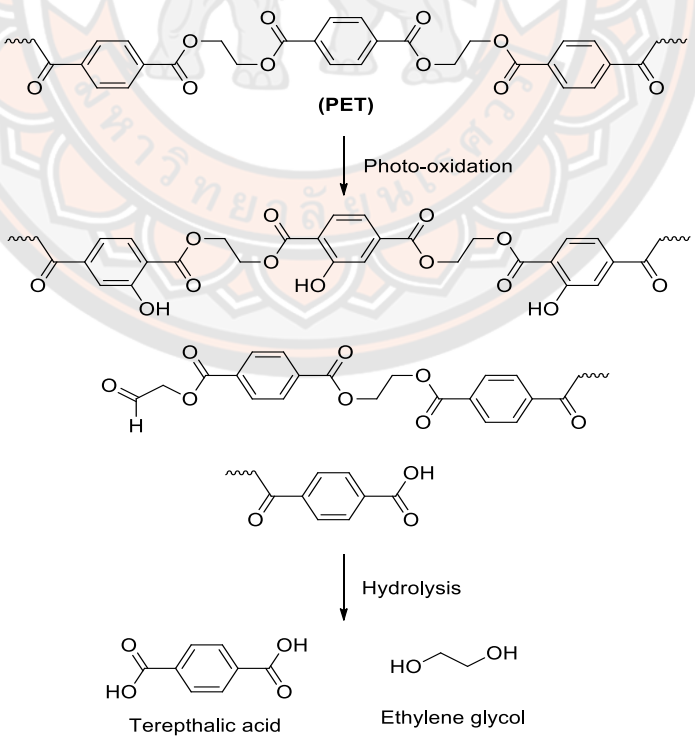


Figure 90 Photo-oxidation and hydrolysis degradation of PET

Enzymatic degradation is being developed as a potential alternative method for the degradation of PET films to their monomers. However, the efficiency of the degradation reaction could be hampered by inaccessibility of the hydrolyzable ester bond. Therefore, in this part, photo-oxidative processing and hydrolysis were used together for the degradation of PET film. In the first step, we attempted to damage the PET film by a photo-oxidative process. The reaction was carried out with an iridium complex solution and light for 24 h, 48 h, and 72 h. In this process the decomposition of the material should be achieved by the combination of light, which is considered one of the primary sources of damage exerted upon polymeric substrates at ambient conditions, and the iridium catalysts. Subsequently, the PET film subjected to the photooxidation pretreatment was hydrolyzed by HiC lipase. The results of these experiments obtained for the weight loss of PET film were 0.58%, 0.42%, and 0.40%, respectively (Figure 91A). These results show that the weight of PET that the effect of the photocatalyzed pretreatment on PET degradation was minimal. Thus, in the next experiment the reactions were improved by dissolving the iridium complex with methanol and setting up as described above. Unfortunately, very little change in the weight of the PET films was observed in this case as well (Figure 91B).

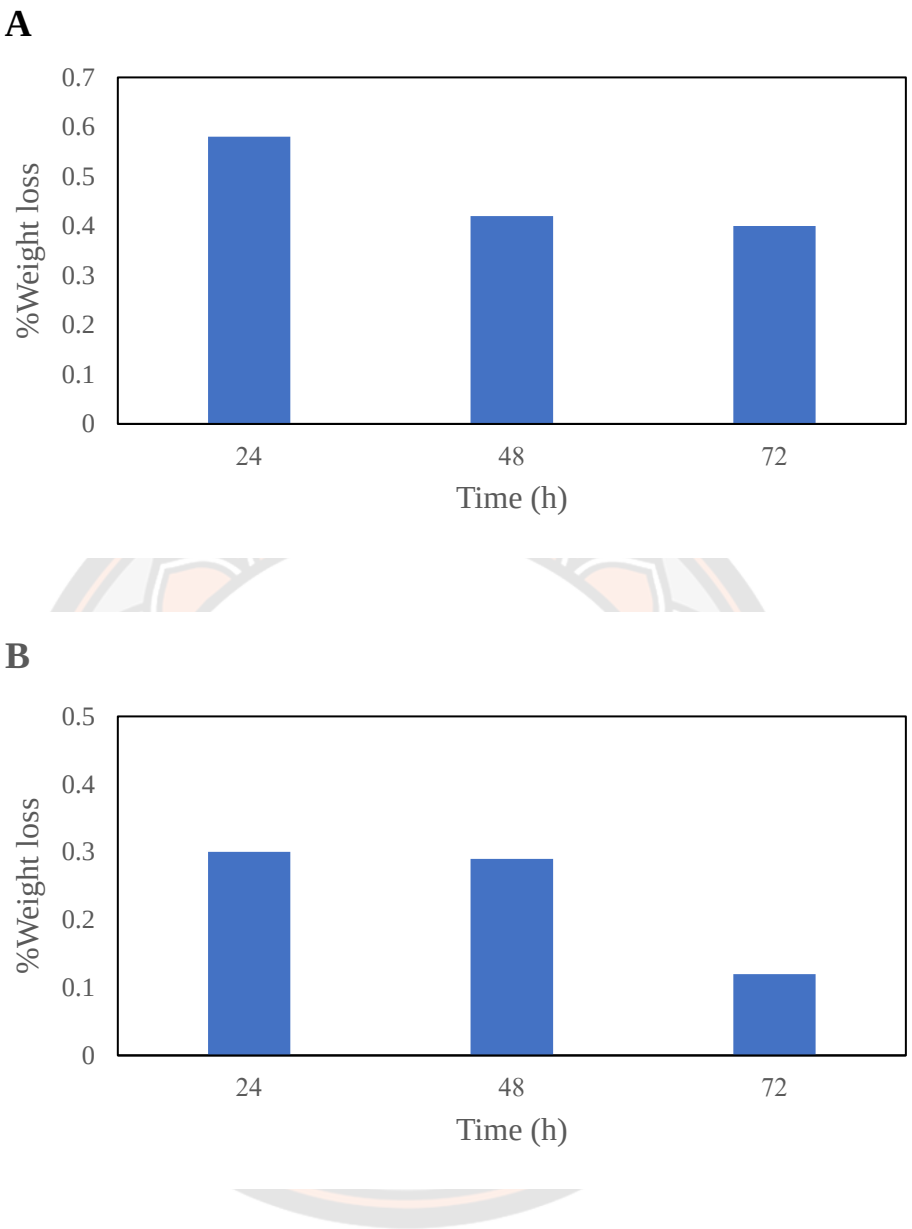
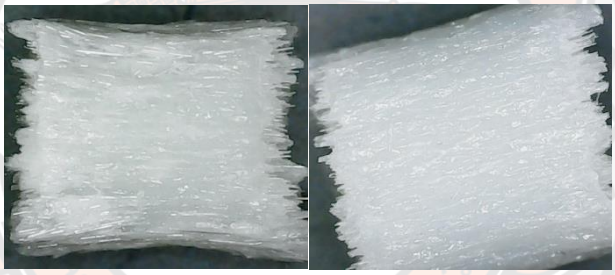


Figure 91 %Weight loss of PET film with photooxidative and enzymatic catalysis A) photooxidative in water solvent B) photooxidative in methanol solvent

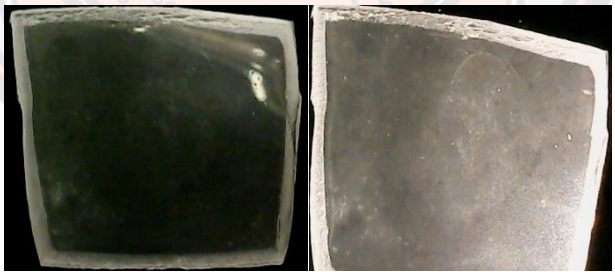
Despite our efforts described above, we were not able to achieve significant PET degradation in our experiments. We have therefore, decided to attempt to used different type of PET (PET granules from Sigma Aldrich). We have carried out the reaction as described above. However even in this case we were not able to achieve significant weight decrease of the PET material.

4.3.3 Improve the surface of the PET film by alkali pretreatment

To address this issue, we decided to test a recently published pretreatment of PET with alkali as an alternative procedure. The reactions were carried out as described above with both PET films and PET granules from Sigma Aldrich being were soaked in 10 M NaOH at room temperature for 24 h. The results after treatment with NaOH are shown in Figure 92. Differences can be observed between the untreated and pretreated PET materials. The NaOH pretreatment leads to an opaque substrate, which is white in color. After that, both PET films were subjected to hydrolysis by Novozym 51032 enzyme. The result show that the weight loss was 1% for both PET films treated with 10 M NaOH. Although the surface of PET film and PET granules have been modified before decomposing with enzymes, the degradation still proceeded to an only very limited extent.



a.) PET granules between untreated (left) and NaOH pre-treated (right)



b.) PET films between untreated (left) and NaOH pre-treated (right)

Figure 92 Photo of PET between untreated and NaOH pre-treated

CHAPTER V

CONCLUSION

In this work we investigated the use of lipase enzymes for various chemical modifications. Initially, we investigated acidolysis as one of the possible reactions for modification of the composition of coconut oil with MCFAs (octanoic acid and decanoic acid) for their ability to achieve the transformation of coconut oil into a product more like MCT oil and give the highest percentage of MCT yield. We started the experiment with the initial enzymatic acidolysis of coconut oil, which has been carried out with octanoic acid (C8:0) as the sole MCFAs reagent. Five commercial lipases were screened for their ability to modify coconut oil. Ultimately, only two lipases (lipase B from *Candida antarctica* immobilized on acrylic resin and Lipozyme TL IM) were selected for testing the optimal conditions: the effect of temperature, the effect of enzyme recycling, and the effect of enzyme loading. Lipozyme TL IM was selected as the best-performing lipase because of its temperature stability, the possibility of its recycling and reuse, and its better cost-effectiveness. Having obtained information enabling us to increase the performance of the acidolysis reaction and how it is affected by various factors, we prepared modified coconut oil (250 mL) for biological testing purposes in mice. We have achieved an increase of octanoic acid content from approximately 8%, in the original coconut oil, to approximately 40%, in the modified oil product. These results demonstrate that the performance of the acidolysis reaction can be maintained in scaled-up reactions with minimum performance degradation. It is well known that most MCTs contain a mixture of octanoic acid (C8:0) and decanoic acid (C10:0). Thus, we carried out the acidolysis reaction with a mixture of these fatty acids (C8:0, C10:0). The acidolysis reaction carried out under optimal conditions can increase the combined content of octanoic acid (C8) and decanoic acid (C10) significantly in the product to 35% compared to the starting coconut oil, which contains only 14.5%. Concomitantly, the content of lauric acid decreased from 48.5 % in the starting coconut oil to 35.2% in the product. The results from the initial experiment have shown that the desired products from these

reactions can indeed be obtained. The modified coconut oil with MCFAs exhibited a more MCT-like character, but the product of the acidolysis reaction had its drawbacks. This could be a result of the purification of the reaction, which is accomplished through a difficult extraction procedure, and the product prepared for the biological testing led to dubious results, due to the presence of impurities (e.g., diglycerides) presumably resulting from the extraction purification procedure. Thus, we decided to modify coconut oil through the interesterification process and use fatty acid esters rather than fatty acids as the starting materials. In the interesterification reaction, two commercial lipases were screened for their ability to modify coconut oil (lipase B from *Candida antarctica* immobilized on acrylic resin and Lipozyme TL IM). Lipozyme TL IM was selected as the best-performing lipase based on initial comparison experiments. As a helpful tool, the response surface method (RSM) was used to optimize the reaction conditions for the incorporation of octanoic acid into the product by utilizing the Box-Benkhen approach. The scale-up of the reaction and the possibility of performing it with esters obtained from coconut oil fatty acid distillate was demonstrated as well. The interesterification modification described in this work achieves the increase of octanoic content from approximately 8%, in coconut oil, to approximately 40%, in the modified oil product. This work has demonstrated the possibility of achieving the transformation of coconut oil into a product more like MCT oil and the possibility of cost-effectively sourcing the needed MCFA esters from the coconut oil fatty acid distillate.

Furthermore, we tested the biological activity of modified coconut oils in mice by evaluating their effect on food intake, body weight (BW), and lipid profiles. The results found that modified oil can reduce energy intake by reducing food intake as well as improving fat accumulation in the liver. However, modified oils led to high levels of TC and LDL in plasma and liver, suggesting that this modified oil may be hazardous to mice. Additional studies on the dosage and duration of the modified oil diet are required.

Secondly, this work aimed to optimize of enzymatic hydrolysis of a mixture of capsaicin and dihydrocapsaicin and to utilize the fatty acids obtained from this reaction (*E*-8-methyl-6-nonenoic and 8-methylnonanoic acids) for modifying coconut oil by acidolysis reactions. Novozym® 435 was selected as a biocatalyst in the hydrolysis of capsaicin to fatty acids because it gives high productivity with the highest

purity. Novozym® 435 in this work can be used more than five times under optimum conditions without loss of enzyme activity. Lipozyme TL IM, an enzyme catalyst described above, was used to modify coconut oil by the acidolysis reaction of coconut oil and fatty acids from capsaicinoids. The factors influencing the performance of the acidolysis reaction include temperature, enzyme loading, and the ratio of coconut oil and fatty acids. Under optimal conditions, modified coconut oil obtained by the acidolysis contained 51.10% of the fatty acids from capsaicinoids.

Thirdly, the possibility of PET degradation using enzymatic methods catalyzed by HiC and a combination of photooxidative pretreatment using an iridium complex was investigated. The progress of the reaction was evaluated by determining the weight loss of the material. The weight of PET films decreased slightly as a result of enzymatic HiC degradation under optimal conditions. The effects of temperature, enzyme loading, reaction time, and continuous enzyme loading were investigated, and it was found that, during the study period, these factors did not increase the extent of degradation of the PET film. The photooxidative pretreatment in combination with enzymatic PET hydrolysis also led to minimal decomposition. PET films experienced only very small weight decrease because of enzymatic degradation. However, degradation still has many limitations, which will be studied in the future to improve the degradation reaction.

In addition, this research also demonstrates the cost-effective use of local natural products and shows the potential for a sustainable future for the world with help from lipases. First, the locally available coconut oil is plentiful and easy to find. Its modification can make it more nutritious. This work also demonstrated coconut oil fatty acid distillate, left after deodorizing coconut oil, as a source of octanoic acid that can be used to modify the composition of coconut oil by lipases. This increased the octanoic content from about 8% in coconut oil to about 39% in the modified oil product. This demonstrates the utilization of waste oil materials. In the second, we demonstrated the benefits of chili peppers, a plant used in local cuisine, which is ubiquitous in Thailand. Its main spice compounds are capsaicin and dihydrocapsaicin. They affect energy metabolism and metabolic response—moreover, capsaicin has been registered by the Food and Drug Administration. In this work, we have improved the quality of the chili to make it less spicy through hydrolysis by lipases to obtain fatty acids, which have

been reported to be beneficial to energy metabolism. After that, we used these fatty acids to modify coconut oil. In addition, the process is environmentally friendly. We have also demonstrated the way to increase the value of local plants by opening the way to natural supplements to lose weight and improve energy metabolism in the body. Thirdly, we have realized the importance of nature locally and found that plastic has greatly harmed the living things on this planet. So, we are trying to find a way to decompose plastic. Even though it was difficult to decompose, we found that it could be degraded to a small extent by the enzymatic biodegradation method. This is a good sign that enables us to develop this process further.

Finally, this work demonstrated that lipases have the potential for many applications. They can catalyze and speed up various processes and increase productivity and purity. In addition, lipase enzymes can be reused and recycled, reducing costs. The reaction can be performed under mild conditions and is harmless and environmentally friendly. However, a complete understanding of the different lipases requires greater input from the research efforts.

REFERENCES

REFERENCES

1. Gandhi NN. Applications of lipase. Journal of the American Oil Chemists' Society. 1997;74(6):621-34.
2. Hou CT, &Shimada, Y., . Lipases, in Encyclopedia of Microbiology (Third Edition). Academic Press: Oxford 2009:385-92.
3. Filho DG, Silva AG, Guidini CZ. Lipases: sources, immobilization methods, and industrial applications. Applied Microbiology and Biotechnology. 2019;103:7399-423.
4. Sheldon RA, Woodley JM. Role of biocatalysis in sustainable chemistry. Chemical reviews. 2018;118(2):801-38.
5. Chapman J, Ismail AE, Dinu CZ. Industrial applications of enzymes: Recent advances, techniques, and outlooks. Catalysts. 2018;8(6):238.
6. de María PD, de Gonzalo G, Alcántara AR. Biocatalysis as useful tool in asymmetric synthesis: An assessment of recently granted patents (2014–2019). Catalysts. 2019;9(10):802.
7. Chen X, Hu Z, Zheng L, Dong P. Method for preparing levetiracetam. Google Patents; 2022.
8. Markham A, Goa KL. Valsartan: a review of its pharmacology and therapeutic use in essential hypertension. Drugs. 1997;54:299-311.
9. Danielle Gonçalves F, Amanda Gonçalves S, Carla Zanella G. Lipases: sources, immobilization methods, and industrial applications. Applied Microbiology and Biotechnology. 2019;103(18):7399-423.
10. Yamane T. Enzyme technology for the lipids industry: an engineering overview. Journal of the american oil chemists' society. 1987;64(12):1657-62.
11. Houde A, Kademi A, Leblanc D. Lipases and their industrial applications: an overview. Applied biochemistry and biotechnology. 2004;118:155-70.
12. Sonntag N. Fat splitting. Journal of the American Oil Chemists' Society. 1979;56(11Part1):729A-32A.
13. Akoh CC. Lipase-catalyzed synthesis of partial glyceride. Biotechnology letters. 1993;15:949-54.

14. Araujo R, Casal M, Cavaco-Paulo A. Application of enzymes for textile fibres processing. Biocatalysis and Biotransformation. 2008;26(5):332-49.

15. Lipase database. [Internet]. Available from: http://www.au-kbc.org/beta/bioproj2/uses.html.

16. Bajpai P. Application of enzymes in the pulp and paper industry. Biotechnology progress. 1999;15(2):147-57.

17. Puskas JE, Sen MY, Kasper JR. Green polymer chemistry: Telechelic poly (ethylene glycol) s via enzymatic catalysis. Journal of Polymer Science Part A: Polymer Chemistry. 2008;46(9):3024-8.

18. Puskas JE, Sen MY, Seo KS. Green polymer chemistry using nature's catalysts, enzymes. Journal of Polymer Science Part A: Polymer Chemistry. 2009;47(12):2959-76.

19. Sahoo B, Gao W, Gross R, editors. Lipase-catalyzed esterification of polyacrylic acid with extraordinary selectivity. ABSTRACTS OF PAPERS OF THE AMERICAN CHEMICAL SOCIETY; 2003: AMER CHEMICAL SOC 1155 16TH ST, NW, WASHINGTON, DC 20036 USA.

20. Kadokawa J-i, Kobayashi S. Polymer synthesis by enzymatic catalysis. Current Opinion in Chemical Biology. 2010;14(2):145-53.

21. Gupta R, Rathi P, Bradoo S. Lipase mediated upgradation of dietary fats and oils. Critical reviews in food science and nutrition. 2003;43(6):635-44.

22. Singh R, Singh A, Sachan S. Enzymes used in the food industry: Friends or foes? Enzymes in food biotechnology: Elsevier; 2019. p. 827-43.

23. Gotor-Fernández V, Brieva R, Gotor V. Lipases: Useful biocatalysts for the preparation of pharmaceuticals. Journal of Molecular Catalysis B: Enzymatic. 2006;40(3-4):111-20.

24. Maugard T, Rejasse B, Legoy MD. Synthesis of water-soluble retinol derivatives by enzymatic method. Biotechnology progress. 2002;18(3):424-8.

25. Noureddini H, Gao X, Philkana R. Immobilized Pseudomonas cepacia lipase for biodiesel fuel production from soybean oil. Bioresource technology. 2005;96(7):769-77.

26. Jegannathan KR, Abang S, Poncelet D, Chan ES, Ravindra P. Production of biodiesel using immobilized lipase—a critical review. Critical reviews in biotechnology. 2008;28(4):253-64.

27. de Oliveira D, Di Luccio M, Faccio C, Rosa CD, Bender JP, Lipke N, et al., editors. Optimization of enzymatic production of biodiesel from castor oil in organic solvent medium. Proceedings of the Twenty-Fifth Symposium on Biotechnology for Fuels and Chemicals Held May 4–7, 2003, in Breckenridge, CO; 2004: Springer.

28. Abigor R, Uadia P, Foglia T, Haas M, Jones K, Okpefa E, et al. Lipase-catalysed production of biodiesel fuel from some Nigerian lauric oils. Portland Press Ltd.; 2000.

29. Nagy I, Friston D, Valente JS, Perez JVT, Andreou AP. Pharmacology of the capsaicin receptor, transient receptor potential vanilloid type-1 ion channel. Capsaicin as a Therapeutic Molecule. 2014:39-76.

30. Activation of TRPV1 by capsaicin [Internet]. 2011. Available from: https://commons.wikimedia.org/wiki/File:Activation_of_TRPV1_by_capsaicin.jpg.

31. Basith S, Cui M, Hong S, Choi S. Harnessing the therapeutic potential of capsaicin and its analogues in pain and other diseases. Molecules. 2016;21(8):966.

32. Duarte DR, Castillo E, Bárzana E, López-Munguía A. Capsaicin hydrolysis by Candida antarctica lipase. Biotechnology letters. 2000;22:1811-4.

33. Torres-Gavilán A, Castillo E, López-Munguía A. The amidase activity of Candida antarctica lipase B is dependent on specific structural features of the substrates. Journal of Molecular Catalysis B: Enzymatic. 2006;41(3-4):136-40.

34. Kanprakobkit W, &Kielar, F. , editor HYDROLYSIS OF CAPSAICIN USING IMMOBILIZED LIPASE B FROM Candida antarctica Paper. The 43rd Congress on Science and Technology of Thailand (STT 43) 2017 2017, October 17-19; Science and Technology of Thailand.

35. Babu AS, Veluswamy SK, Arena R, Guazzi M, Lavie CJ. Virgin coconut oil and its potential cardioprotective effects. Postgraduate medicine. 2014;126(7):76-83.

36. Marina A, Man YC, Amin I. Virgin coconut oil: emerging functional food oil. Trends in Food Science & Technology. 2009;20(10):481-7.

37. What is Coconut Oil? [Internet]. Available from: https://coconutsandkettlebells.com/what-is-coconut-oil/.

38. Reger MA, Henderson ST, Hale C, Cholerton B, Baker LD, Watson GS, et al. Effects of β-hydroxybutyrate on cognition in memory-impaired adults. Neurobiology of aging. 2004;25(3):311-4.

39. Chatterjee P, Fernando M, Fernando B, Dias CB, Shah T, Silva R, et al. Potential of coconut oil and medium chain triglycerides in the prevention and treatment of Alzheimer's disease. Mechanisms of Ageing and Development. 2020;186:111209.

40. Sung M-H, Liao F-H, Chien Y-W. Medium-chain triglycerides lower blood lipids and body weight in streptozotocin-induced type 2 diabetes rats. Nutrients. 2018;10(8):963.

41. Cassiday L. Coconut oil boom. Inform. 2016;27(5):6-13.

42. Bornscheuer UT. Enzymes in lipid modification. Annual review of food science and technology. 2018;9:85-103.

43. Kim BH, Akoh CC. Recent research trends on the enzymatic synthesis of structured lipids. Journal of Food Science. 2015;80(8):C1713-C24.

44. Nunes PA, Pires-Cabral P, Guillén M, Valero F, Luna D, Ferreira-Dias S. Production of MLM-type structured lipids catalyzed by immobilized heterologous Rhizopus oryzae lipase. Journal of the American Oil Chemists' Society. 2011;88:473-80.

45. Caballero E, Soto C, Olivares A, Altamirano C. Potential use of avocado oil on structured lipids MLM-type production catalysed by commercial immobilised lipases. Plos one. 2014;9(9):e107749.

46. Ifeduba EA, Akoh CC. Modification of stearidonic acid soybean oil by immobilized Rhizomucor miehei lipase to incorporate caprylic acid. Journal of the American Oil Chemists' Society. 2014;91(6):953-65.

47. Koletzko B. Human milk lipids. Annals of Nutrition and Metabolism. 2016;69(Suppl. 2):27-40.

48. Zou X, Jin Q, Guo Z, Xu X, Wang X. Preparation and characterization of human milk fat substitutes based on triacylglycerol profiles. Journal of the American Oil Chemists' Society. 2016;93(6):781-92.

49. Zou X-G, Hu J-N, Zhao M-L, Zhu X-M, Li H-Y, Liu X-R, et al. Lipozyme RM IM-catalyzed acidolysis of Cinnamomum camphora seed oil with oleic acid to

produce human milk fat substitutes enriched in medium-chain fatty acids. Journal of agricultural and food chemistry. 2014;62(43):10594-603.

50. Mohamed IO. Lipase-catalyzed acidolysis of palm mid fraction oil with palmitic and stearic fatty acid mixture for production of cocoa butter equivalent. Applied biochemistry and biotechnology. 2013;171:655-66.

51. Kim S, Kim IH, Akoh CC, Kim BH. Enzymatic production of cocoa butter equivalents high in 1-palmitoyl-2-oleoyl-3-stearin in continuous packed bed reactors. Journal of the American Oil Chemists' Society. 2014;91(5):747-57.

52. Ghazani SM, Marangoni AG. Facile lipase-catalyzed synthesis of a chocolate fat mimetic. Scientific Reports. 2018;8(1):1-18.

53. Fernandes G. Dietary lipids and risk of autoimmune disease. Clinical immunology and immunopathology. 1994;72(2):193-7.

54. Qiu W, Lu H, Qi Y, Wang X. Dietary fat intake and ovarian cancer risk: a meta-analysis of epidemiological studies. Oncotarget. 2016;7(24):37390.

55. Ornla-Ied P, Sonwai S, Lertthirasuntorn S. Trans-free margarine fat produced using enzymatic interesterification of rice bran oil and hard palm stearin. Food science and biotechnology. 2016;25:673-80.

56. Xu Y, Zhu X, Ma X, Xiong H, Zeng Z, Peng H, et al. Enzymatic production of trans-free shortening from coix seed oil, fully hydrogenated palm oil and Cinnamomum camphora seed oil. Food bioscience. 2018;22:1-8.

57. Compton DL, Laszlo JA, Evans KO. Influence of solid supports on acyl migration in 2-monoacylglycerols: purification of 2-MAG via flash chromatography. Journal of the American Oil Chemists' Society. 2013;90:1397-403.

58. Wang X, Wang X, Jin Q, Wang T. Improved synthesis of monopalmitin on a large scale by two enzymatic methods. Journal of the American Oil Chemists' Society. 2013;90:1455-63.

59. Dhara R, Singhal RS. Process optimization of enzyme catalyzed production of dietary diacylglycerol (DAG) using TLIM as biocatalyst. Journal of Oleo Science. 2014;63(2):169-76.

60. Meng Z, Lu S, Geng W, Huang J, Wang X, Liu Y. Preliminary study on acyl incorporation and migration in the production of 1, 3-diacylglycerol by immobilized

Lipozyme RM IM-catalyzed esterification. Food Science and Technology Research. 2014;20(2):175-82.

61. Rao R, Manohar B, Sambaiah K, Lokesh BR. Enzymatic acidolysis in hexane to produce n− 3 or n− 6 FA-enriched structured lipids from coconut oil: Optimization of reactions by response surface methodology. Journal of the American Oil Chemists' Society. 2002;79:885-90.

62. Nandi S, Gangopadhyay S, Ghosh S. Production of medium chain glycerides from coconut and palm kernel fatty acid distillates by lipase-catalyzed reactions. Enzyme and Microbial Technology. 2005;36(5-6):725-8.

63. Hamam F, Shahidi F. Synthesis of structured lipids containing medium-chain and omega-3 fatty acids. Journal of agricultural and food chemistry. 2006;54(12):4390-6.

64. Maurelli S, Blasi F, Cossignani L, Bosi A, Simonetti MS, Damiani P. Production and structural analysis of triacylglycerols containing capric acid and conjugated linoleic acid isomers obtained by enzymatic acidolysis. Journal of the Science of Food and Agriculture. 2009;89(15):2595-600.

65. Öztürk T, Ustun G, Aksoy HA. Production of medium-chain triacylglycerols from corn oil: optimization by response surface methodology. Bioresource technology. 2010;101(19):7456-61.

66. Chnadhapuram M, Sunkireddy YR. Preparation of palm olein enriched with medium chain fatty acids by lipase acidolysis. Food chemistry. 2012;132(1):216-21.

67. Kavadia MR, Yadav MG, Odaneth AA, Lali AM. Synthesis of designer triglycerides by enzymatic acidolysis. Biotechnology Reports. 2018;18:e00246.

68. Sivakanthan S, Madhujith T. Current trends in applications of enzymatic interesterification of fats and oils: A review. Lwt. 2020;132:109880.

69. Korma SA, Zou X, Ali AH, Abed SM, Jin Q, Wang X. Preparation of structured lipids enriched with medium-and long-chain triacylglycerols by enzymatic interesterification for infant formula. Food and Bioproducts Processing. 2018;107:121-30.

70. Cui H, Li J, Xu X, Li J, Lu M, Song H, et al. Enzymatic interesterification of beef tallow/coconut oil blends to produce a superior margarine base stock. International Journal of Food Science & Technology. 2022;57(2):908-19.

71. Almeida LC, Barbosa MS, de Jesus FA, Santos RM, Fricks AT, Freitas LS, et al. Enzymatic transesterification of coconut oil by using immobilized lipase on biochar: An experimental and molecular docking study. Biotechnology and Applied Biochemistry. 2021;68(4):801-8.

72. Zhang Z, Lee WJ, Sun X, Wang Y. Enzymatic interesterification of palm olein in a continuous packed bed reactor: Effect of process parameters on the properties of fats and immobilized Thermomyces lanuginosus lipase. LWT. 2022;162:113459.

73. Seymour RB. Polymer science before and after 1899: notable developments during the lifetime of Maurits Dekker. Journal of Macromolecular Science—Chemistry. 1989;26(8):1023-32.

74. Shah AA, Hasan F, Hameed A, Ahmed S. Biological degradation of plastics: a comprehensive review. Biotechnology advances. 2008;26(3):246-65.

75. Srikanth M, Sandeep T, Sucharitha K, Godi S. Biodegradation of plastic polymers by fungi: a brief review. Bioresources and Bioprocessing. 2022;9(1):42.

76. Kleeberg I, Welzel K, VandenHeuvel J, Müller R-J, Deckwer W-D. Characterization of a new extracellular hydrolase from Thermobifida fusca degrading aliphatic− aromatic Copolyesters. Biomacromolecules. 2005;6(1):262-70.

77. Marten E, Müller R-J, Deckwer W-D. Studies on the enzymatic hydrolysis of polyesters. II. Aliphatic–aromatic copolyesters. Polymer degradation and stability. 2005;88(3):371-81.

78. Eberl A, Heumann S, Kotek R, Kaufmann F, Mitsche S, Cavaco-Paulo A, et al. Enzymatic hydrolysis of PTT polymers and oligomers. Journal of Biotechnology. 2008;135(1):45-51.

79. Hooker J, Hinks D, Montero G, Icherenska M. Enzyme-catalyzed hydrolysis of poly (ethylene terephthalate) cyclic trimer. Journal of applied polymer science. 2003;89(9):2545-52.

80. Vertommen M, Nierstrasz V, Van Der Veer M, Warmoeskerken M. Enzymatic surface modification of poly (ethylene terephthalate). Journal of biotechnology. 2005;120(4):376-86.

81. Müller RJ, Schrader H, Profe J, Dresler K, Deckwer WD. Enzymatic degradation of poly (ethylene terephthalate): rapid hydrolyse using a hydrolase from T. fusca. Macromolecular rapid communications. 2005;26(17):1400-5.

82. Ronkvist ÅM, Xie W, Lu W, Gross RA. Cutinase-catalyzed hydrolysis of poly (ethylene terephthalate). Macromolecules. 2009;42(14):5128-38.

83. Ribitsch D, Heumann S, Trotscha E, Herrero Acero E, Greimel K, Leber R, et al. Hydrolysis of polyethyleneterephthalate by p-nitrobenzylesterase from Bacillus subtilis. Biotechnology progress. 2011;27(4):951-60.

84. Ribitsch D, Herrero Acero E, Przylucka A, Zitzenbacher S, Marold A, Gamerith C, et al. Enhanced cutinase-catalyzed hydrolysis of polyethylene terephthalate by covalent fusion to hydrophobins. Applied and Environmental Microbiology. 2015;81(11):3586-92.

85. Barth M, Wei R, Oeser T, Then J, Schmidt J, Wohlgemuth F, et al. Enzymatic hydrolysis of polyethylene terephthalate films in an ultrafiltration membrane reactor. Journal of membrane science. 2015;494:182-7.

86. Yoshida S, Hiraga K, Takehana T, Taniguchi I, Yamaji H, Maeda Y, et al. A bacterium that degrades and assimilates poly (ethylene terephthalate). Science. 2016;351(6278):1196-9.

87. Barth M, Honak A, Oeser T, Wei R, Belisário-Ferrari MR, Then J, et al. A dual enzyme system composed of a polyester hydrolase and a carboxylesterase enhances the biocatalytic degradation of polyethylene terephthalate films. Biotechnology Journal. 2016;11(8):1082-7.

88. Carniel A, Valoni É, Junior JN, da Conceição Gomes A, de Castro AM. Lipase from Candida antarctica (CALB) and cutinase from Humicola insolens act synergistically for PET hydrolysis to terephthalic acid. Process Biochemistry. 2017;59:84-90.

89. de Castro AM, Carniel A, Sirelli L, Dias ML, de Menezes SMC, Junior LSC, et al. Enzyme-catalyzed simultaneous hydrolysis-glycolysis reactions reveals tunability on PET depolymerization products. Biochemical Engineering Journal. 2018;137:239-46.

90. Day M, Wiles D. Photochemical degradation of poly (ethylene terephthalate). II. Effect of wavelength and environment on the decomposition process. Journal of Applied Polymer Science. 1972;16(1):191-202.

91. Fechine G, Souto-Maior R, Rabello M. Structural changes during photodegradation of poly (ethylene terephthalate). Journal of materials science. 2002;37:4979-84.

92. Chelliah A, Subramaniam M, Gupta R, Gupta A. Evaluation on the thermo-oxidative degradation of PET using prodegradant additives. Indian J Sci Technol. 2017;10:2-5.

93. Giraldo-Narcizo S, Guenani N, Sánchez-Pérez AM, Guerrero A. Accelerated Polyethylene Terephthalate (PET) Enzymatic Degradation by Room Temperature Alkali Pre-treatment for Reduced Polymer Crystallinity. ChemBioChem. 2023;24(1):e202200503.

APPENDIX

APPENDIX A

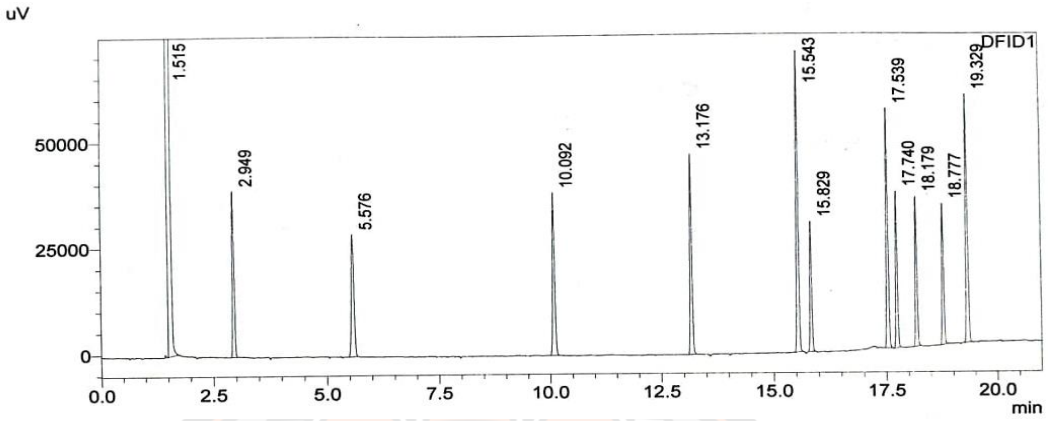


Figure 93 Gas chromatogram of a standard mixture of fatty acid methyl esters

Table 29 Retention times of fatty acid methyl esters in standard mixture

Fatty acid symbol	Fatty acid trivial name	Retention time (min)
C8	Caprylic acid	2.95
C10	Capric acid	5.58
C12	Lauric acid	10.09
C14	Myristic acid	13.18
C16:0	Palmitic acid	15.83
C16:1	Palmitoleic	15.54
C18:0	Stearic acid	18.78
C18:1	Oleic acid	18.18
C18:2	Linoleic acid	17.74
C18:3	Linolenic acid	17.54
C20:0	Arachidic acid	19.33

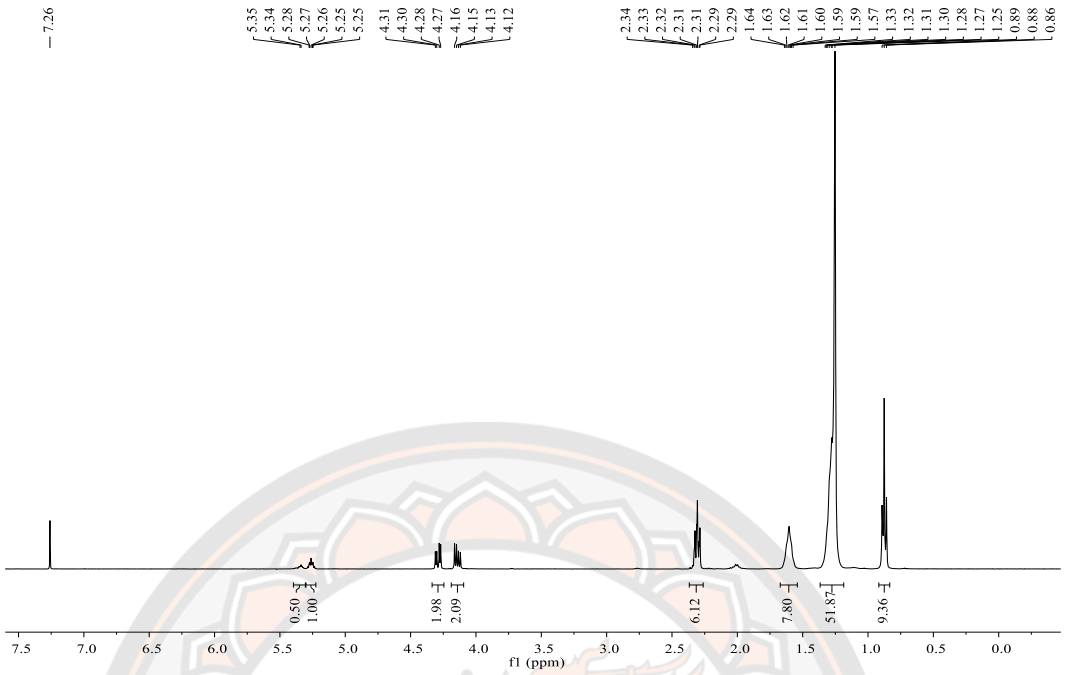


Figure 94 The ^{1}H-NMR spectrum of the original coconut oil (VCO) (naturel brand)

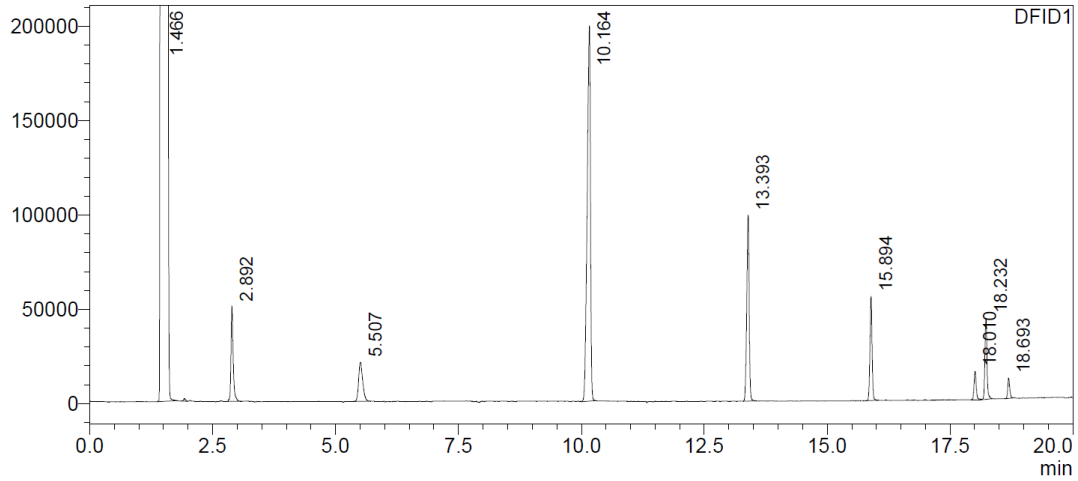


Figure 95 GC spectrum of the original coconut oil (VCO) (naturel brand)

Table 30 Fatty acid composition of the original coconut oil (VCO) (naturel brand) determined by gas chromatography

Fatty acid symbol	Fatty acid trivial name	Retention time (min)	Specifications (%)
C8	Caprylic acid	2.892	8.20
C10	Capric acid	5.507	6.30
C12	Lauric acid	10.164	48.50
C14	Myristic acid	13.393	17.80
C16:0	Palmitic acid	15.894	8.60
C18:2	Linoleic acid	18.010	2.40
C18:1	Oleic acid	18.232	6.60
C18:0	Stearic acid	18.693	1.60

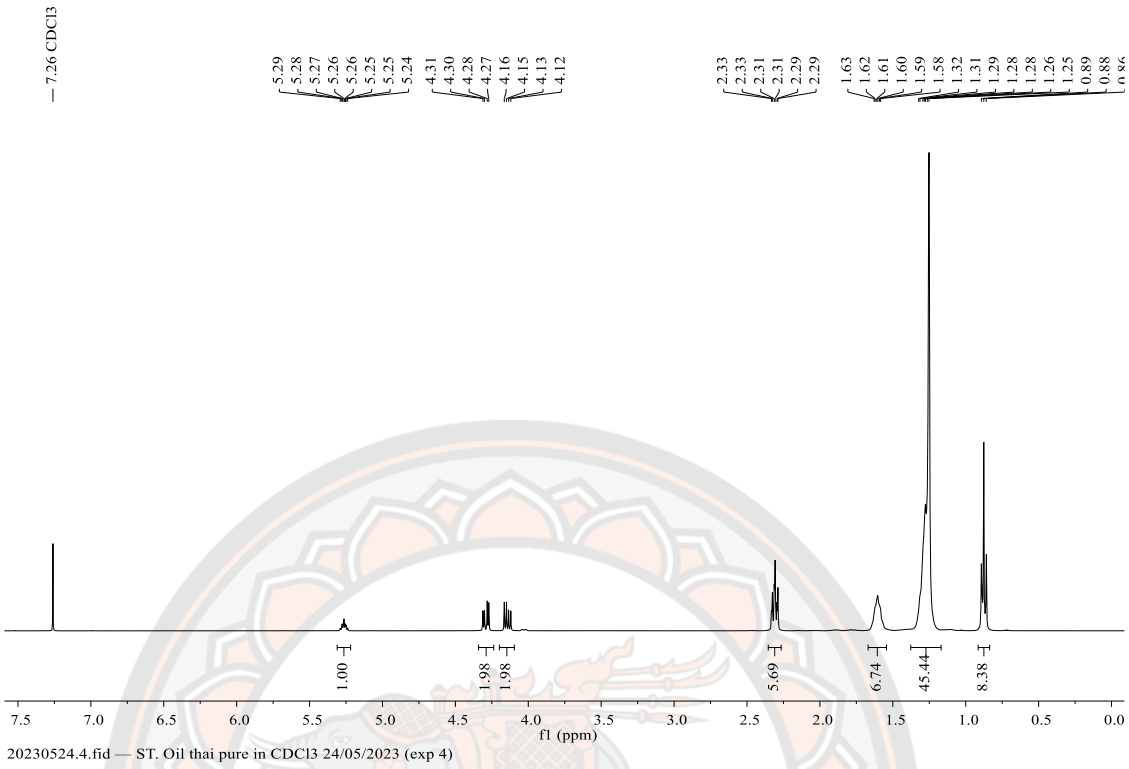


Figure 96 The ^{1}H-NMR spectrum of the original coconut oil (VCO) (Thai Pure Coconut Co., Ltd.)

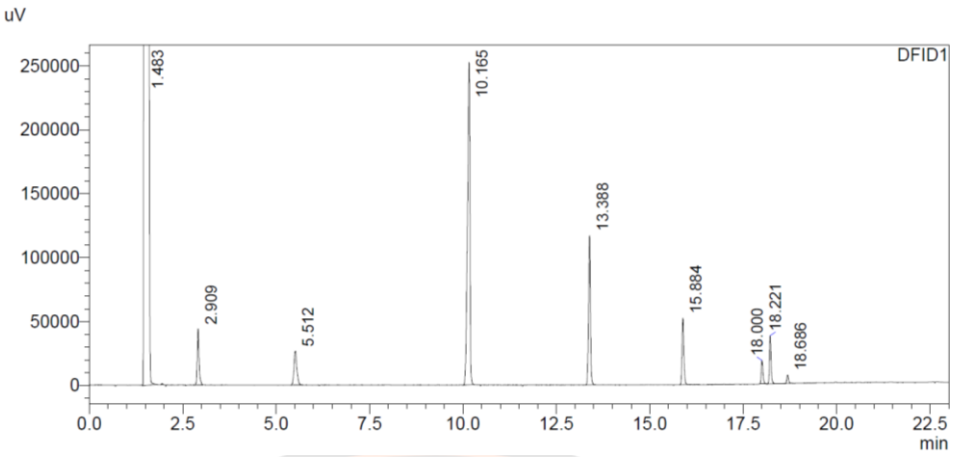


Figure 97 GC spectrum of the original coconut oil (VCO) (Thai Pure Coconut Co., Ltd.)

Table 31 Fatty acid composition of original coconut oil (VCO) (Thai Pure Coconut Co., Ltd.) determined by gas chromatography

Fatty acid symbol	Fatty acid trivial name	Retention time (min)	Specifications (%)
C8:0	Caprylic acid	2.839	6.70
C10:0	Capric acid	5.405	6.40
C12:0	Lauric acid	10.087	51.60
C14:0	Myristic acid	13.333	18.40
C16:0	Palmitic acid	15.840	8.00
C18:2	Linoleic acid	17.961	2.70
C18:1	Oleic acid	18.180	5.10
C18:0	Stearic acid	18.642	0.90

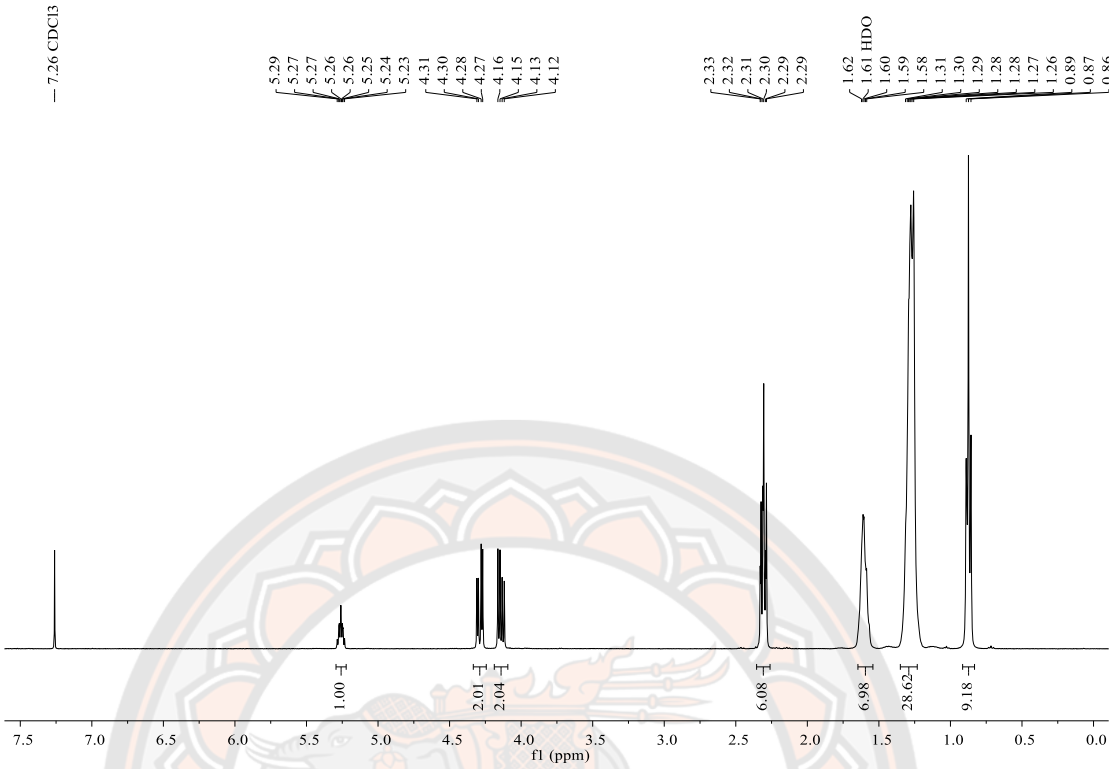


Figure 98 The ^{1}H-NMR spectrum of 100% pure coconut MCT oil (Healtholicious)

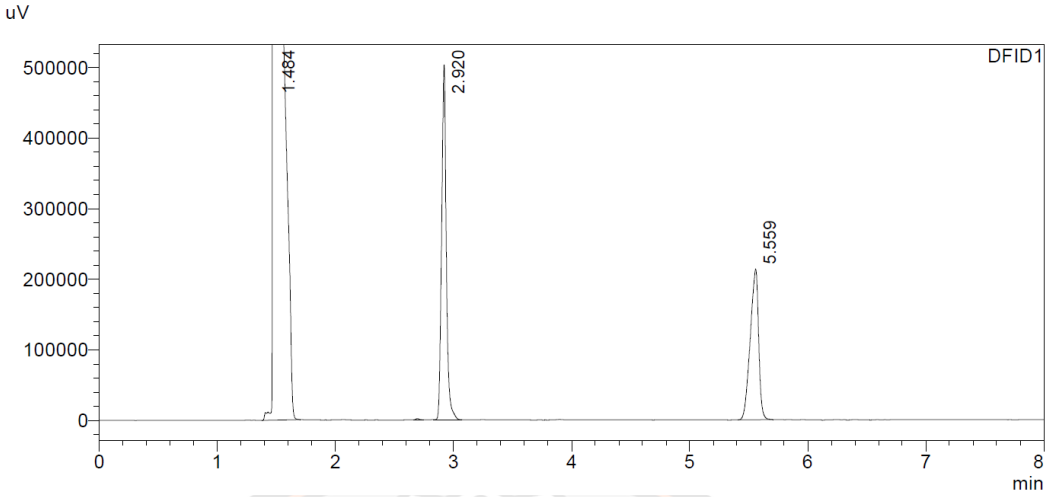


Figure 99 GC spectrum of 100% pure coconut MCT oil (Healtholicious)

Table 32 Fatty acid composition of 100% pure coconut MCT oil (Healtholicious) determined by gas chromatography

Fatty acid symbol	Fatty acid trivial name	Retention time (min)	Specifications (%)
C8:0	Caprylic acid	2.839	56.70
C10:0	Capric acid	5.405	43.30

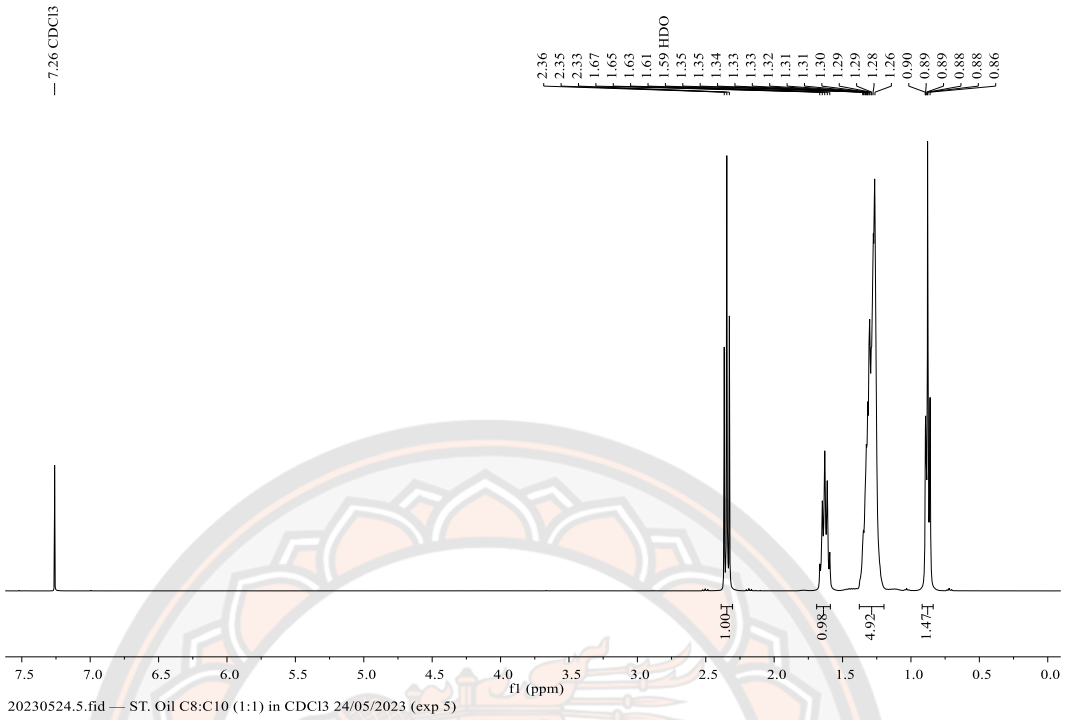


Figure 100 The ^{1}H-NMR spectrum of the MCT oil (octanoic acid: decanoic acid, 50:50)

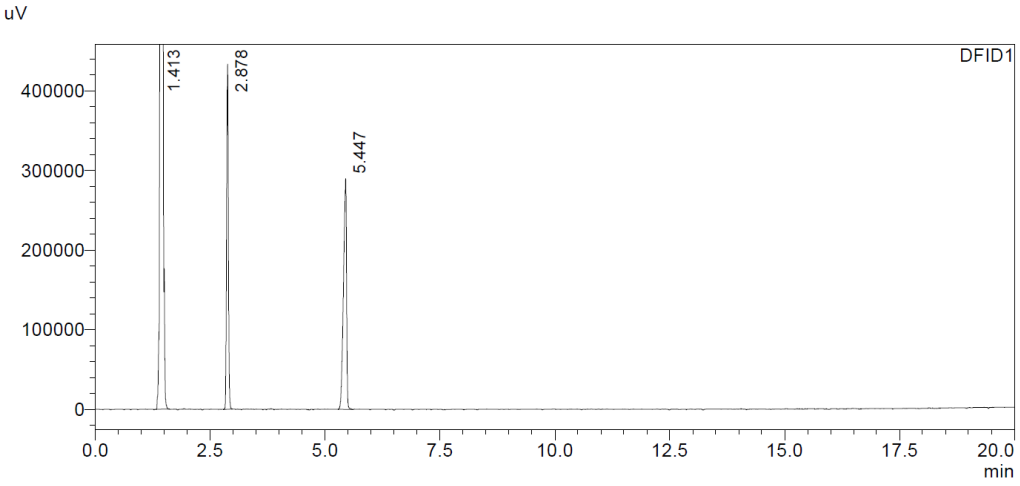


Figure 101 GC spectrum of the MCT oil (octanoic acid: decanoic acid, 50:50)

Table 33 Fatty acid composition of the MCT oil (octanoic acid: decanoic acid, 50:50) determined by gas chromatography

Fatty acid symbol	Fatty acid trivial name	Retention time (min)	Specifications (%)
C8	Caprylic acid	2.878	46.00
C10	Capric acid	5.447	54.00

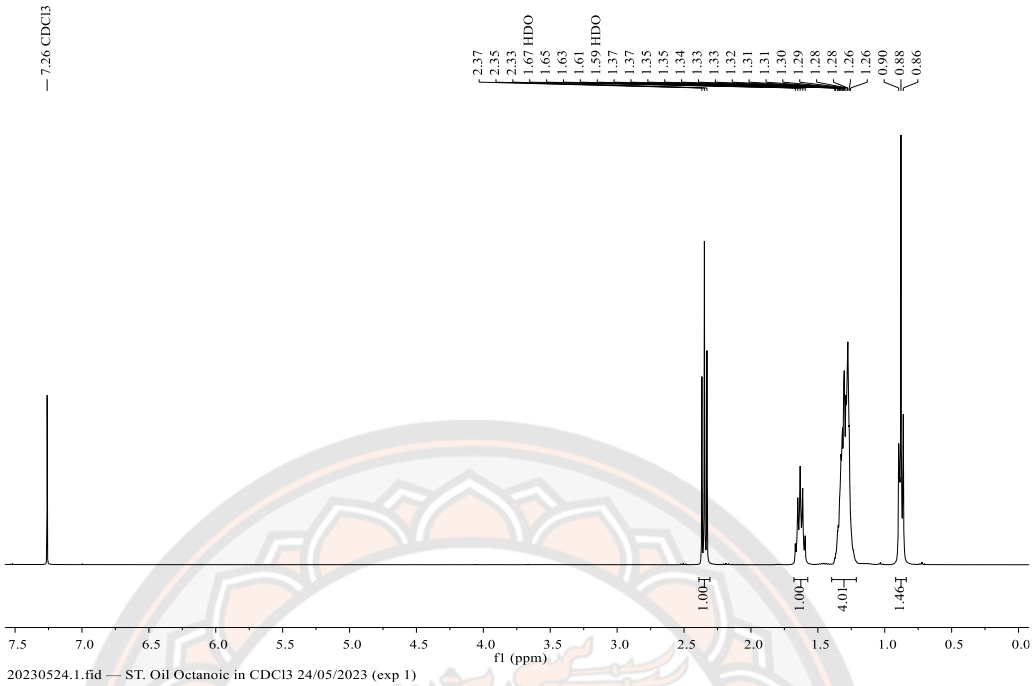


Figure 102 The ^{1}H-NMR spectrum of the octanoic acid (C8:0)

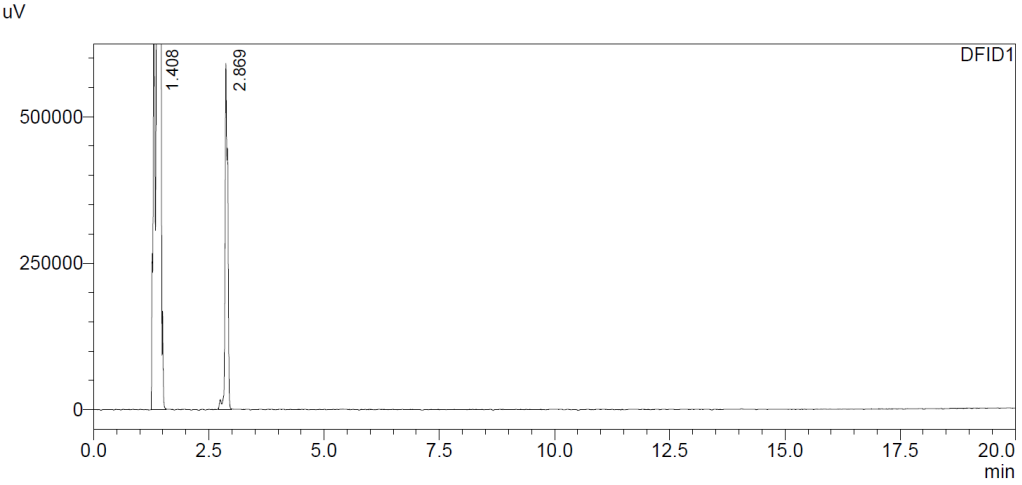


Figure 103 GC spectrum of the octanoic acid (C8:0)

Table 34 %Content of the octanoic acid (C8:0) determined by gas chromatography

Fatty acid symbol	Fatty acid trivial name	Retention time (min)	Specifications (%)
C8	Caprylic acid	2.869	100

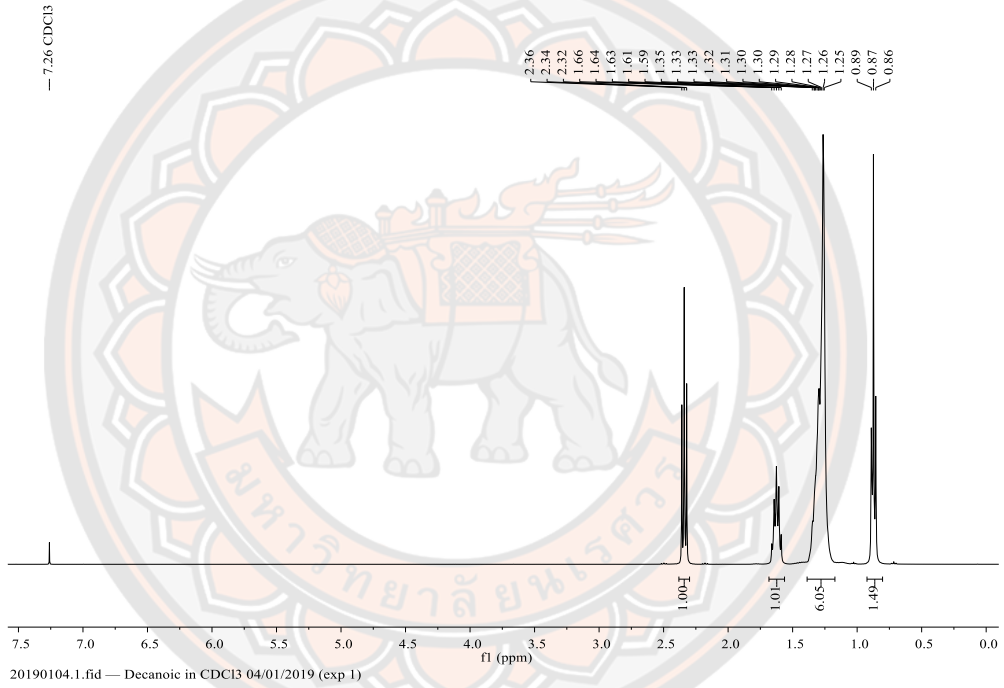


Figure 104 The ^{1}H-NMR spectrum of the decanoic acid (C10:0)

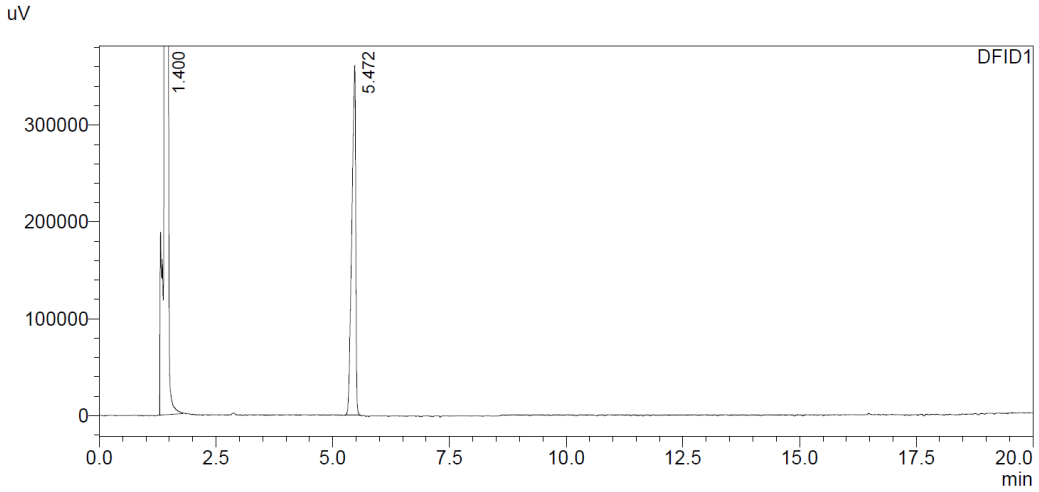


Figure 105 GC spectrum of the decanoic acid (C10:0)

Table 35 %Content of the decanoic acid (C10:0) determined by gas chromatography

Fatty acid symbol	Fatty acid trivial name	Retention time (min)	Specifications (%)
C10	Capric acid	5.472	100

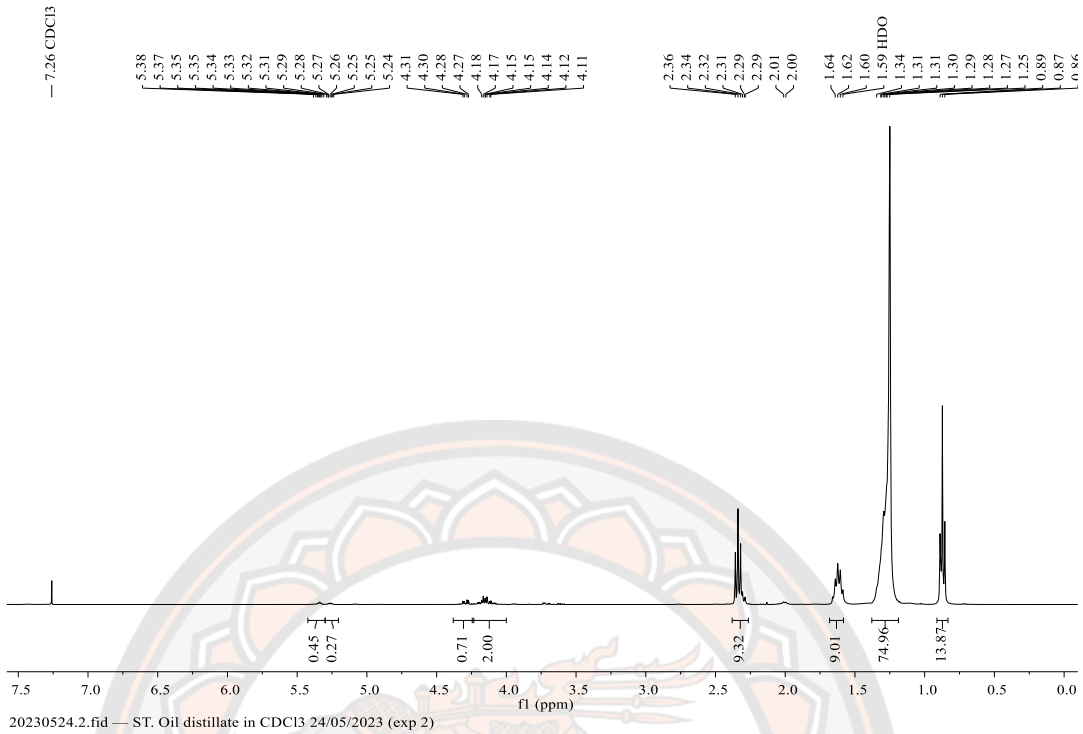


Figure 106 The ^{1}H-NMR spectrum of coconut oil fatty acid distillate

The appendix relates to Part I: Initial experiments: coconut oil acidolysis with fatty acid from commercial

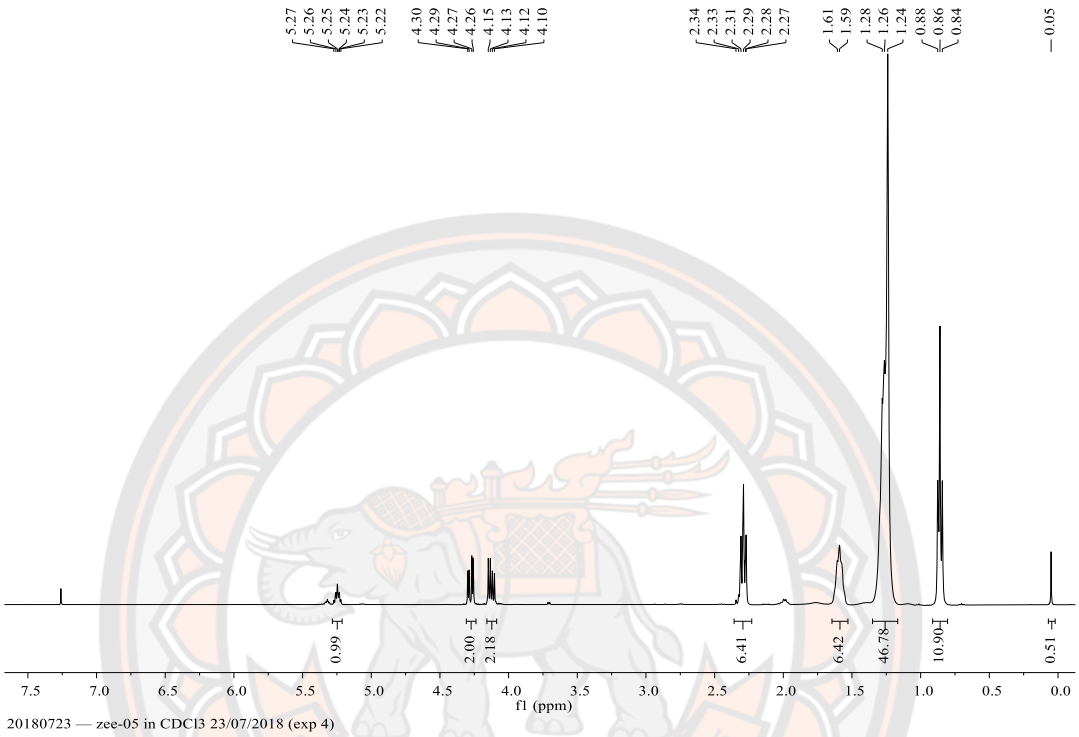


Figure 107 ^{1}H-NMR spectrum of oil obtained by enzyme lipase B from *Candida antarctica* immobilized on acrylic resin catalyzed acidolysis of coconut oil with octanoic acid (initial reaction)

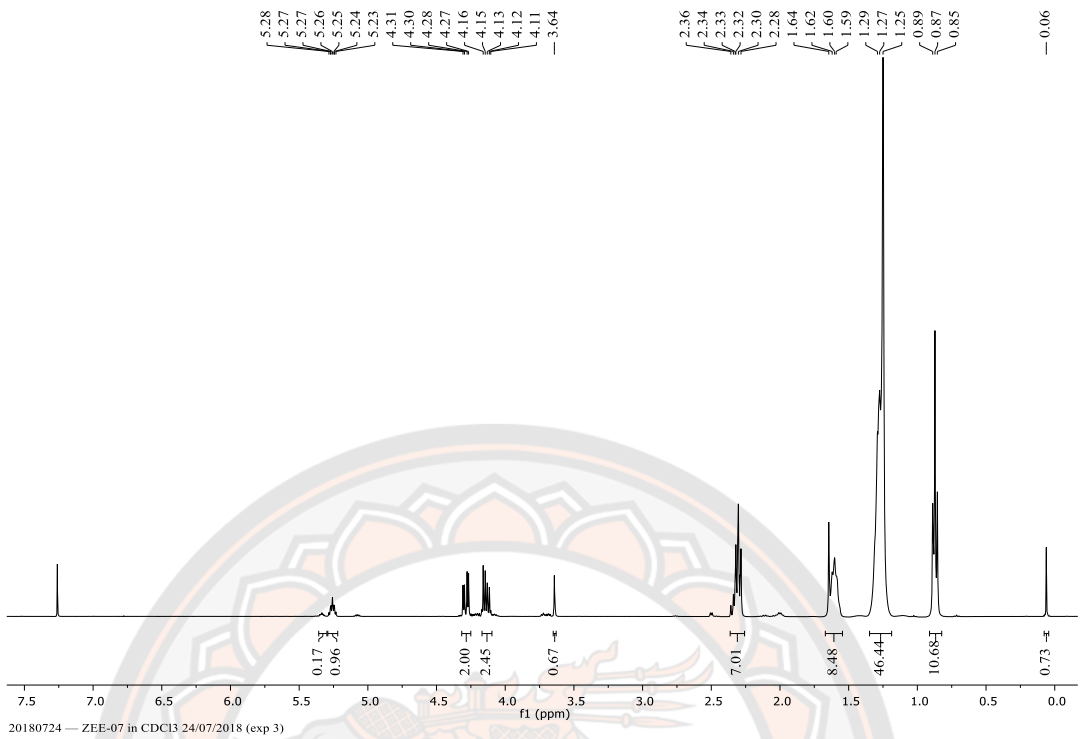


Figure 108 ^{1}H-NMR spectrum of oil obtained by enzyme lipase immobilized on immobead 150, from *Pseudomonus cepacia* catalyzed acidolysis of coconut oil with octanoic acid (initial reaction)

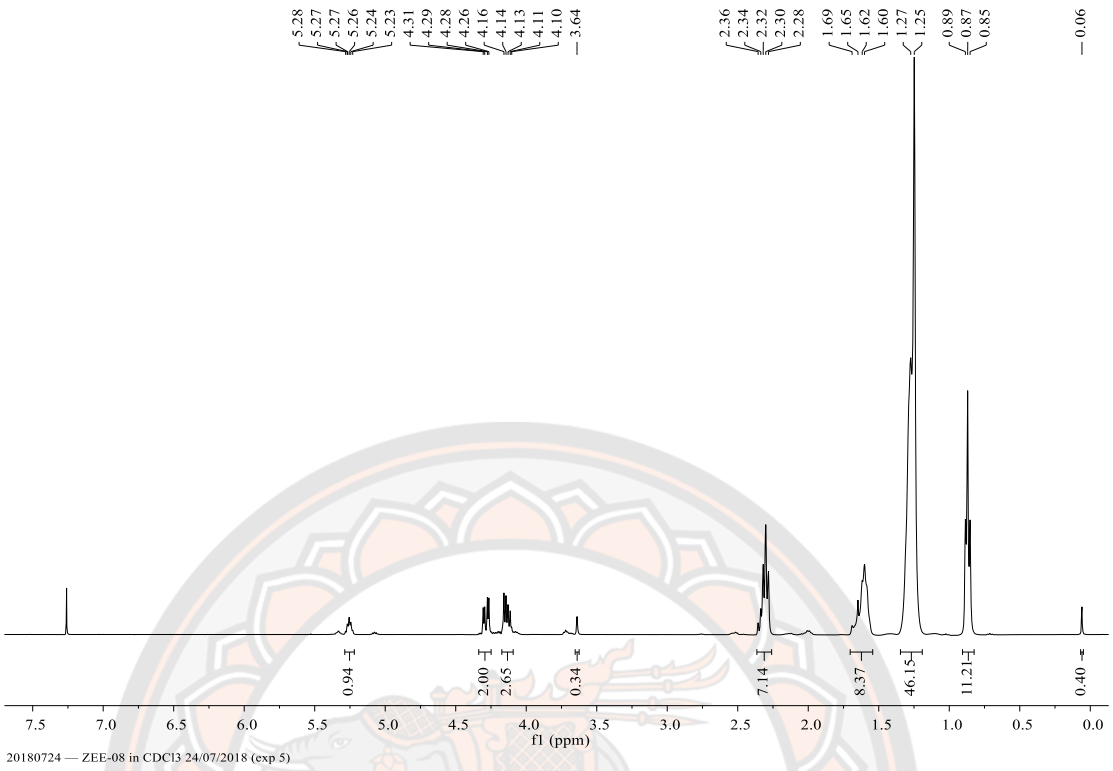


Figure 109 ^{1}H-NMR spectrum of oil obtained by enzyme lipase immobilized on immobead 150, from *Rhizopus oryzae* catalyzed acidolysis of coconut oil with octanoic acid (initial reaction)

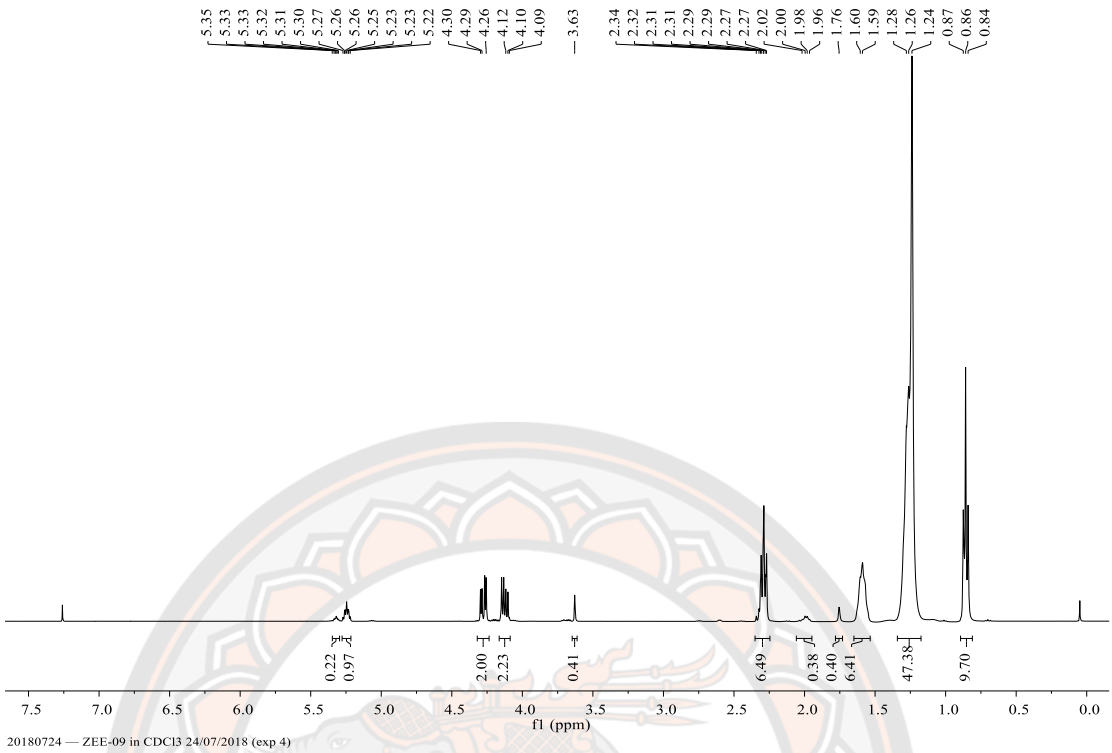


Figure 110 ^{1}H-NMR spectrum of oil obtained by enzyme lipase immobilized on immobead 150, from *Candida rugosa* catalyzed acidolysis of coconut oil with octanoic acid (initial reaction)

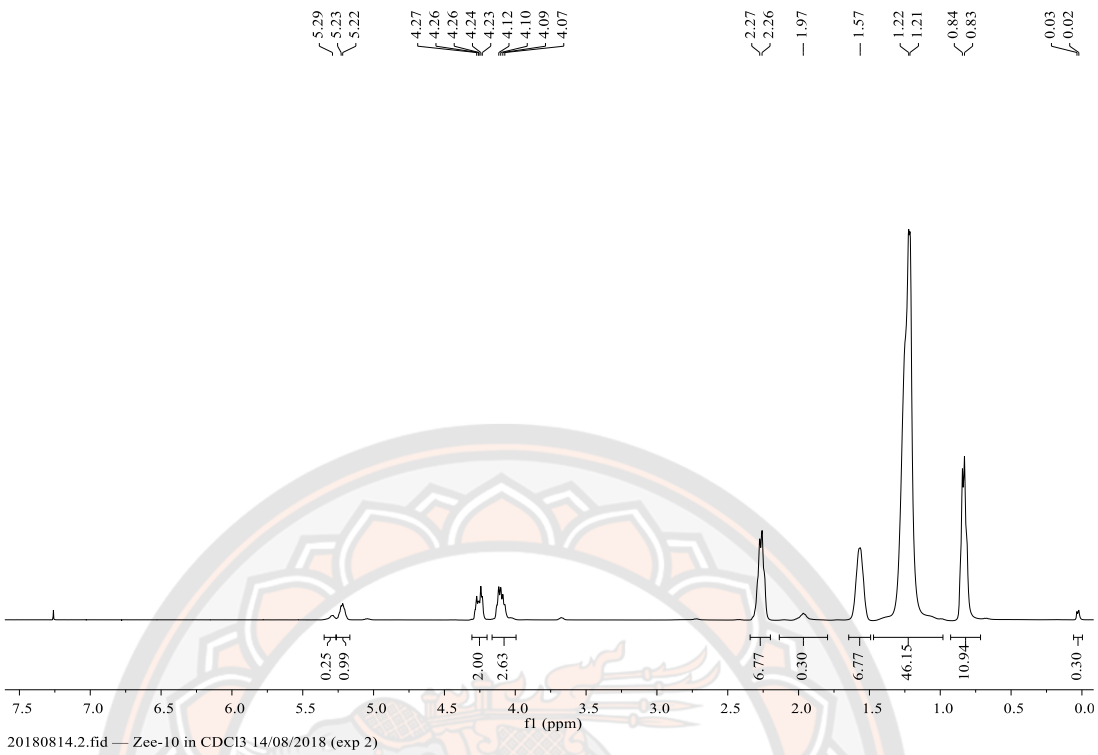


Figure 111 ^{1}H-NMR spectrum of oil obtained by enzyme lipase Lipozyme TLIM catalyzed acidolysis of coconut oil with octanoic acid (initial reaction)

Investigation of the effect of temperatures on coconut oil acidolysis with octanoic acid (C8:0)

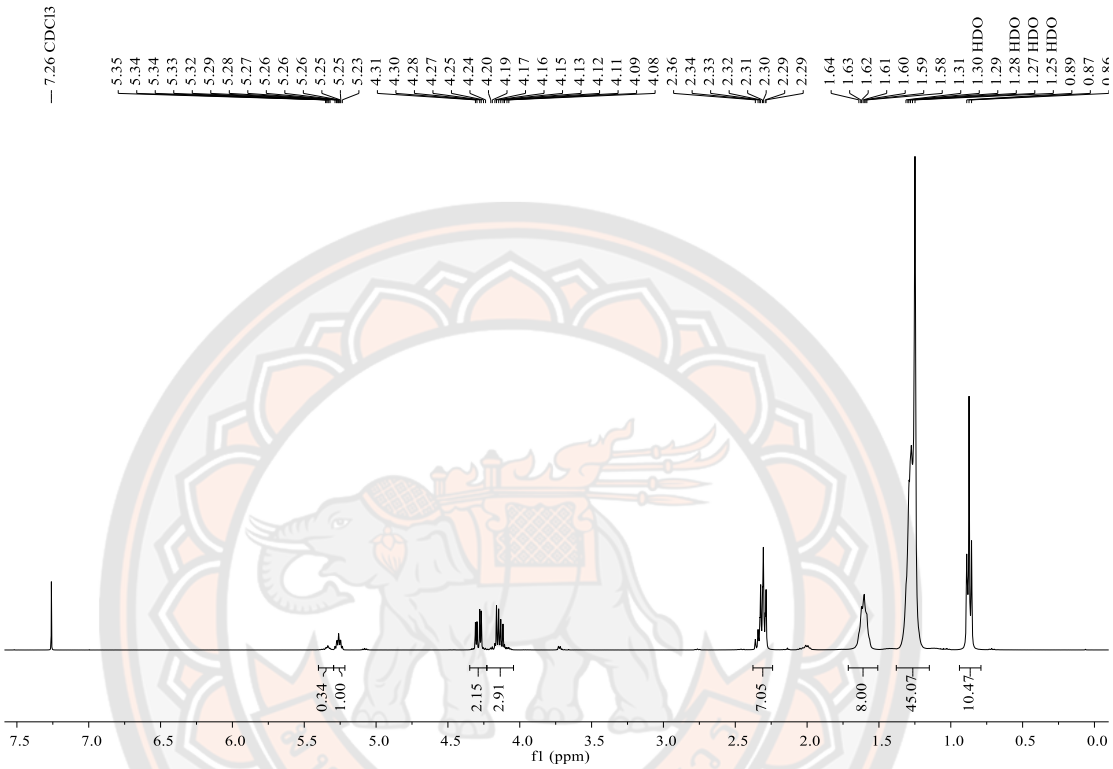


Figure 112 ^{1}H-NMR spectrum of the product of enzyme Lipozyme TL IM catalyzed acidolysis of coconut oil at 45^{o}C

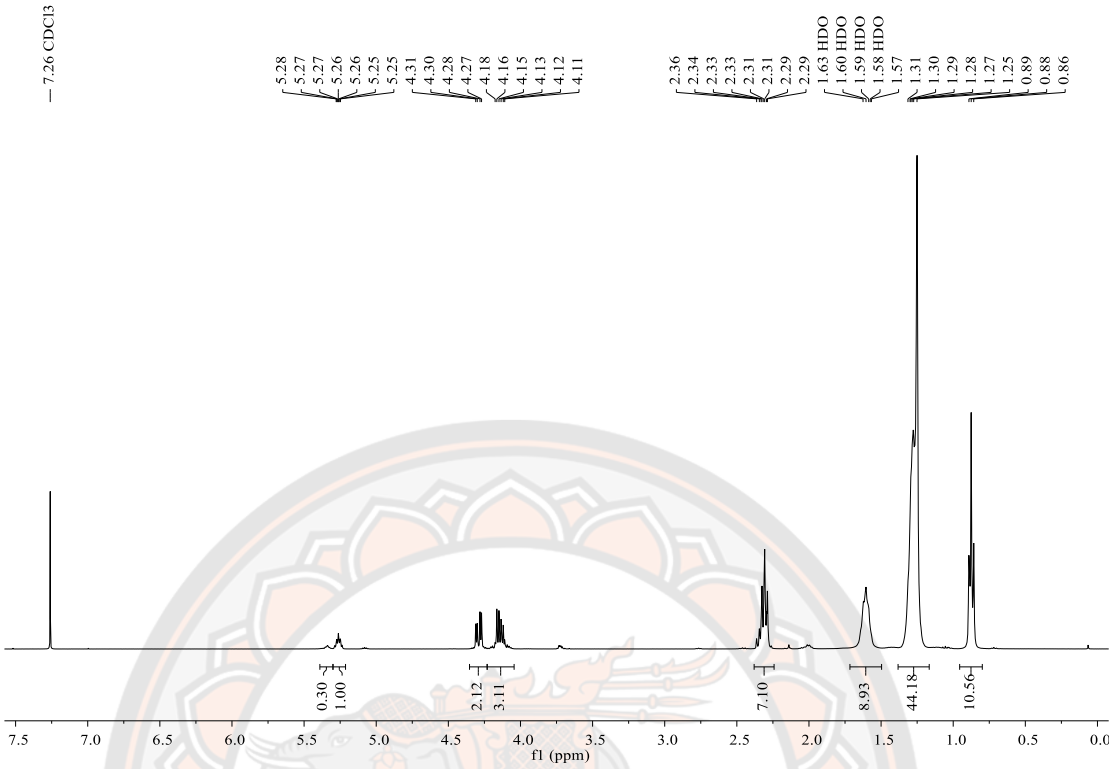


Figure 113 ^{1}H-NMR spectrum of the product of enzyme Lipozyme TL IM catalyzed acidolysis of coconut oil at 50^{o}C

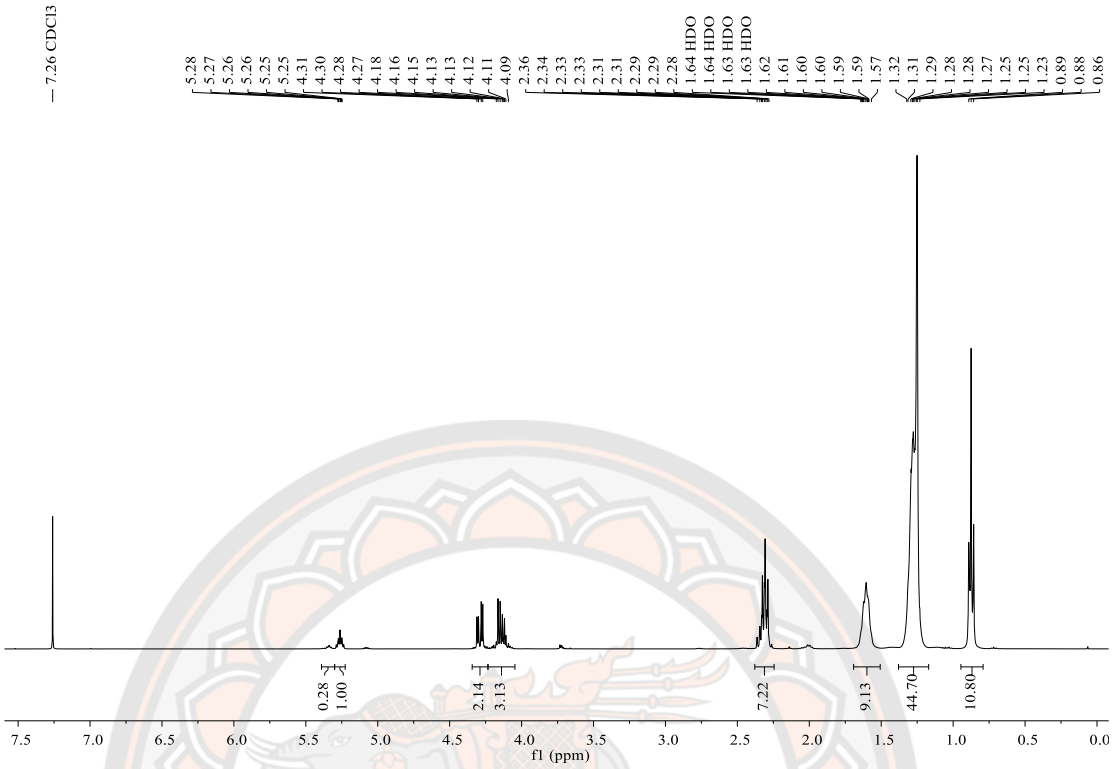


Figure 114 ^{1}H-NMR spectrum of the product of enzyme Lipozyme TL IM catalyzed acidolysis of coconut oil at 55^{o}C

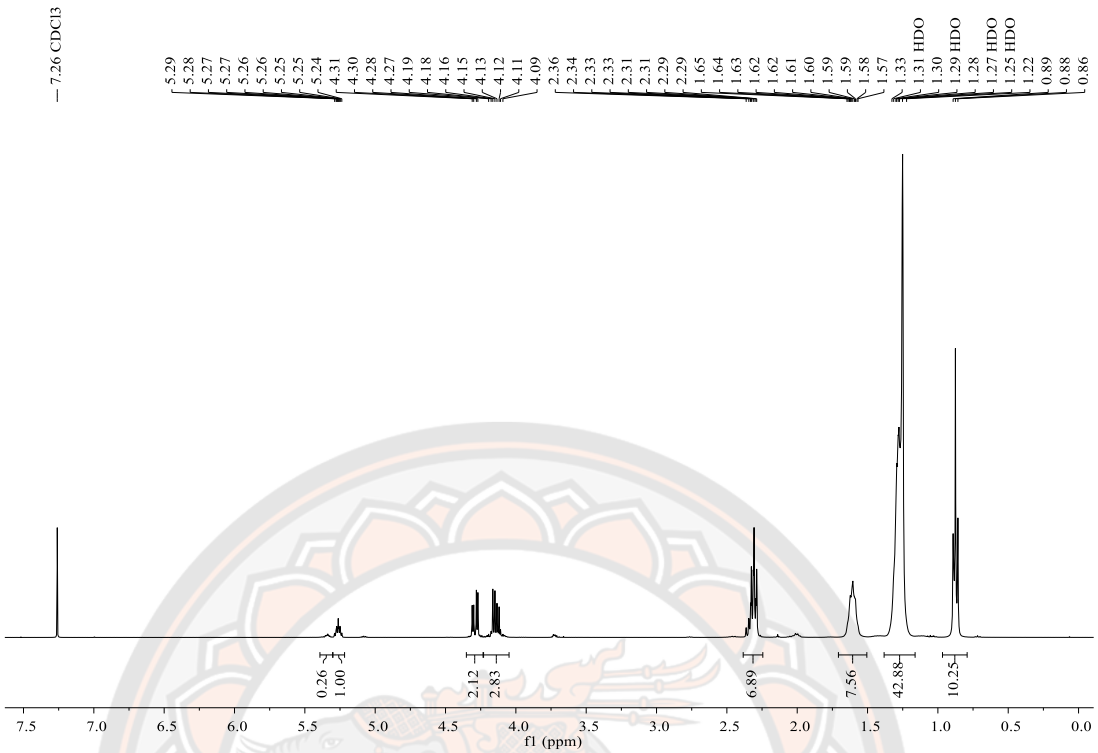


Figure 115 ^{1}H-NMR spectrum of the product of enzyme Lipozyme TL IM catalyzed acidolysis of coconut oil at 60^{o}C

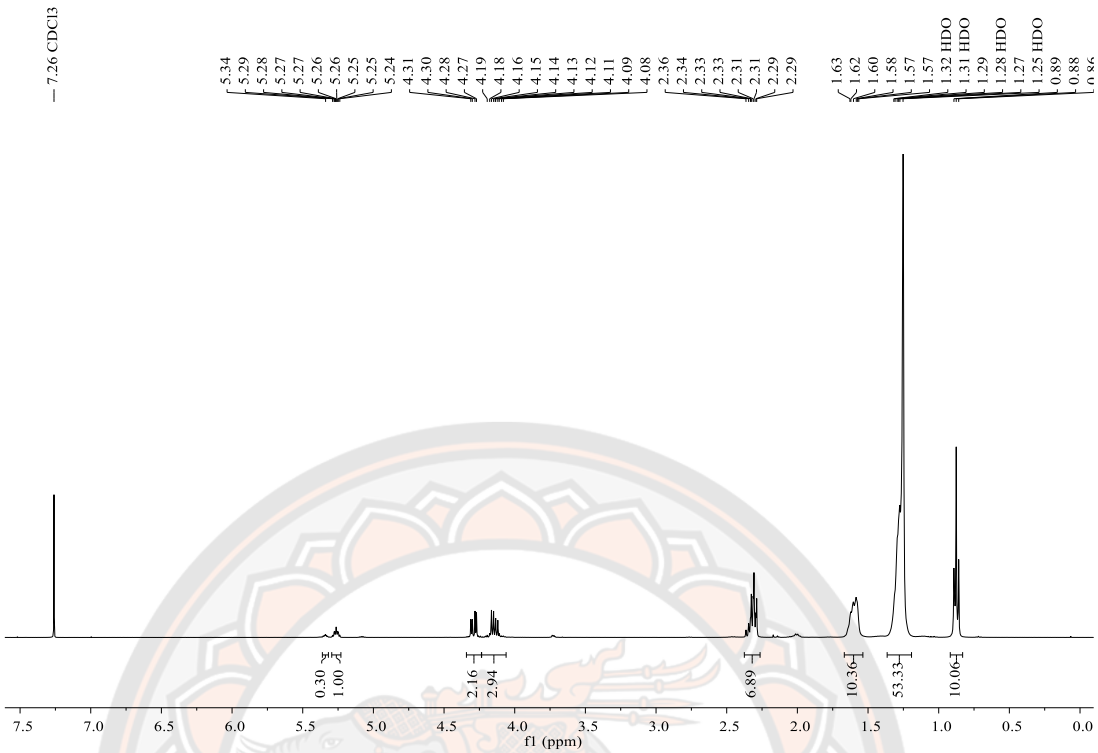


Figure 116 ^{1}H-NMR spectrum of the product of enzyme Lipozyme TL IM catalyzed acidolysis of coconut oil at 65^{o}C

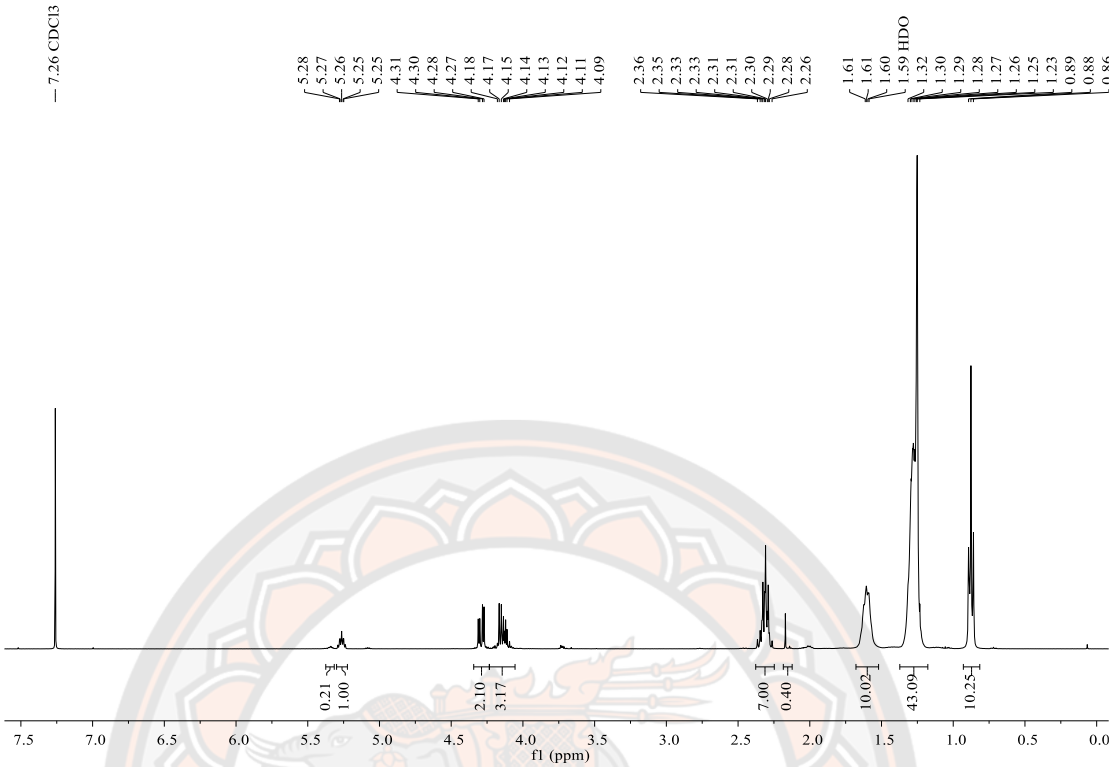


Figure 117 ^{1}H-NMR spectrum of the product of enzyme Lipozyme TL IM catalyzed acidolysis of coconut oil at 70^{o}C

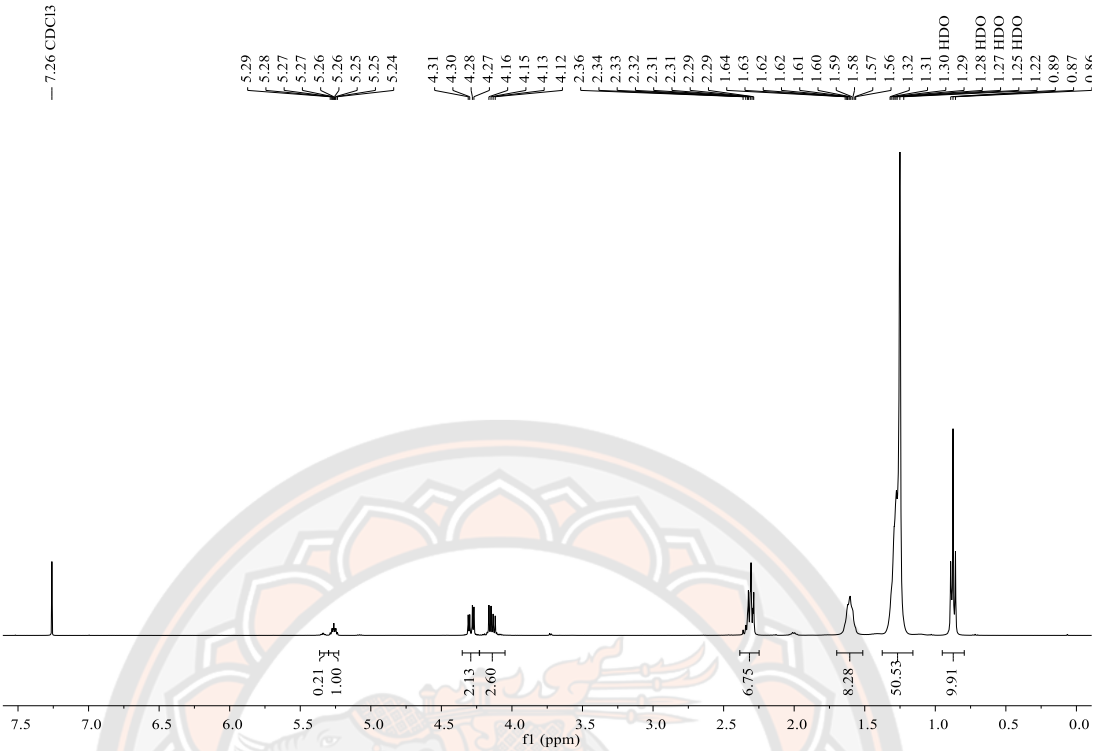


Figure 118 ^{1}H-NMR spectrum of the product of enzyme Lipozyme TL IM catalyzed acidolysis of coconut oil at 75^{o}C

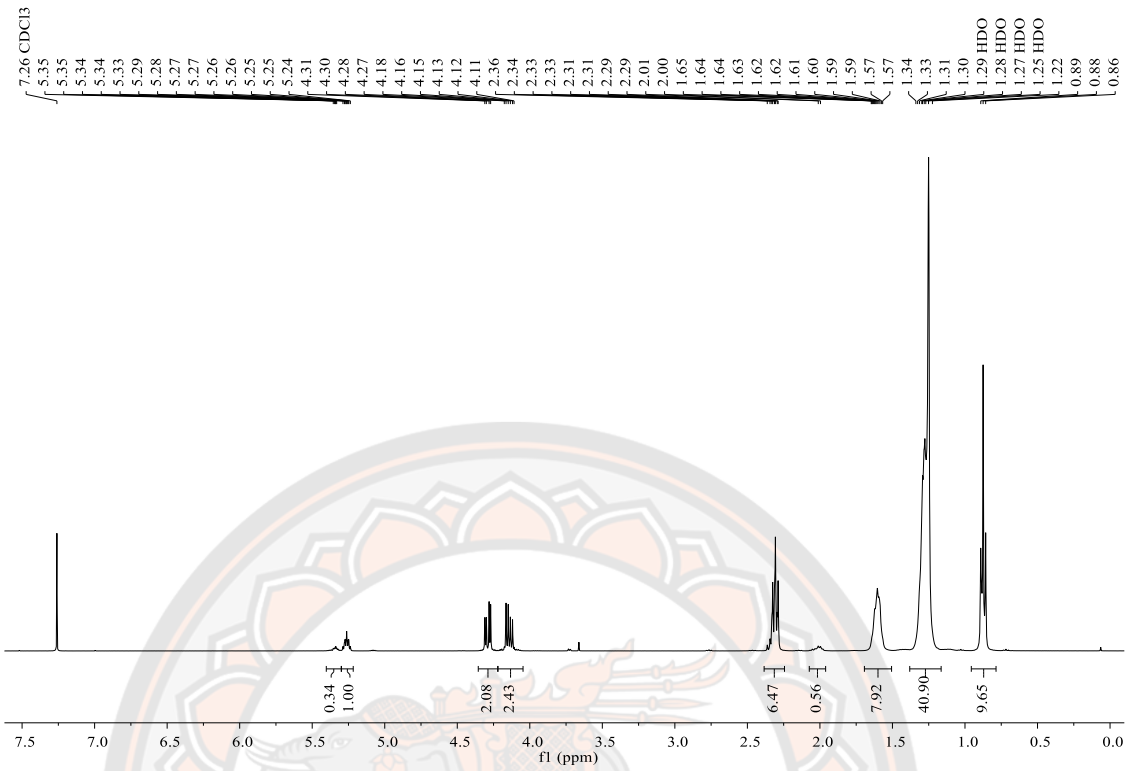


Figure 119 ^{1}H-NMR spectrum of the product of enzyme lipase B from *Candida antarctica* immobilized on acrylic resin catalyzed acidolysis of coconut oil at 45^{o}C

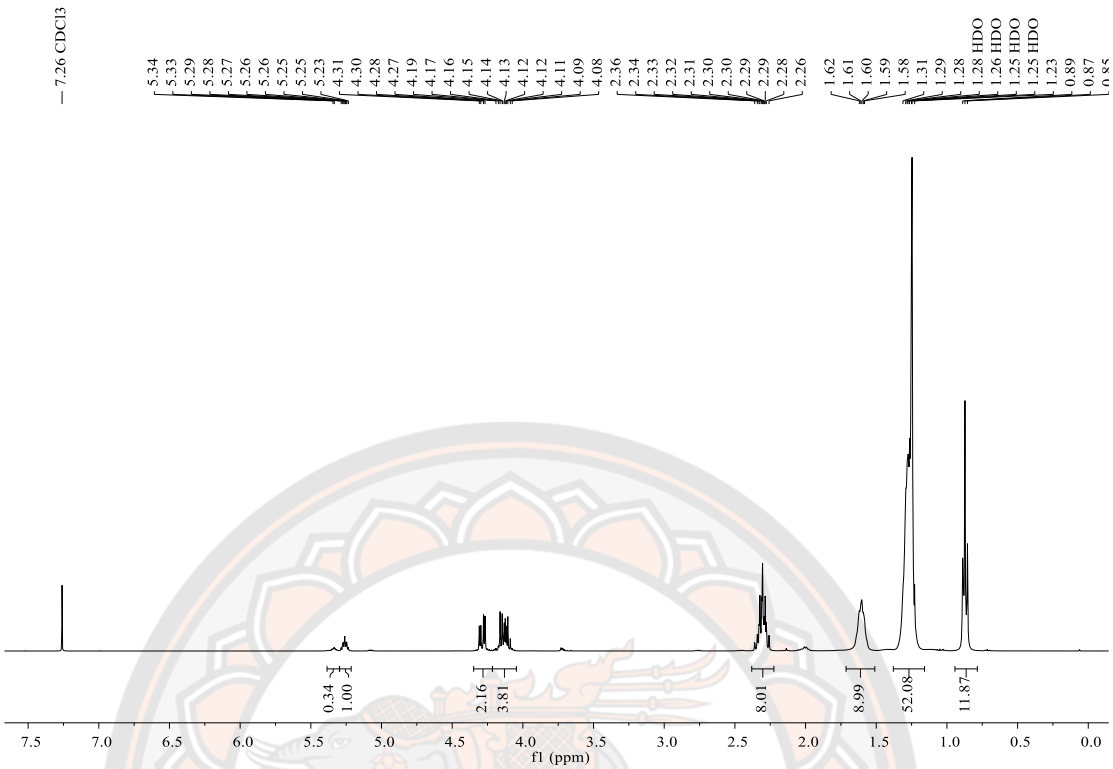


Figure 120 ^{1}H-NMR spectrum of the product of enzyme lipase B from *Candida antarctica* immobilized on acrylic resin catalyzed acidolysis of coconut oil at 50^{o}C

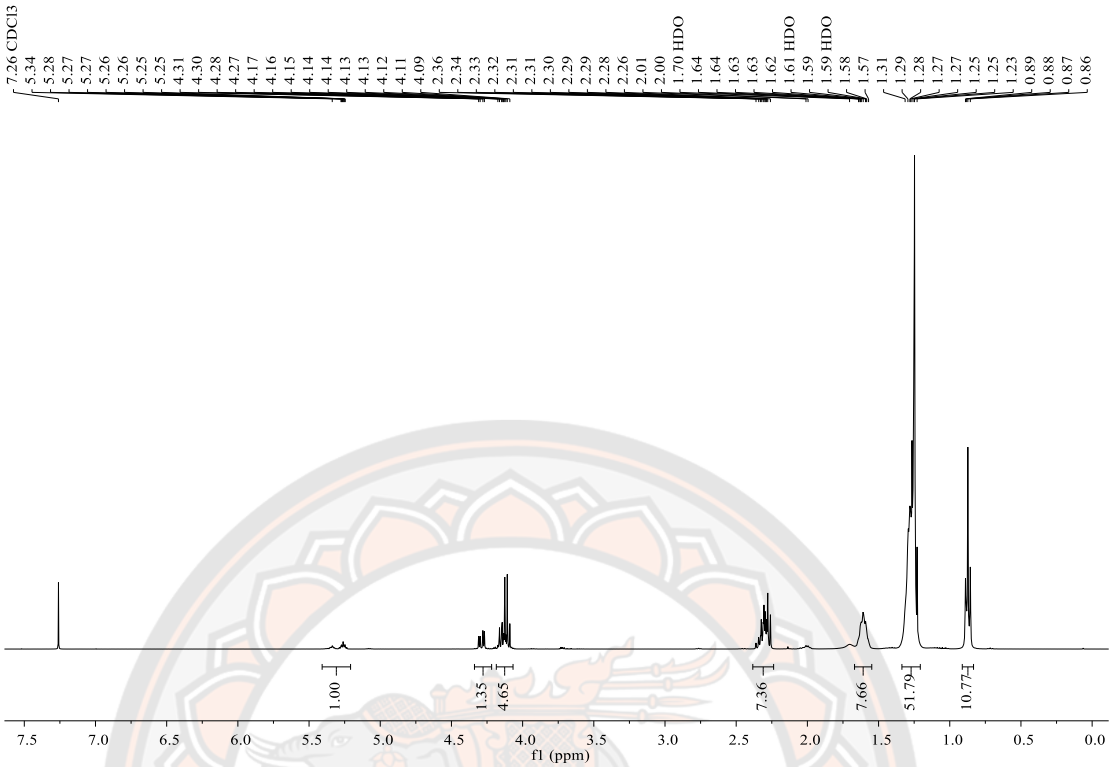


Figure 121 ^{1}H-NMR spectrum of the product of enzyme lipase B from *Candida antarctica* immobilized on acrylic resin catalyzed acidolysis of coconut oil at 55^{o}C

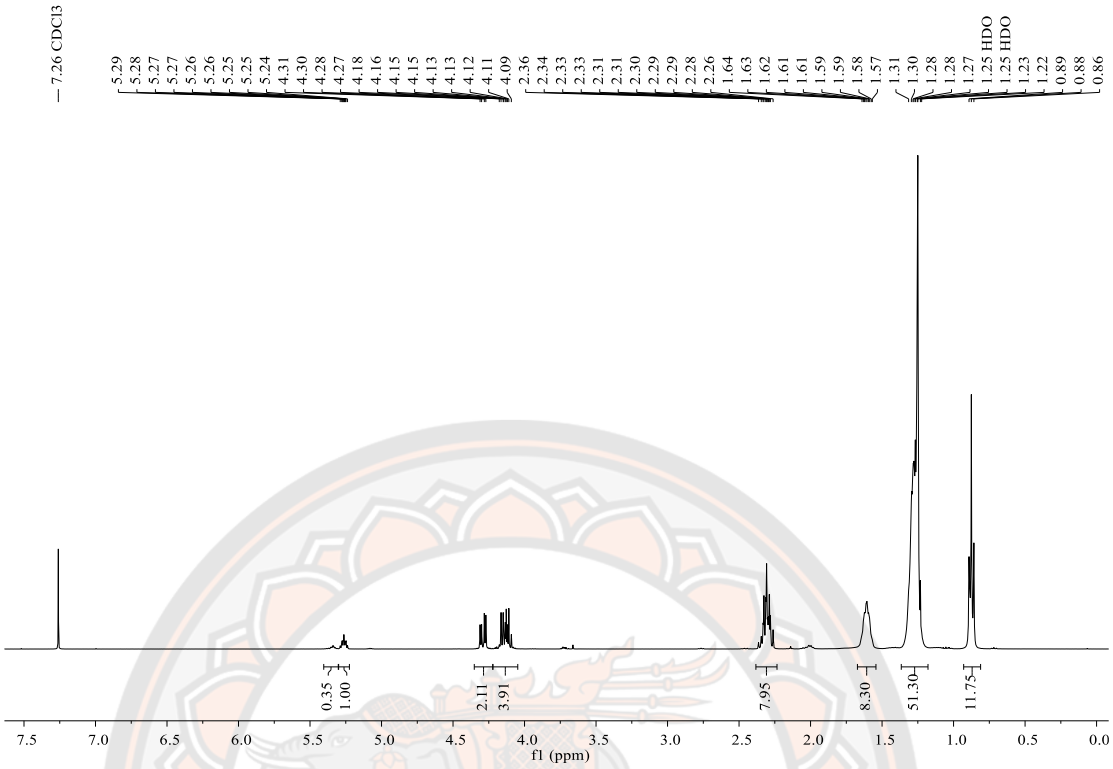


Figure 122 ^{1}H-NMR spectrum of the product of enzyme lipase B from *Candida antarctica* immobilized on acrylic resin catalyzed acidolysis of coconut oil at 60^{o}C

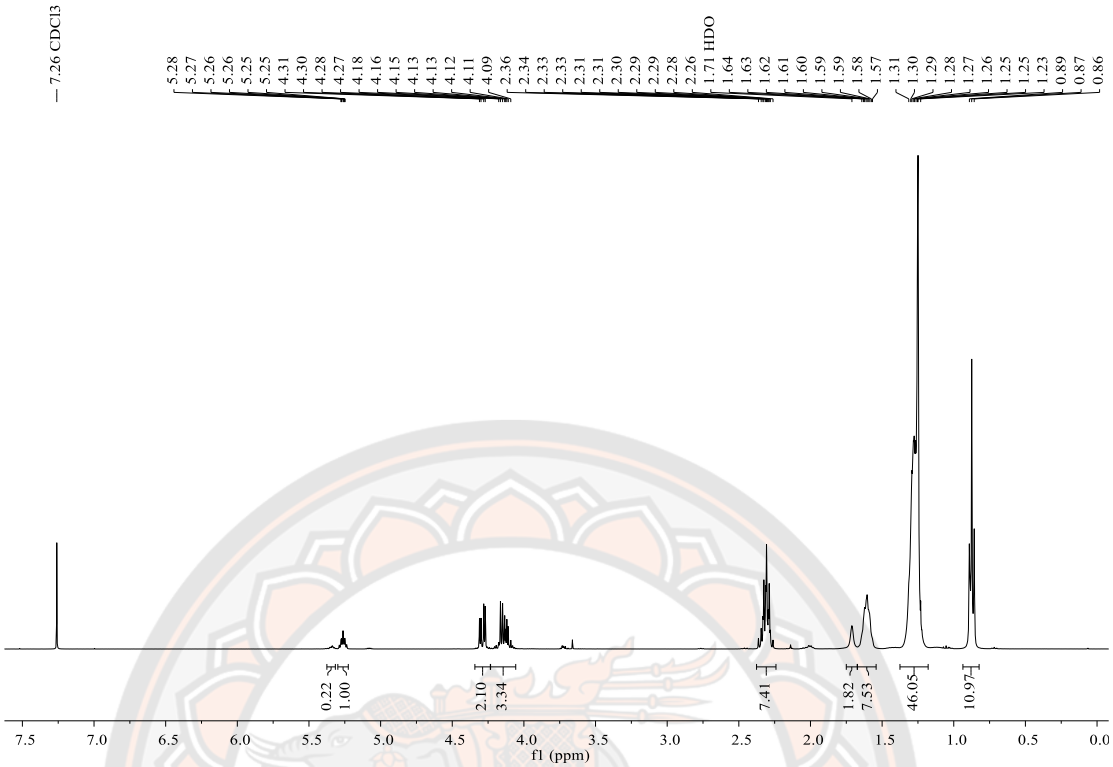


Figure 123 ^{1}H-NMR spectrum of the product of enzyme lipase B from *Candida antarctica* immobilized on acrylic resin catalyzed acidolysis of coconut oil at 65^{o}C

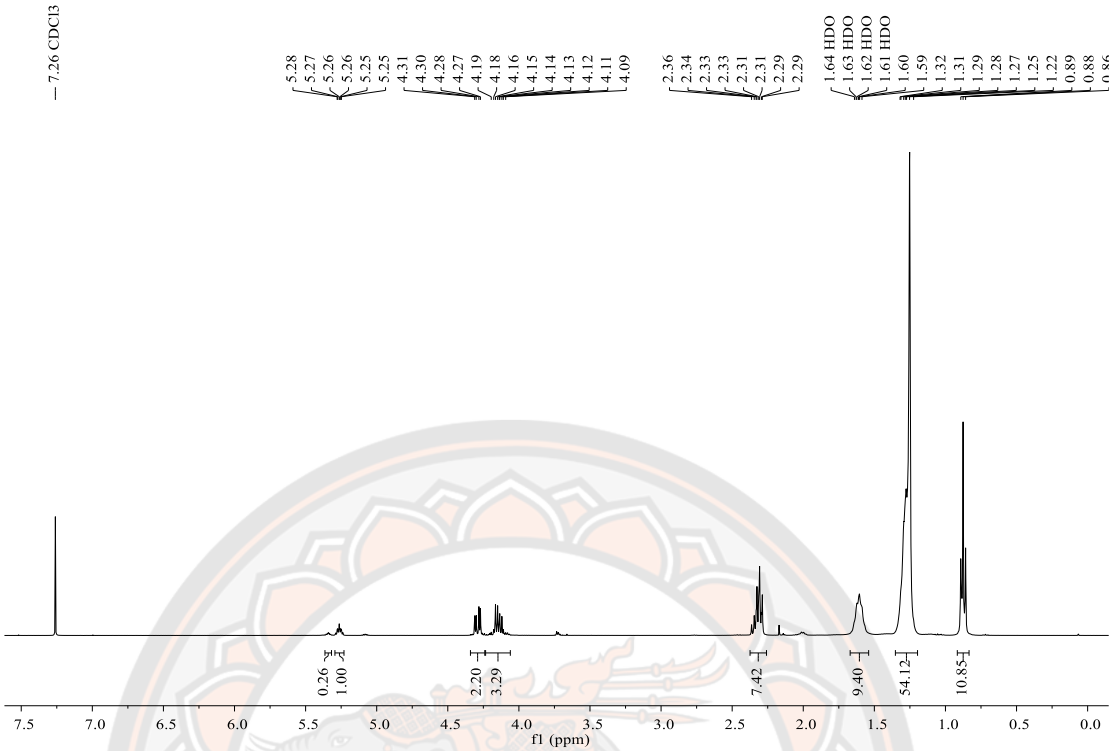


Figure 124 ^{1}H-NMR spectrum of the product of enzyme lipase B from *Candida antarctica* immobilized on acrylic resin catalyzed acidolysis of coconut oil at 70^{o}C

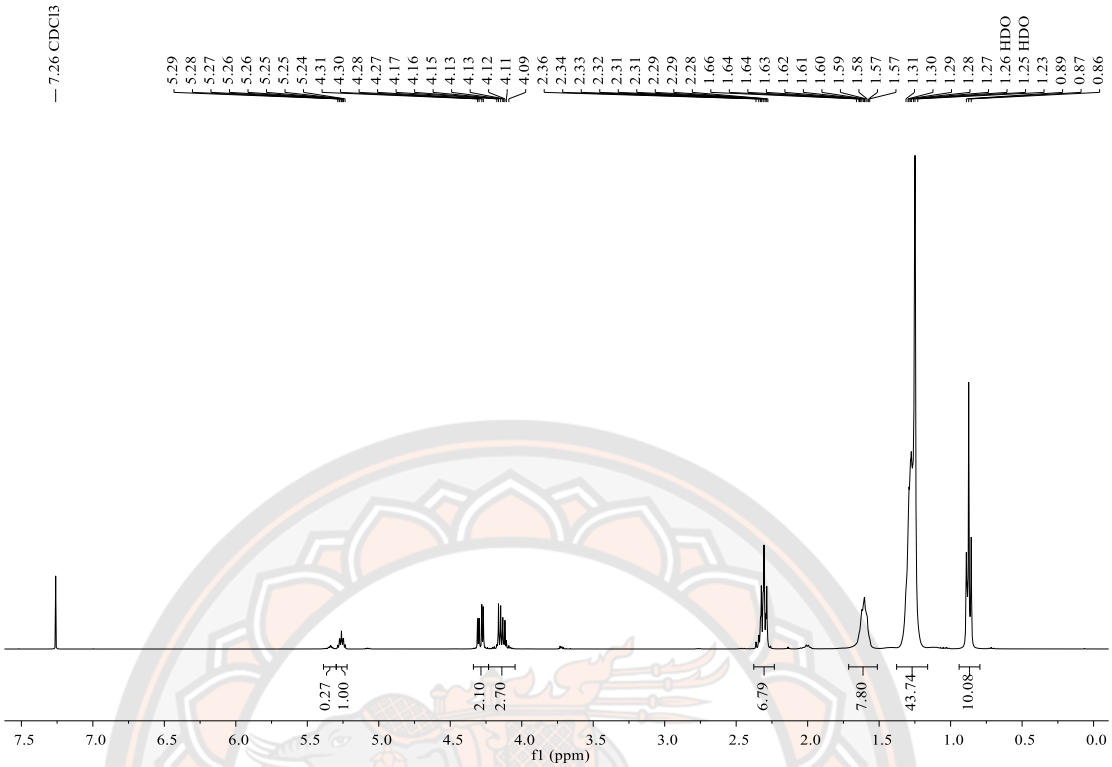


Figure 125 ^{1}H-NMR spectrum of the product of enzyme lipase B from *Candida antarctica* immobilized on acrylic resin catalyzed acidolysis of coconut oil at 75^{o}C

Investigation of the potential for enzyme recycling on coconut oil acidolysis with octanoic acid (C8:0)

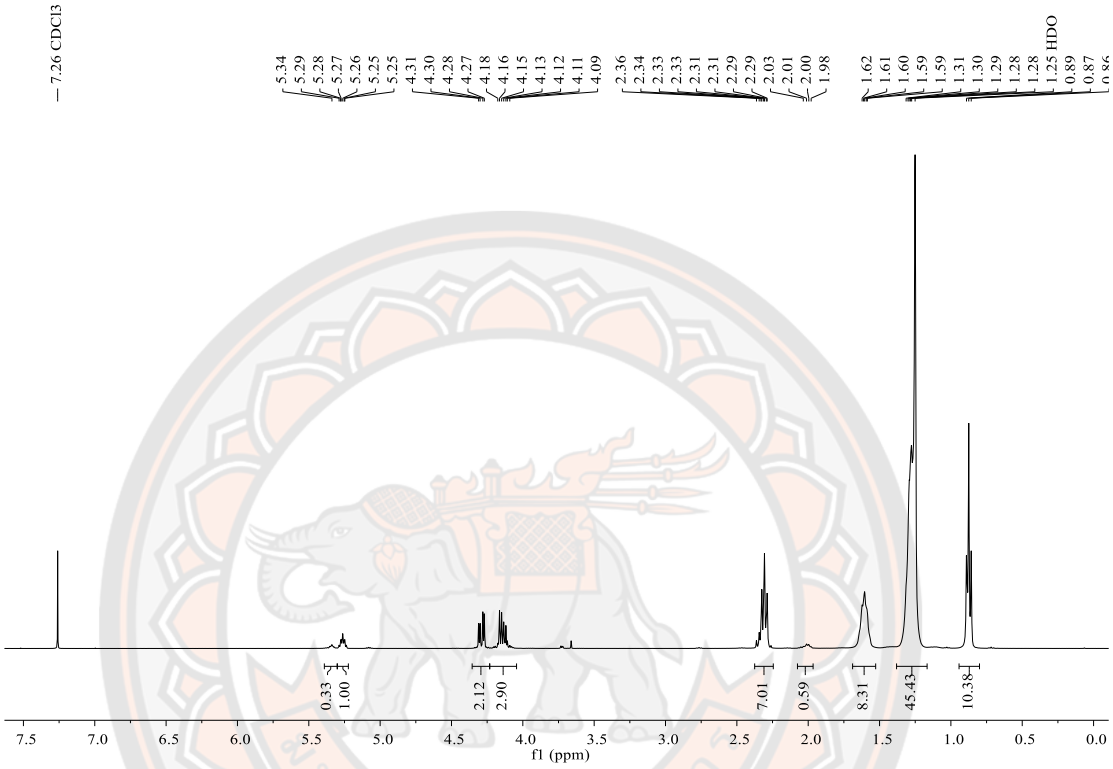


Figure 126 ^{1}H-NMR spectrum of the product of enzyme Lipozyme TL IM catalyzed acidolysis of coconut oil by first-time recycling enzyme

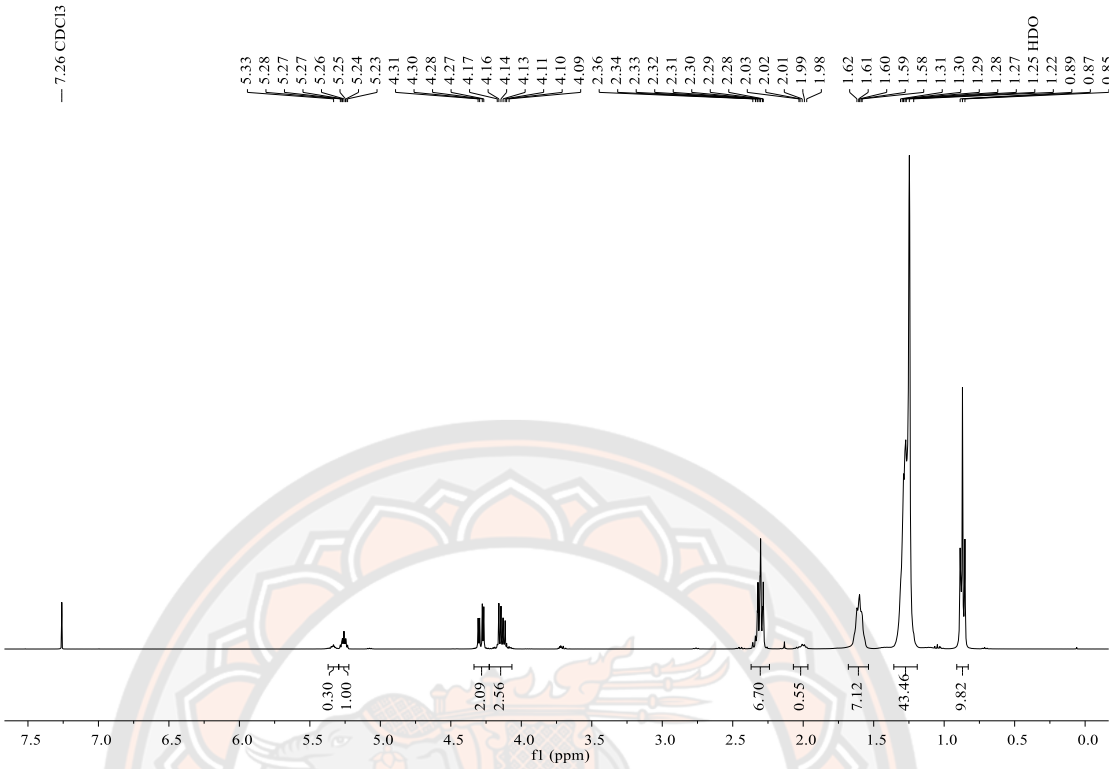


Figure 127 ^{1}H-NMR spectrum of the product of enzyme Lipozyme TL IM catalyzed acidolysis of coconut oil by second-time recycling enzyme

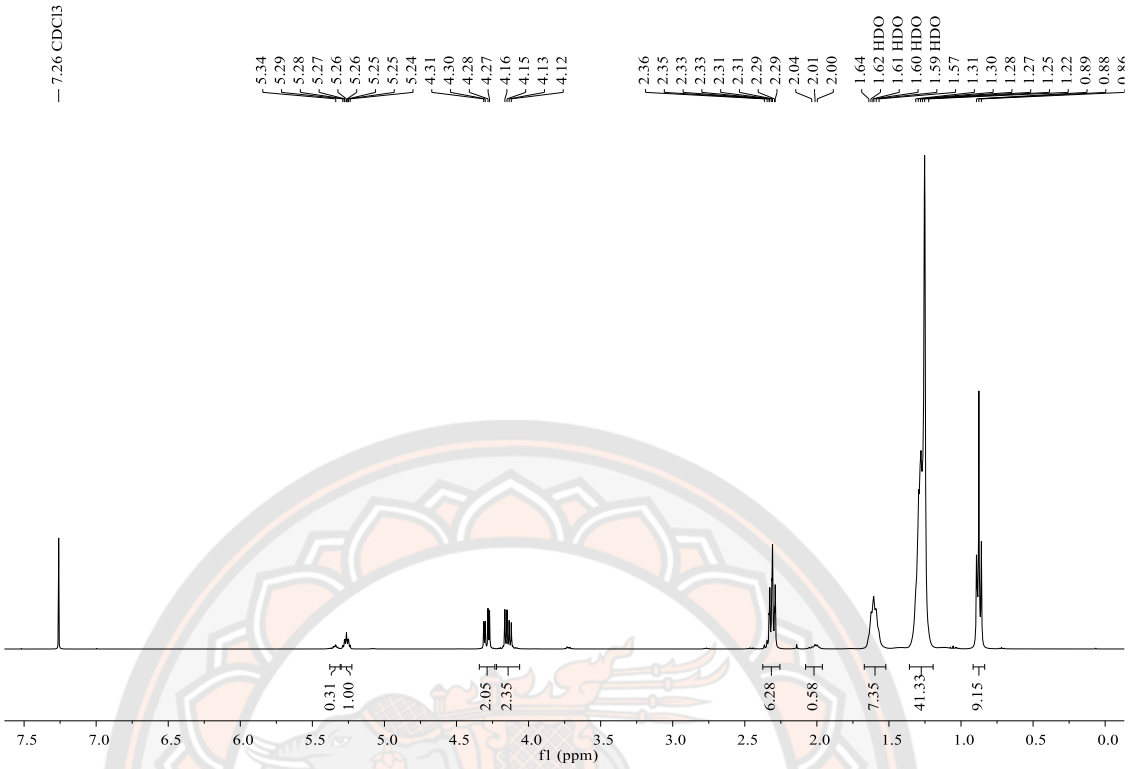


Figure 128 ^{1}H-NMR spectrum of the product of enzyme Lipozyme TL IM catalyzed acidolysis of coconut oil by third-time recycling enzyme

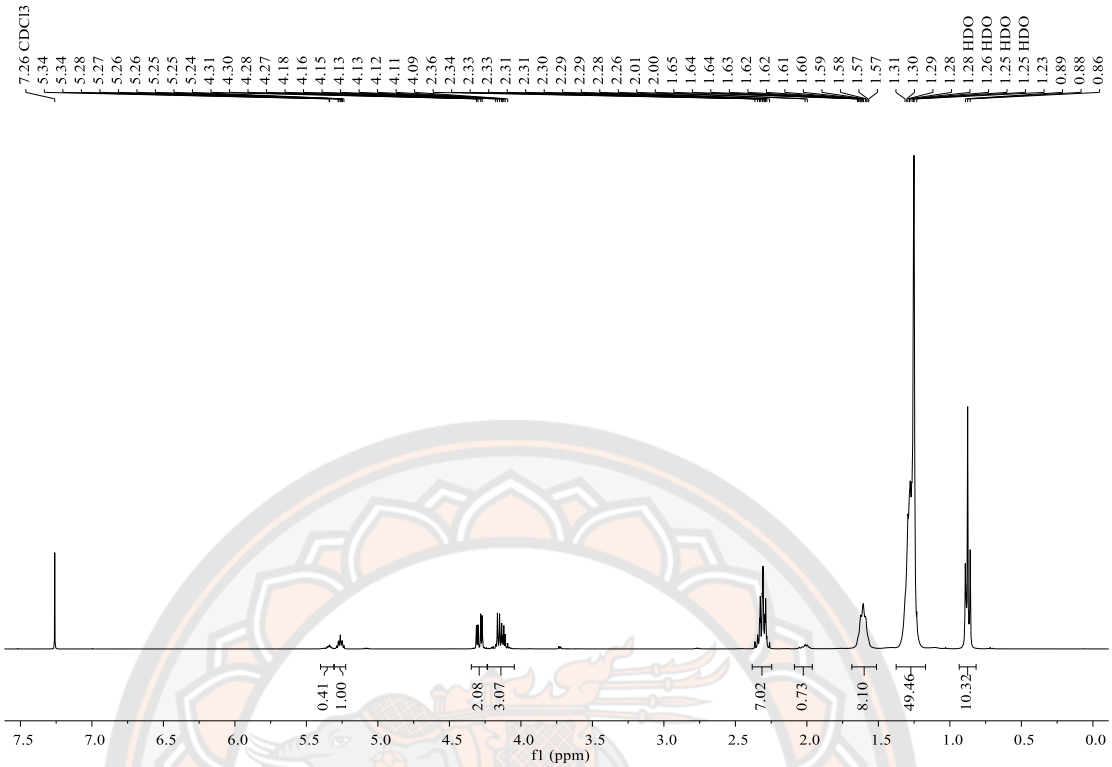


Figure 129 ^{1}H-NMR spectrum of the product of enzyme Lipozyme TL IM catalyzed acidolysis of coconut oil by fourth-time recycling enzyme

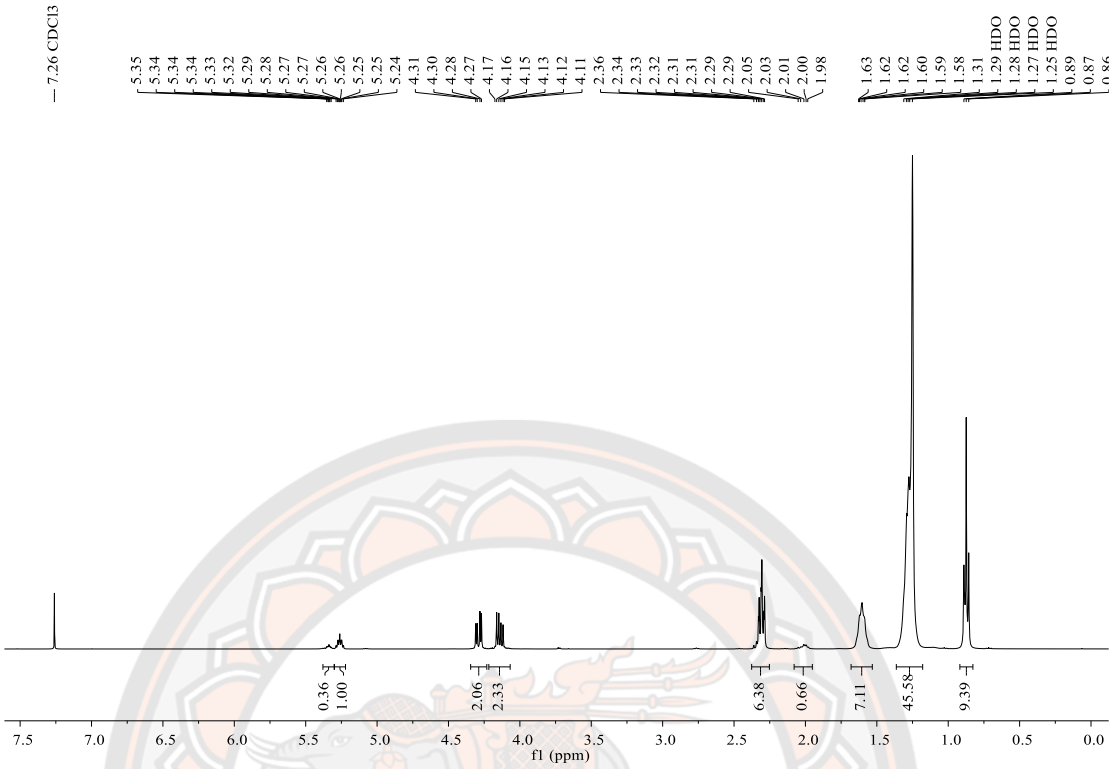


Figure 130 ^{1}H-NMR spectrum of the product of enzyme Lipozyme TL IM catalyzed acidolysis of coconut oil by fifth-time recycling enzyme

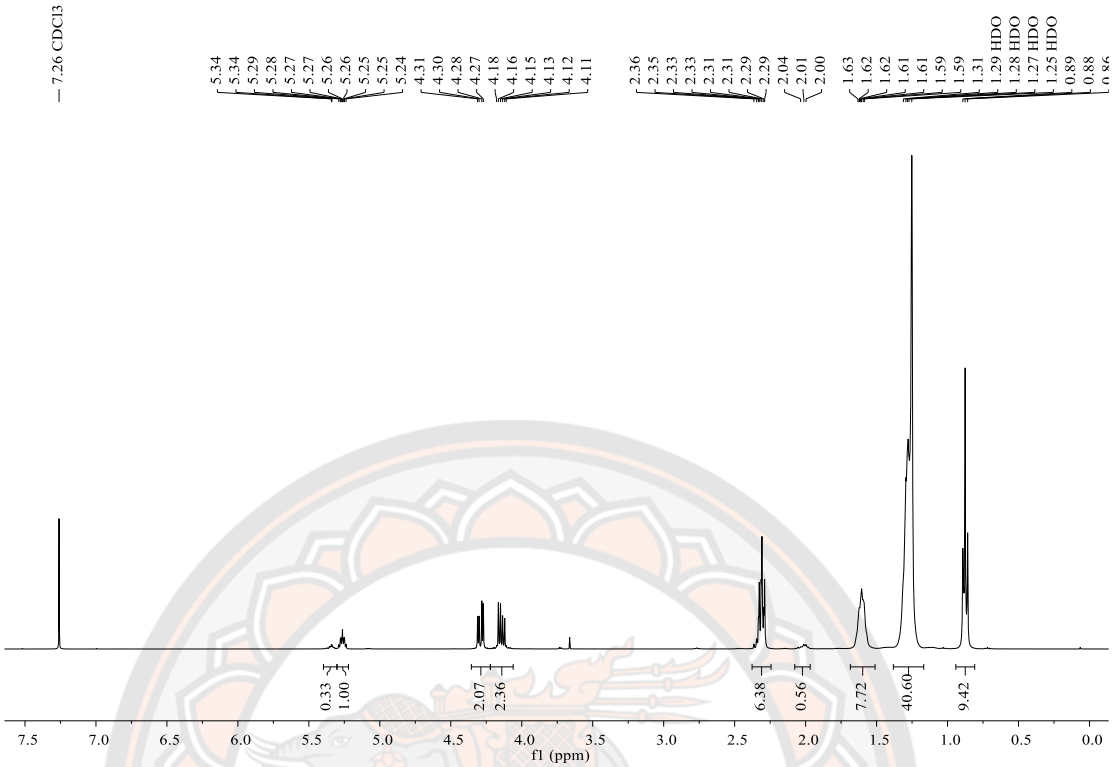


Figure 131 ^{1}H-NMR spectrum of the product of enzyme lipase B from *Candida antarctica* immobilized on acrylic resin catalyzed acidolysis of coconut oil by first-time recycling enzyme

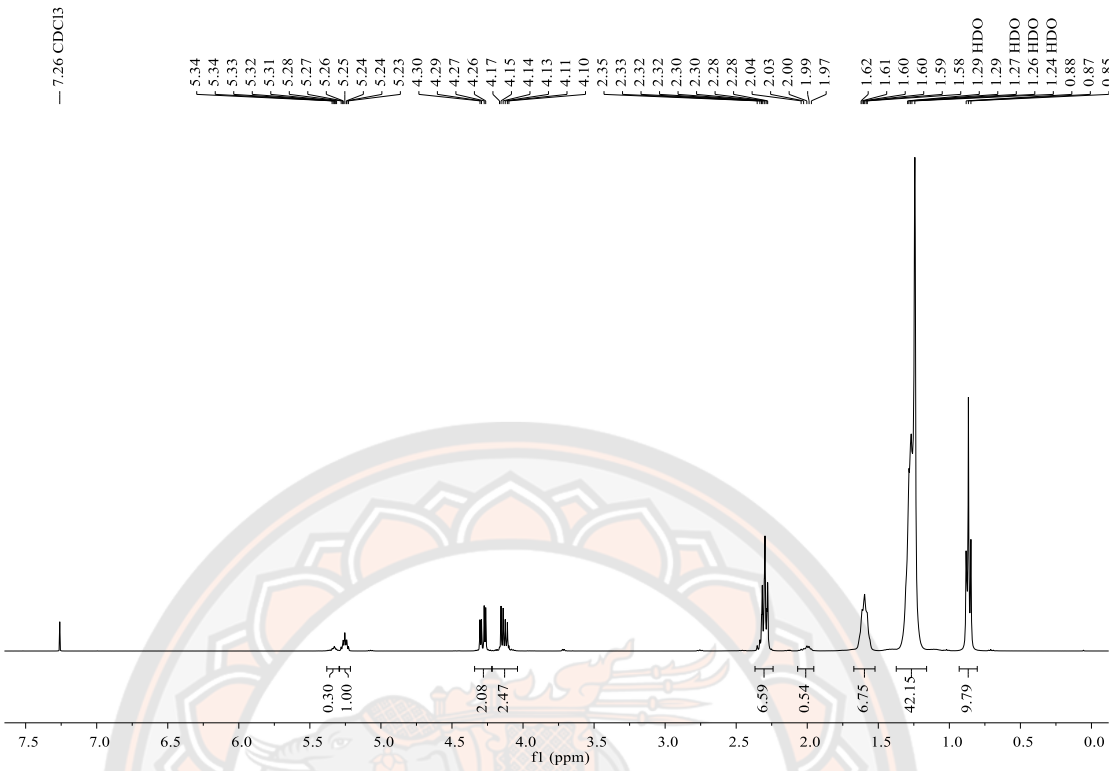


Figure 132 ^{1}H-NMR spectrum of the product of enzyme lipase B from *Candida antarctica* immobilized on acrylic resin catalyzed acidolysis of coconut oil by second-time recycling enzyme

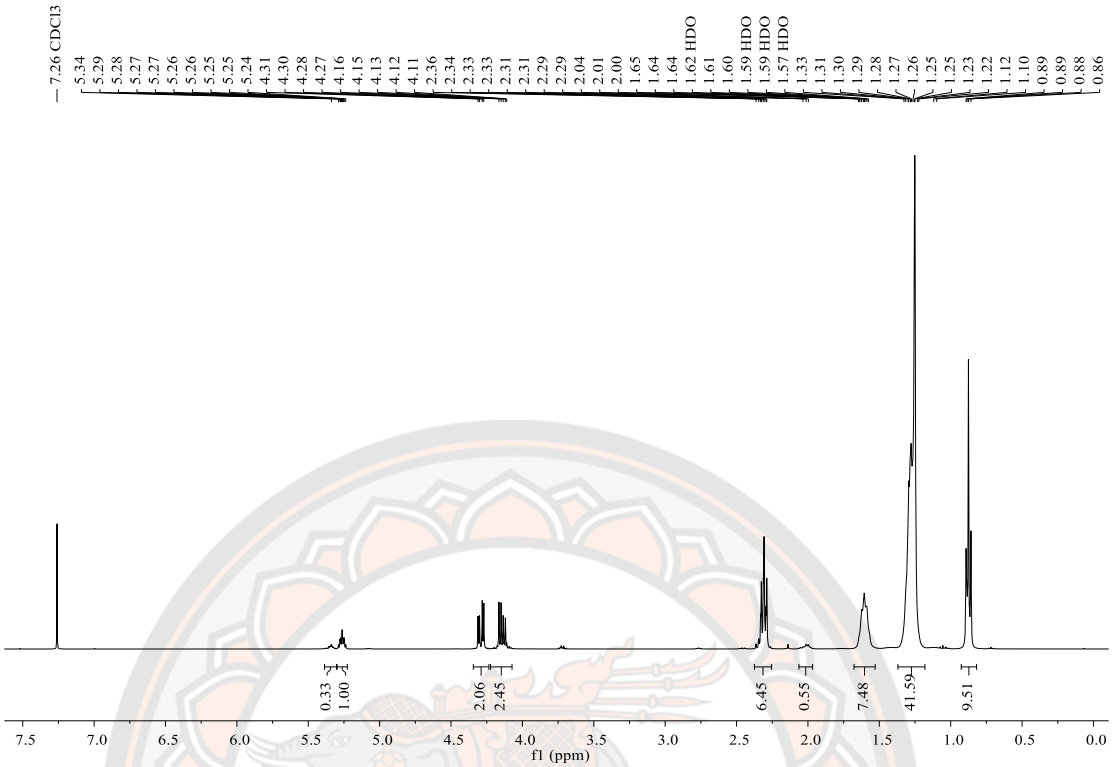


Figure 133 ^{1}H-NMR spectrum of the product of enzyme lipase B from *Candida antarctic*a immobilized on acrylic resin catalyzed acidolysis of coconut oil by third-time recycling enzyme

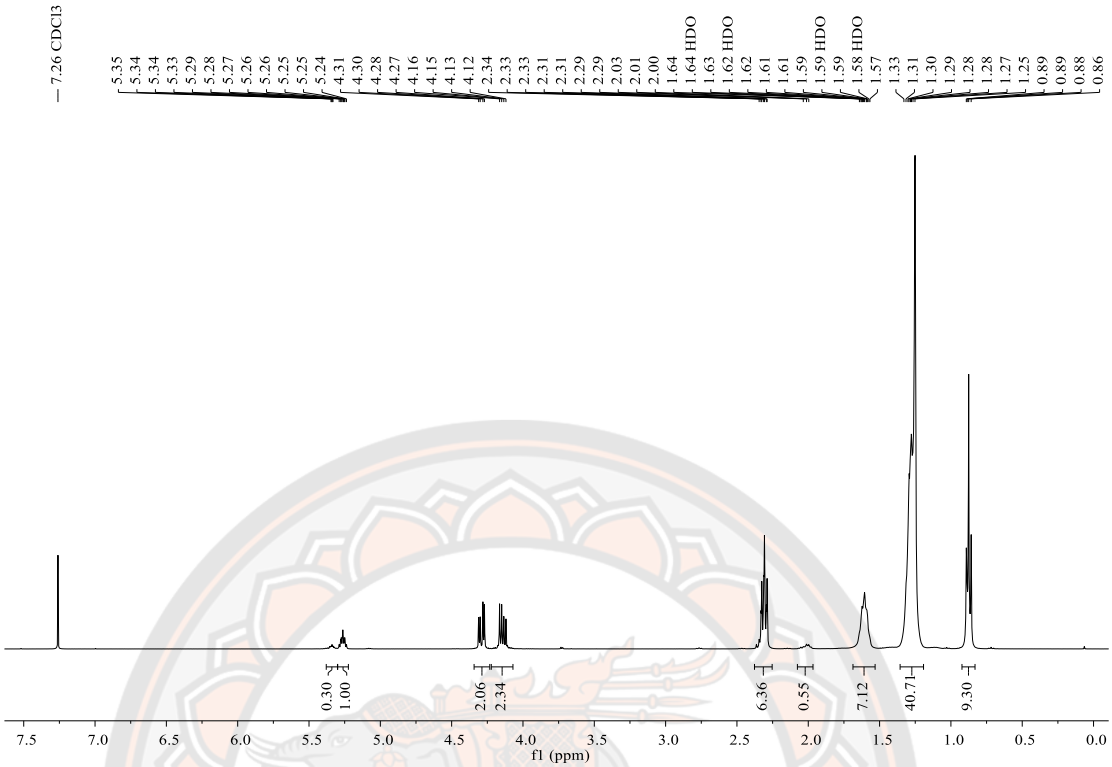


Figure 134 ^{1}H-NMR spectrum of the product of enzyme lipase B from *Candida antarctica* immobilized on acrylic resin catalyzed acidolysis of coconut oil by fourth-time recycling enzyme

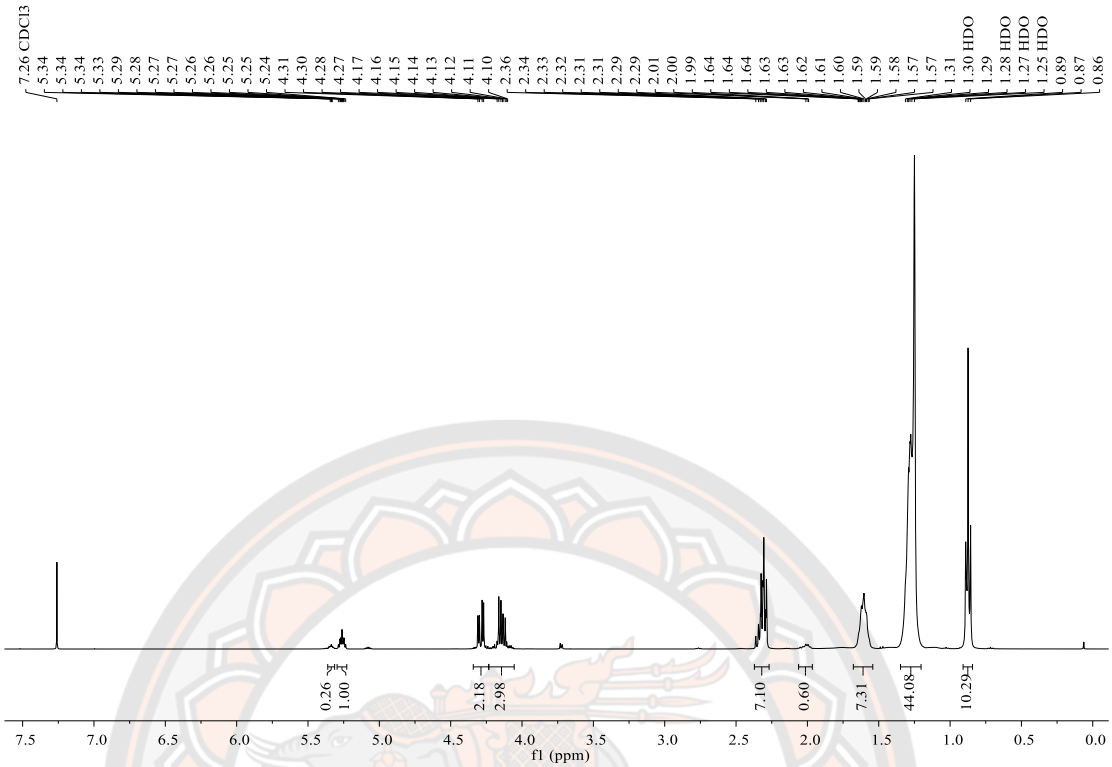


Figure 135 ^{1}H-NMR spectrum of the product of enzyme lipase B from *Candida antarctica* immobilized on acrylic resin catalyzed acidolysis of coconut oil by fifth-time recycling enzyme

Investigation of the potential to decrease the enzyme loading on coconut oil acidolysis with octanoic acid (C8:0)

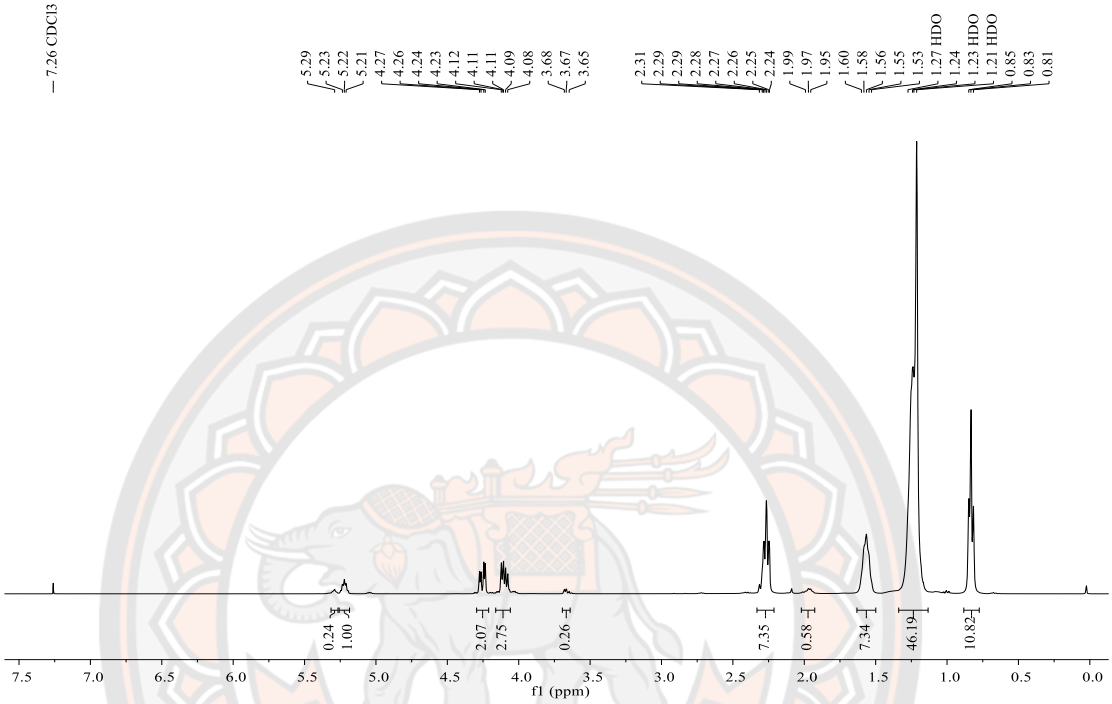


Figure 136 ^{1}H-NMR spectrum of the product of enzyme Lipozyme TL IM catalyzed acidolysis of coconut oil by loading enzyme 10%

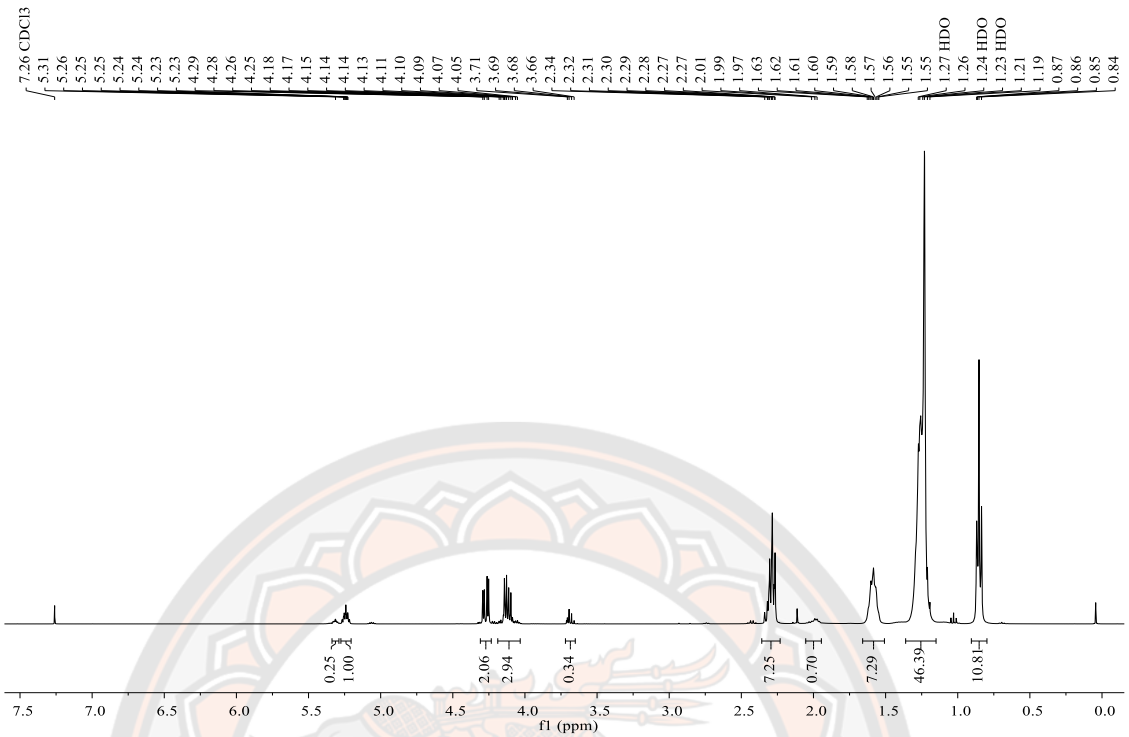


Figure 137 ^{1}H-NMR spectrum of the product of enzyme Lipozyme TL IM catalyzed acidolysis of coconut oil by loading enzyme 5%

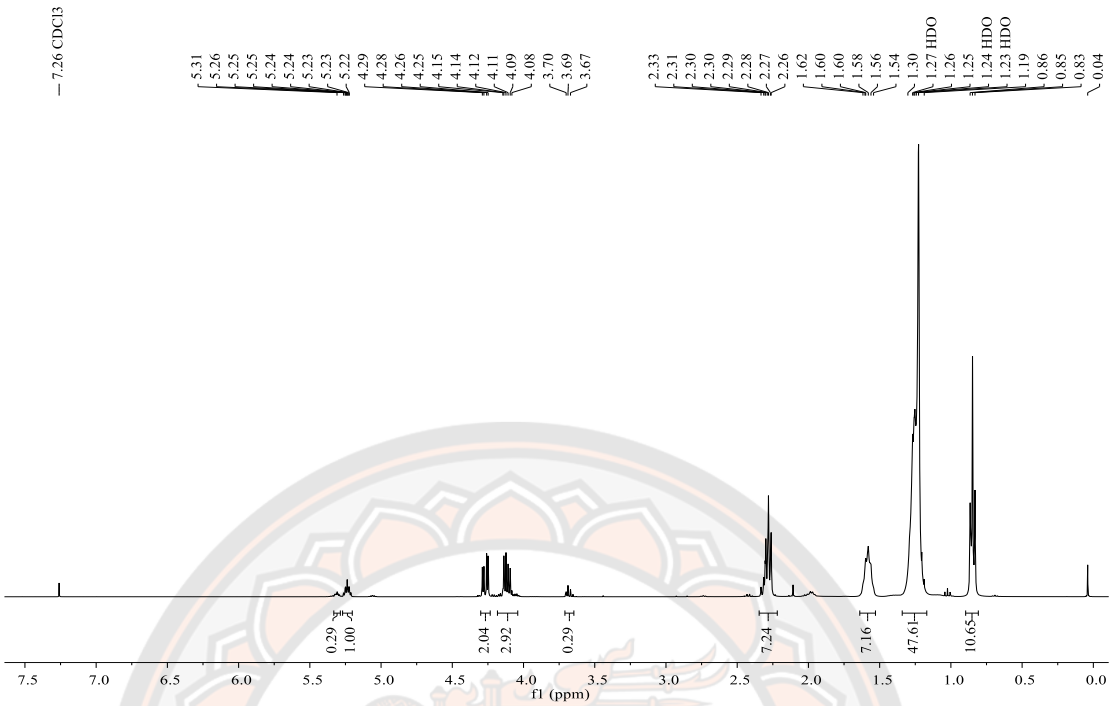


Figure 138 ^{1}H-NMR spectrum of the product of enzyme Lipozyme TL IM catalyzed acidolysis of coconut oil by loading enzyme 2.5%

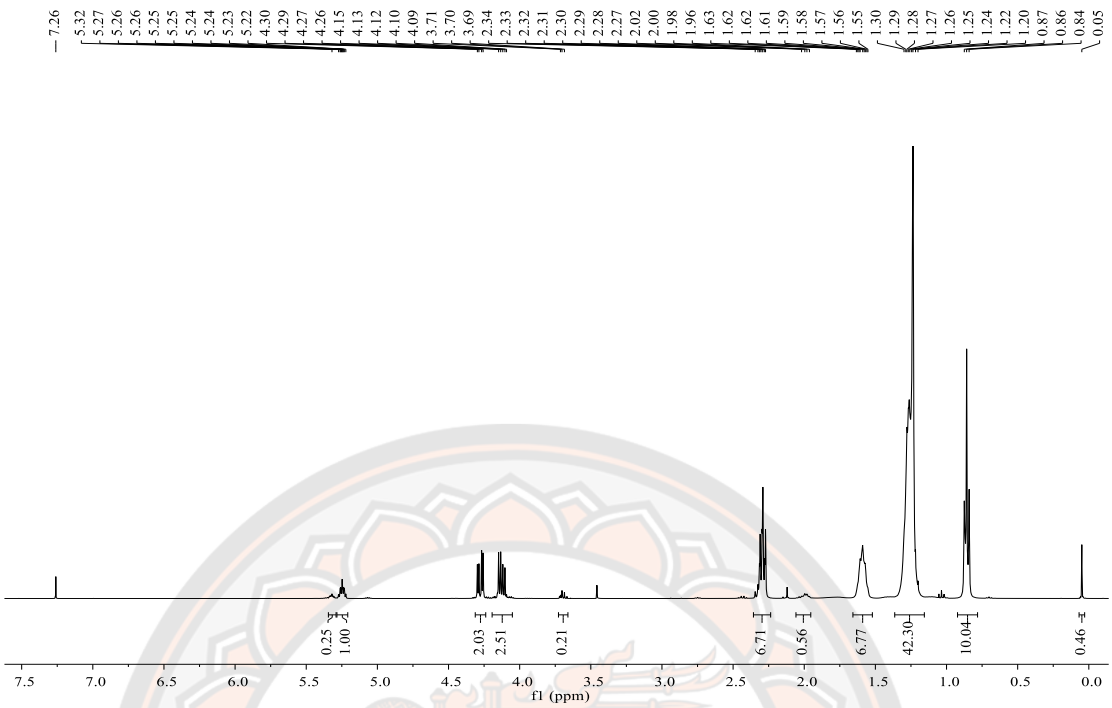


Figure 139 ^{1}H-NMR spectrum of the product of enzyme lipase B from *Candida antarctica* immobilized on acrylic resin catalyzed acidolysis of coconut oil by loading enzyme 10%

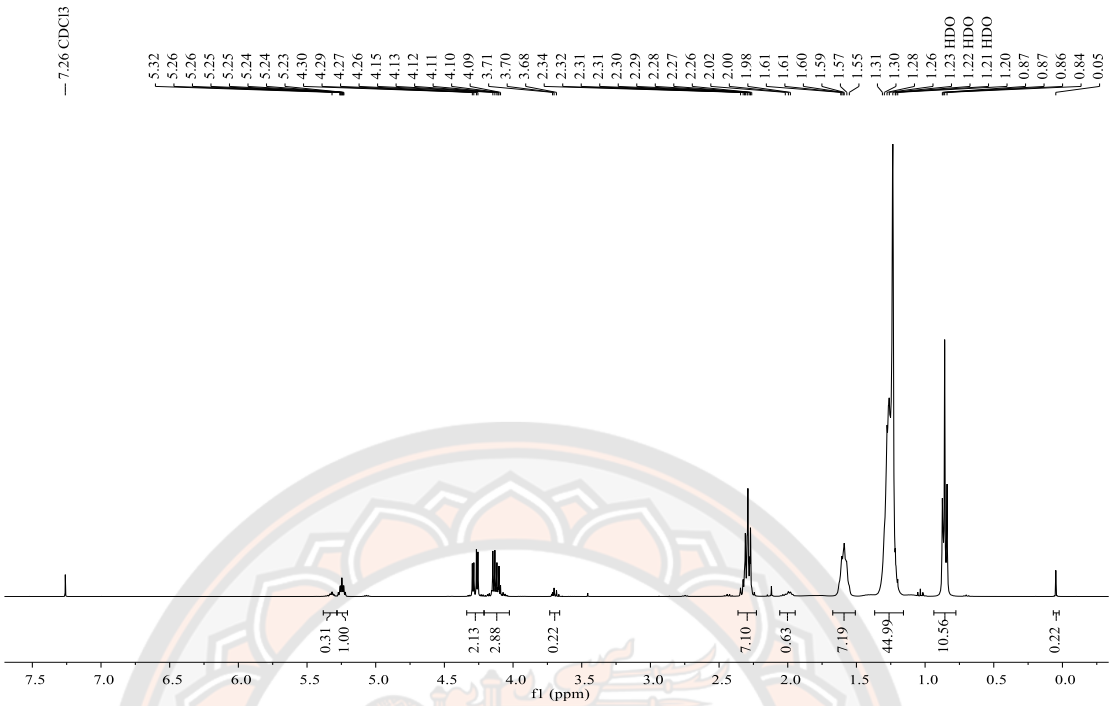


Figure 140 ^{1}H-NMR spectrum of the product of enzyme lipase B from *Candida antarctica* immobilized on acrylic resin catalyzed acidolysis of coconut oil by loading enzyme 5%

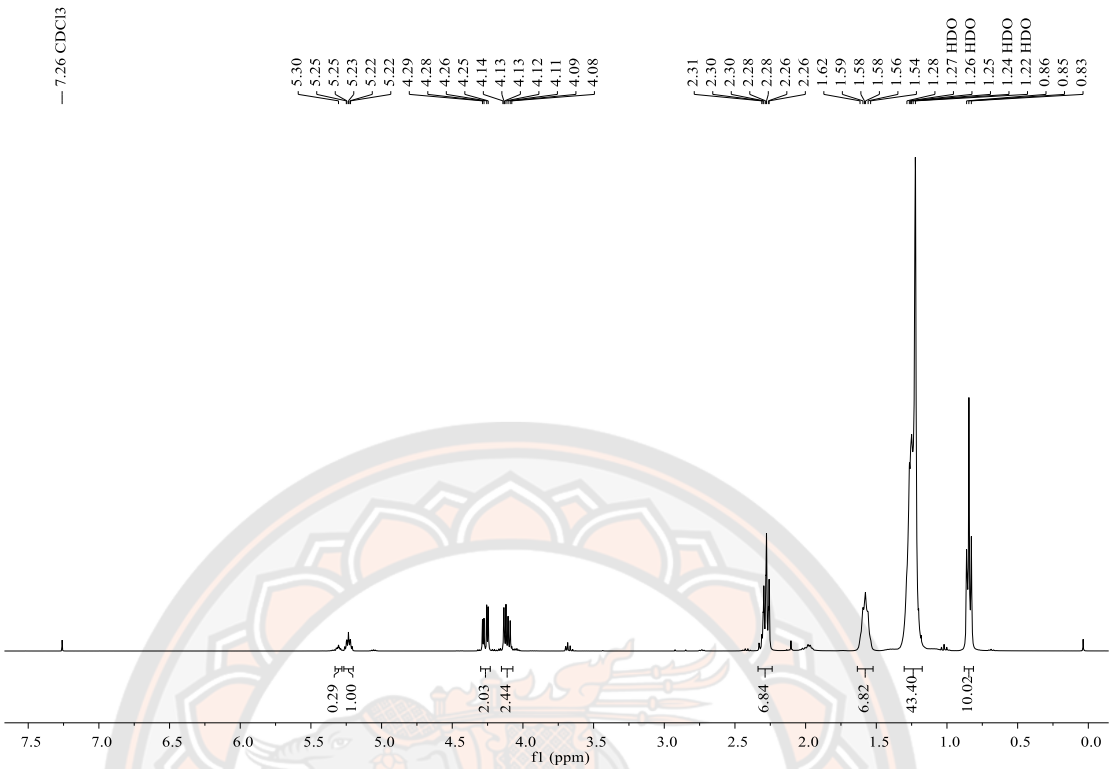


Figure 141 ^{1}H-NMR spectrum of the product of enzyme lipase B from *Candida antarctica* immobilized on acrylic resin catalyzed acidolysis of coconut oil by loading enzyme 2.5%

Investigation of the effect of temperature for coconut oil interesterification by methyl octanoate

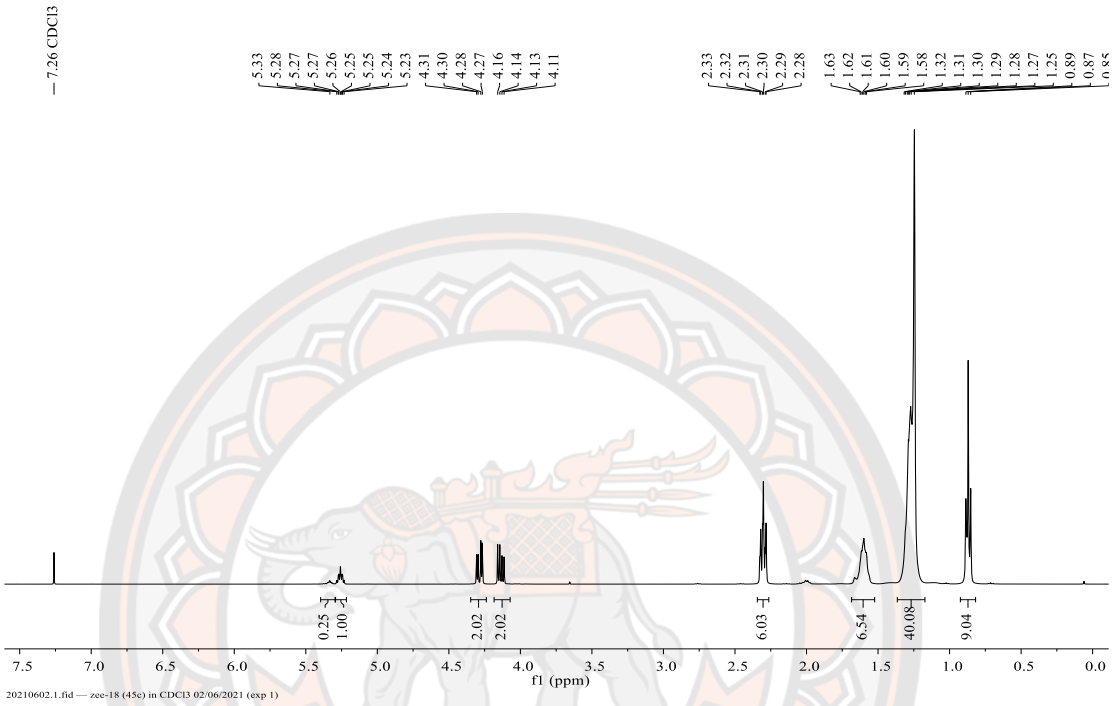


Figure 142 ^{1}H-NMR spectrum of the product of enzyme Lipozyme TL IM catalyzed interesterification of coconut oil at 45^{o}C

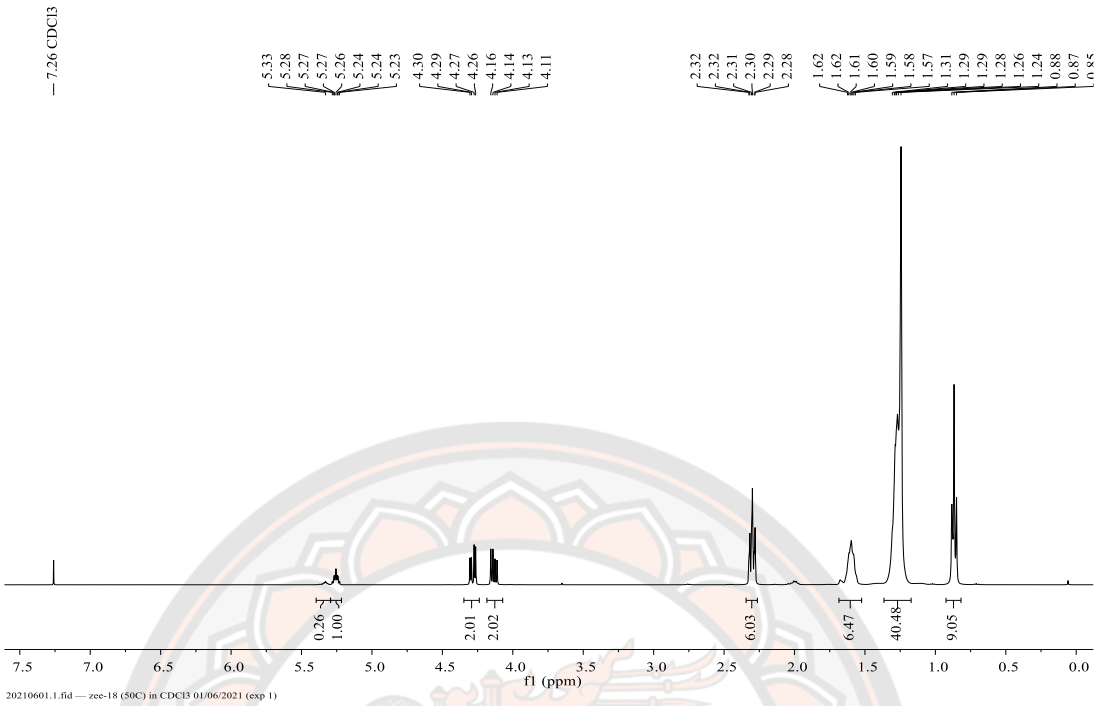


Figure 143 ^{1}H-NMR spectrum of the product of enzyme Lipozyme TL IM catalyzed interesterification of coconut oil at 50ºC

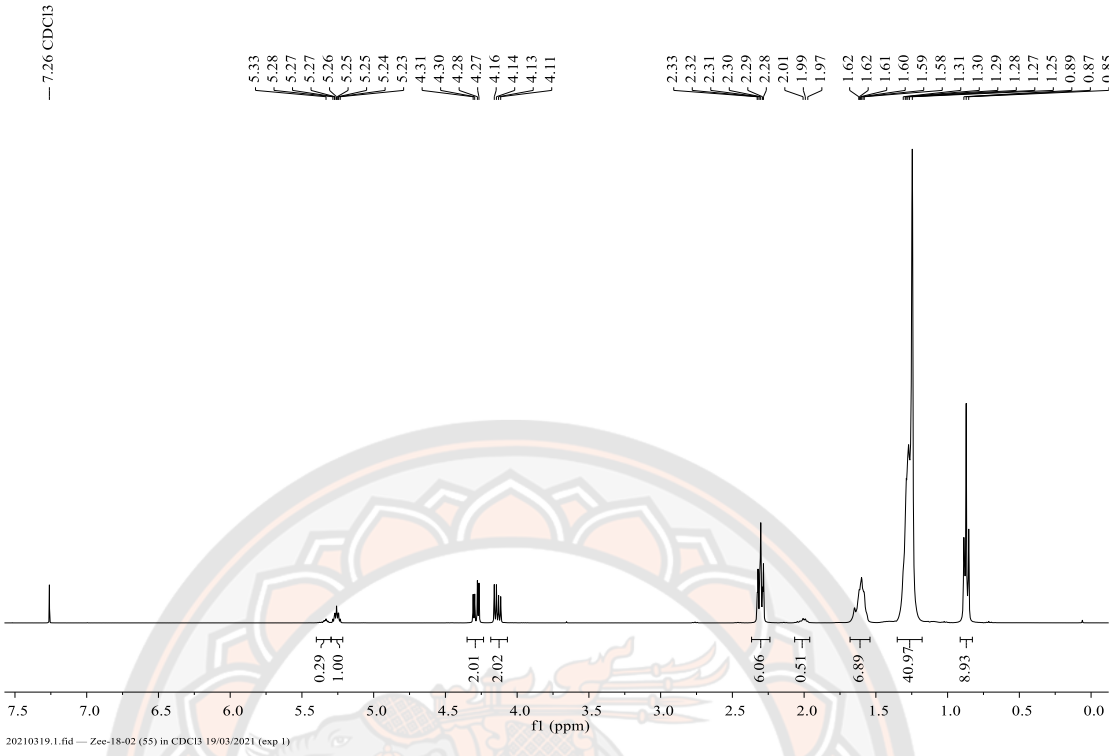


Figure 144 ^{1}H-NMR spectrum of the product of enzyme Lipozyme TL IM catalyzed interesterification of coconut oil at 55ºC

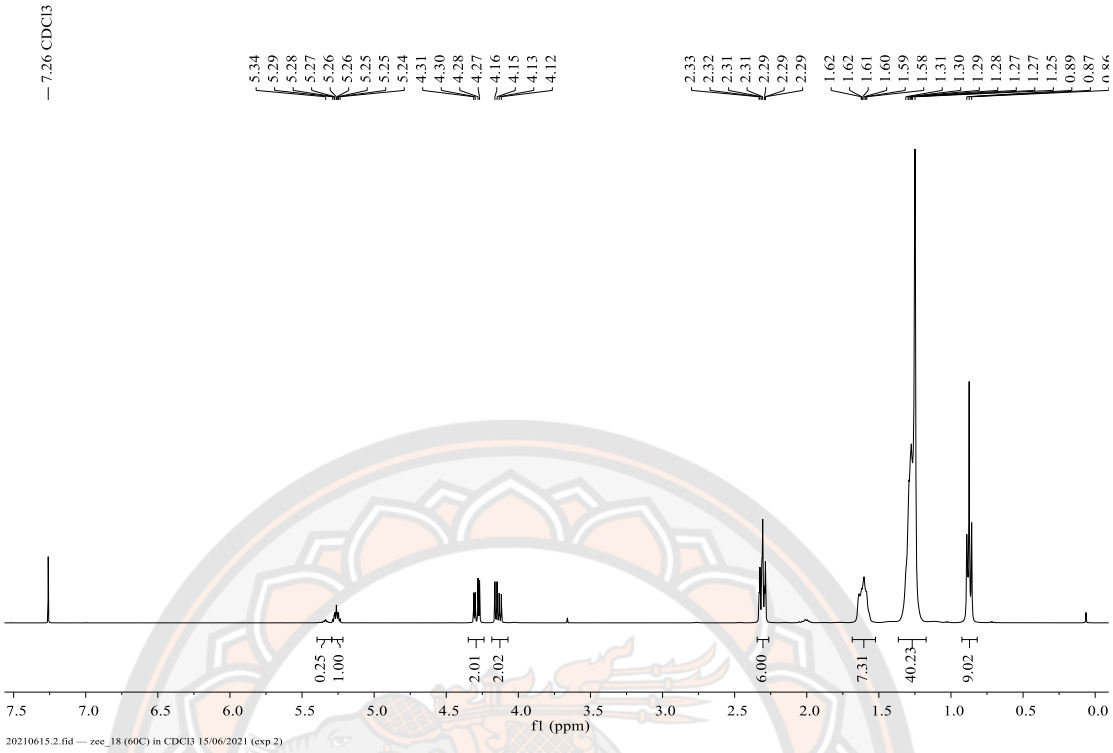


Figure 145 ^{1}H-NMR spectrum of the product of enzyme Lipozyme TL IM catalyzed interesterification of coconut oil at 60^{o}C

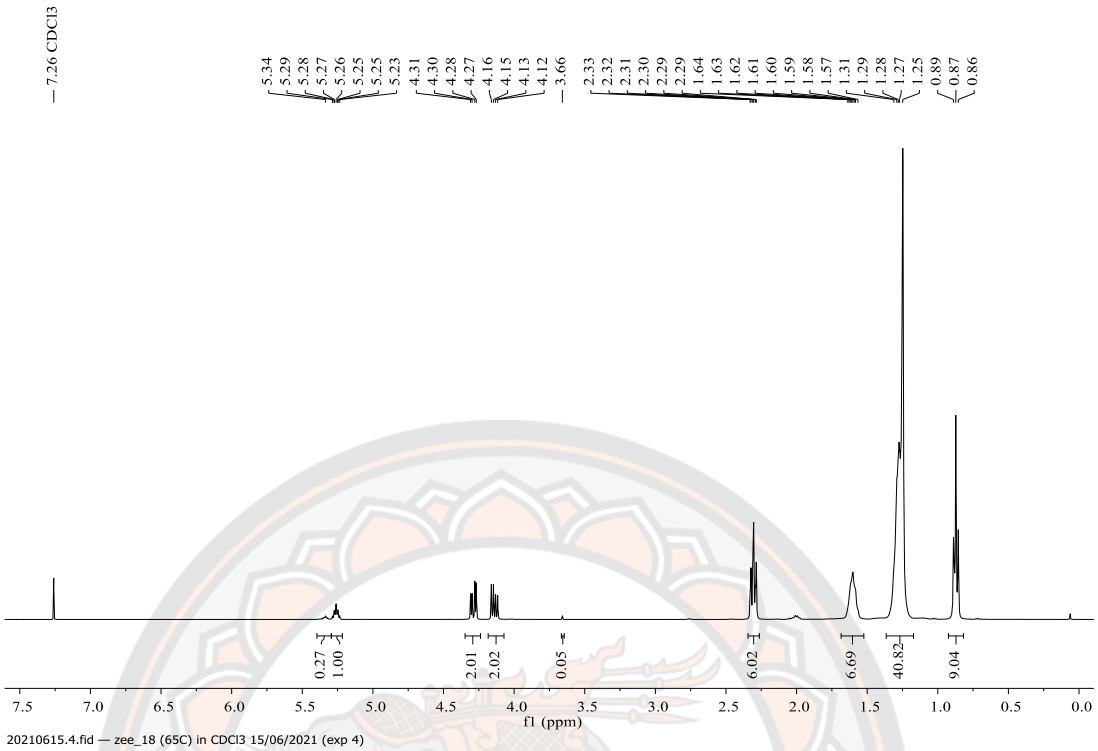


Figure 146 ^{1}H-NMR spectrum of the product of enzyme Lipozyme TL IM catalyzed interesterification of coconut oil at 65ºC

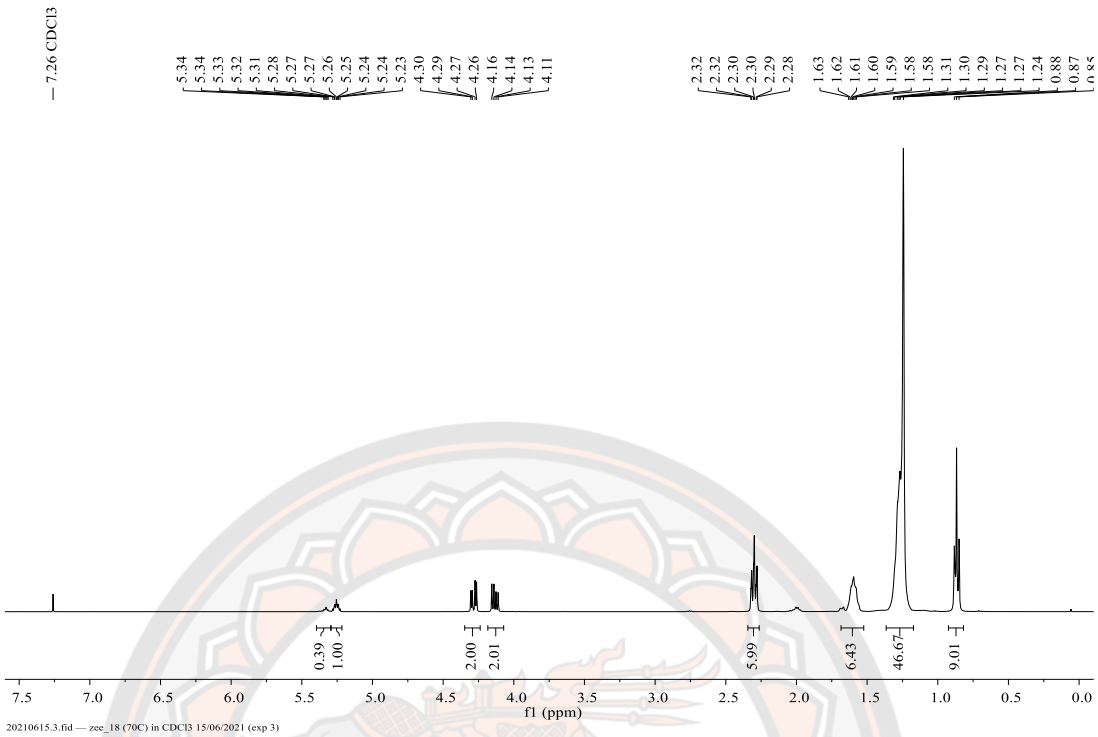


Figure 147 ^{1}H-NMR spectrum of the product of enzyme Lipozyme TL IM catalyzed interesterification of coconut oil at 70^{o}C

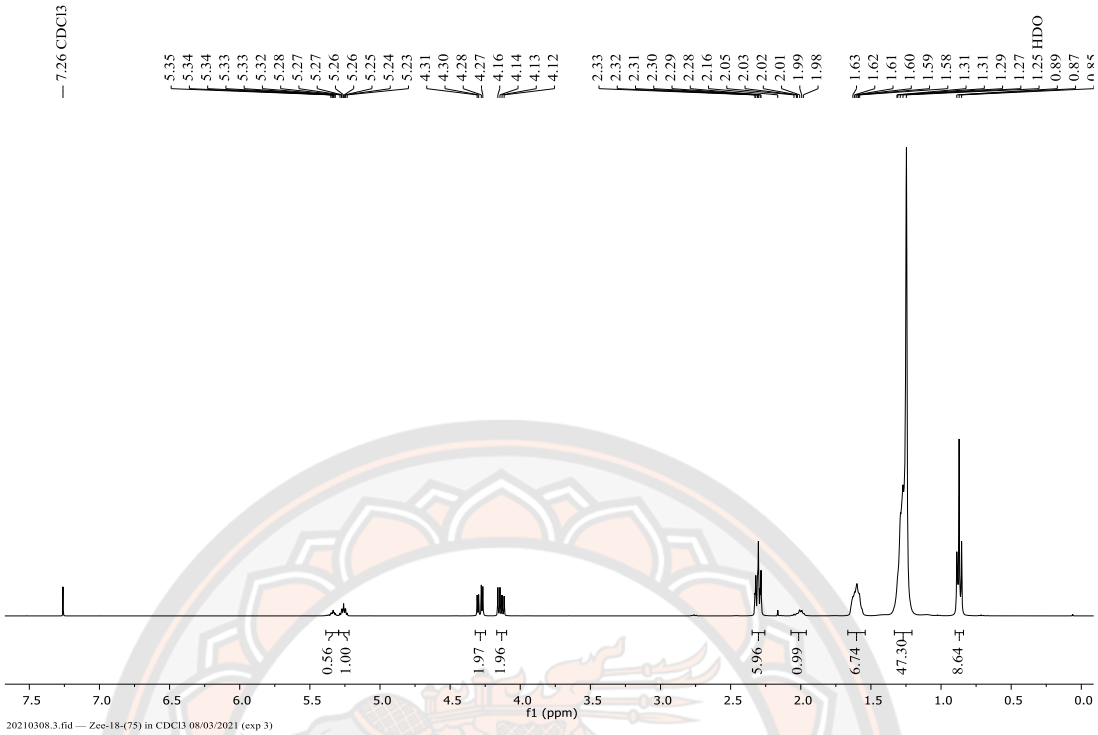


Figure 148 ^{1}H-NMR spectrum of the product of enzyme Lipozyme TL IM catalyzed interesterification of coconut oil at 75^{o}C

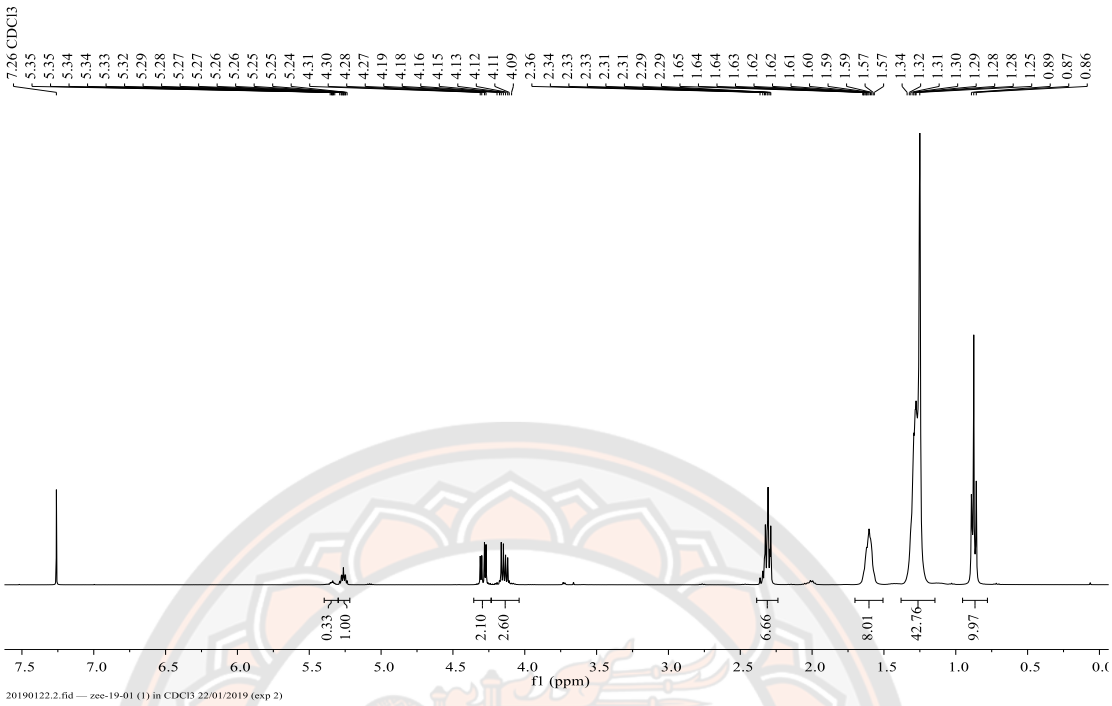


Figure 149 ^{1}H-NMR spectrum of the product of enzyme lipase B from *Candida antarctica* immobilized on acrylic resin catalyzed interesterification of coconut oil at 45^{o}C

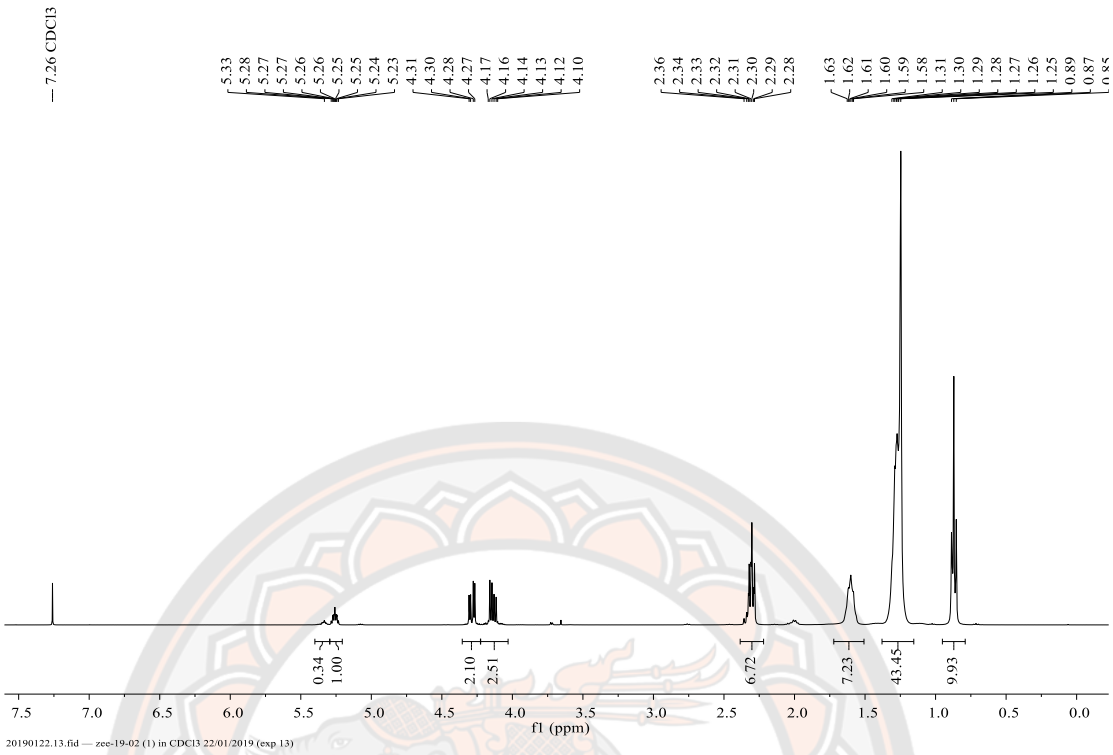


Figure 150 ^{1}H-NMR spectrum of the product of enzyme lipase B from *Candida antarctica* immobilized on acrylic resin catalyzed interesterification of coconut oil at 50^{o}C

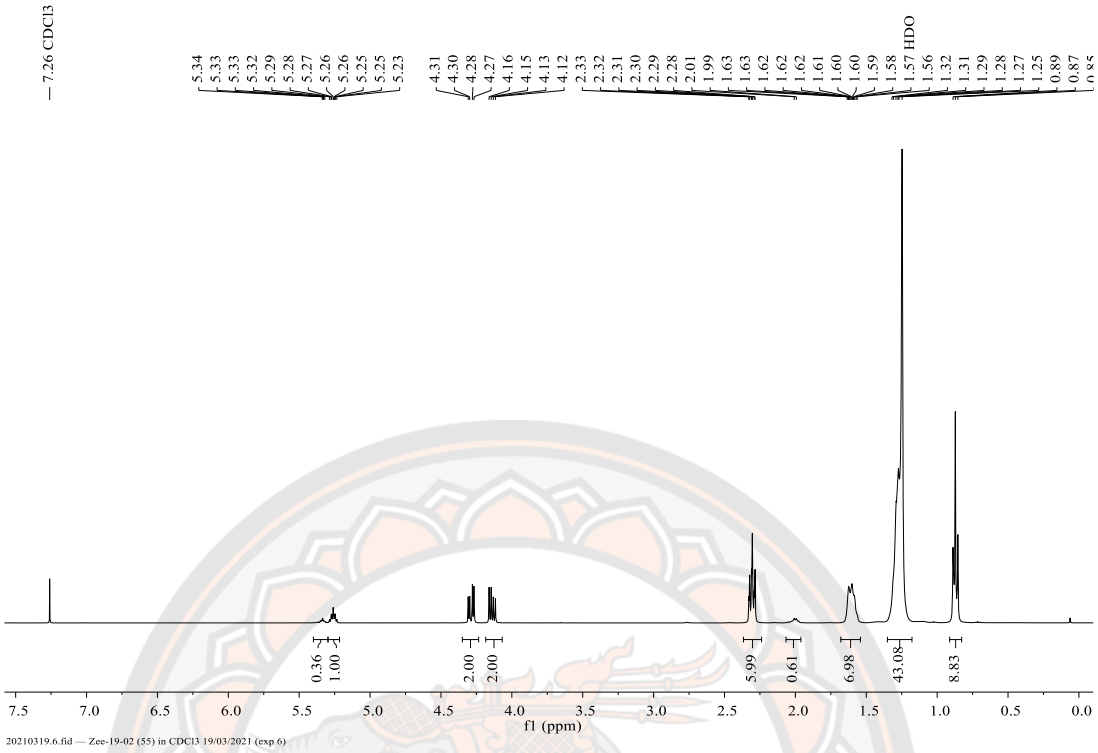


Figure 151 ^{1}H-NMR spectrum of the product of enzyme lipase B from *Candida antarctica* immobilized on acrylic resin catalyzed interesterification of coconut oil at 55^{o}C

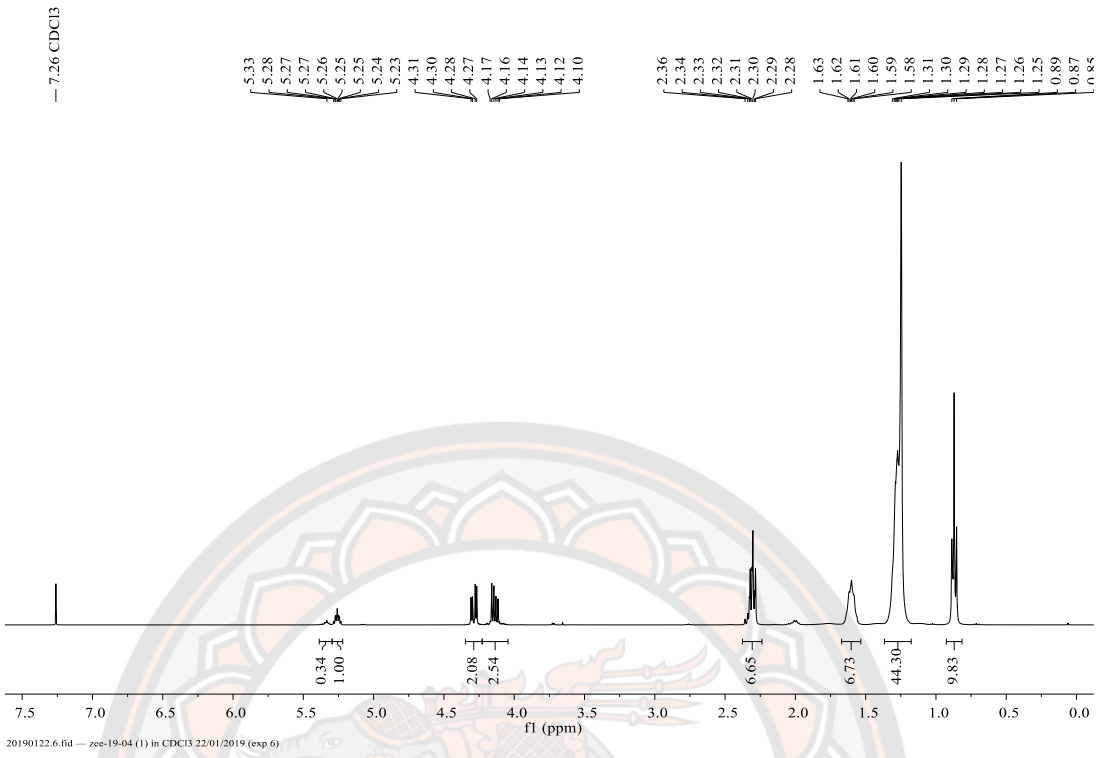


Figure 152 ^{1}H-NMR spectrum of the product of enzyme lipase B from *Candida antarctica* immobilized on acrylic resin catalyzed interesterification of coconut oil at 60ºC

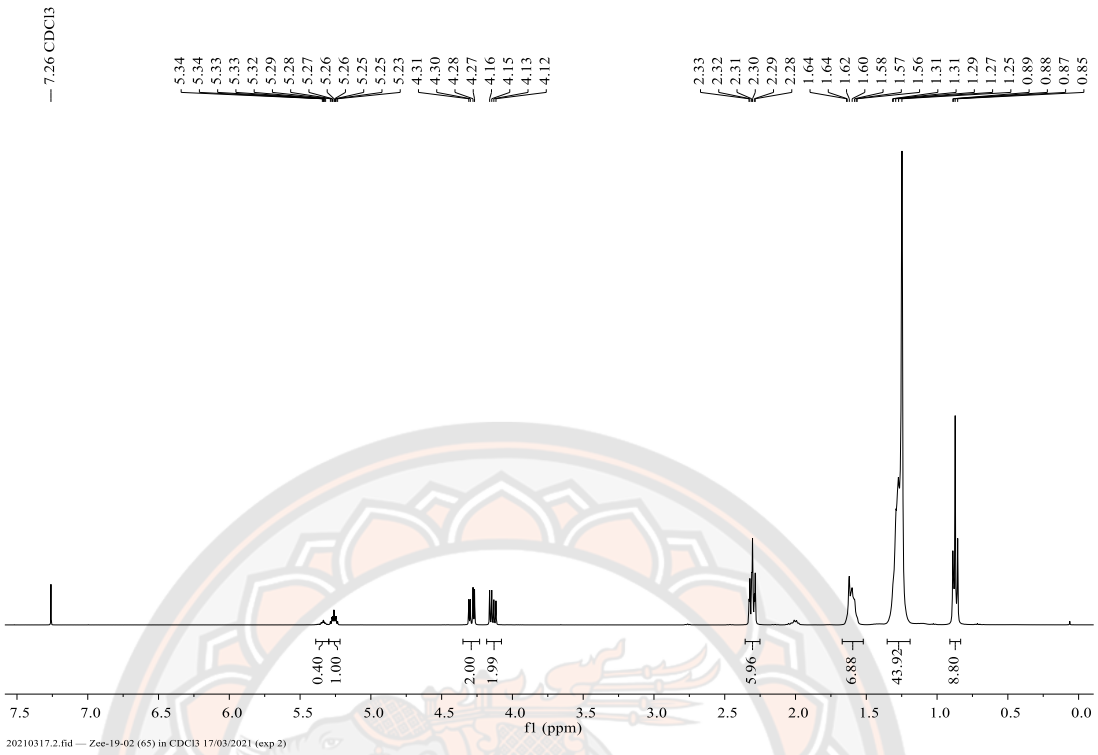


Figure 153 ^{1}H-NMR spectrum of the product of enzyme lipase B from *Candida antarctica* immobilized on acrylic resin catalyzed interesterification of coconut oil at 65^{o}C

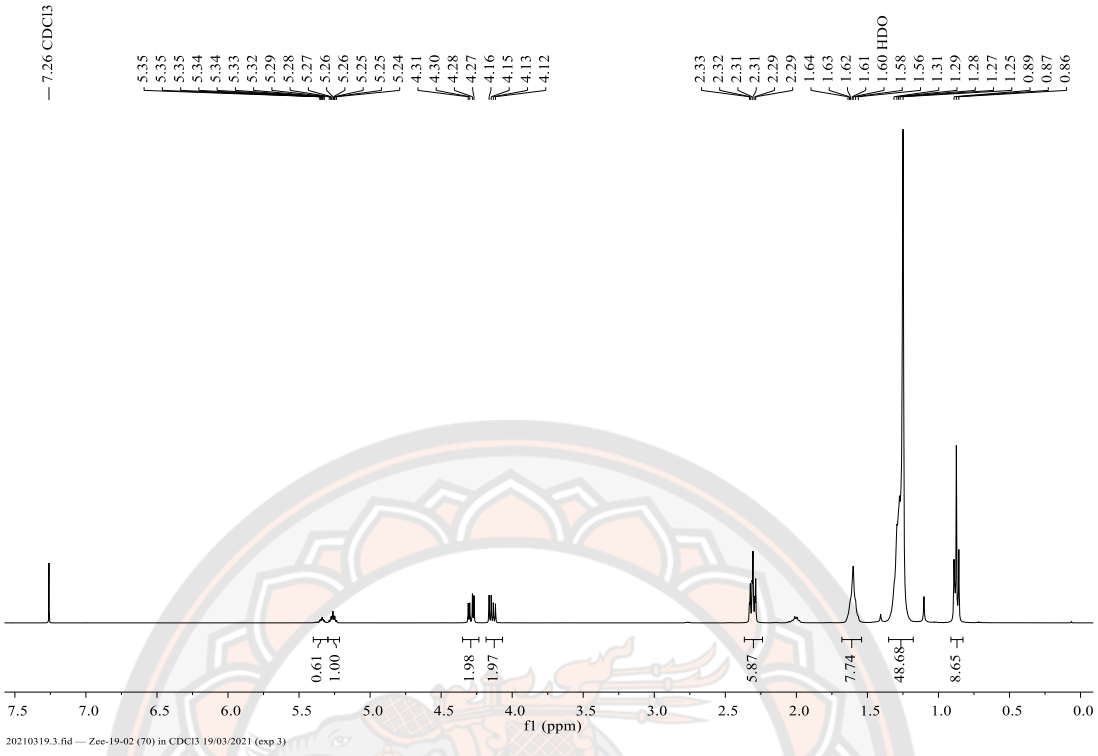


Figure 154 ^{1}H-NMR spectrum of the product of enzyme lipase B from *Candida antarctica* immobilized on acrylic resin catalyzed interesterification of coconut oil at 60^{o}C

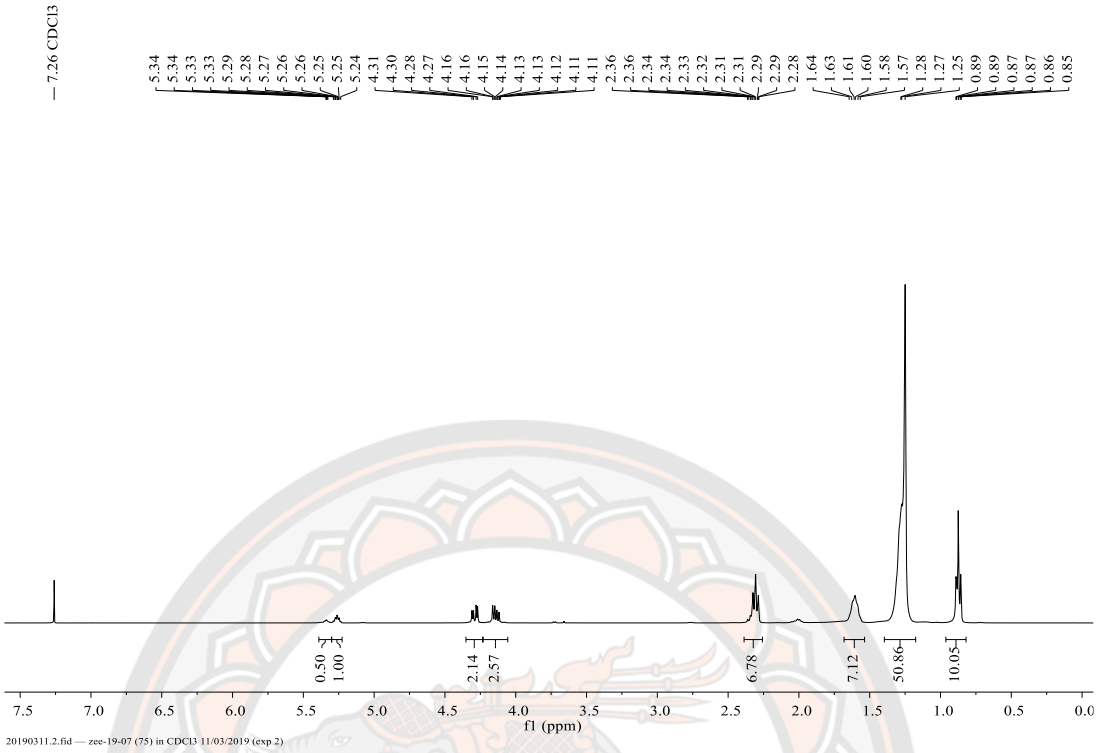


Figure 155 ^{1}H-NMR spectrum of the product of enzyme lipase B from *Candida antarctica* immobilized on acrylic resin catalyzed interesterification of coconut oil at 75^{o}C

Investigation of the potential for enzyme recycling

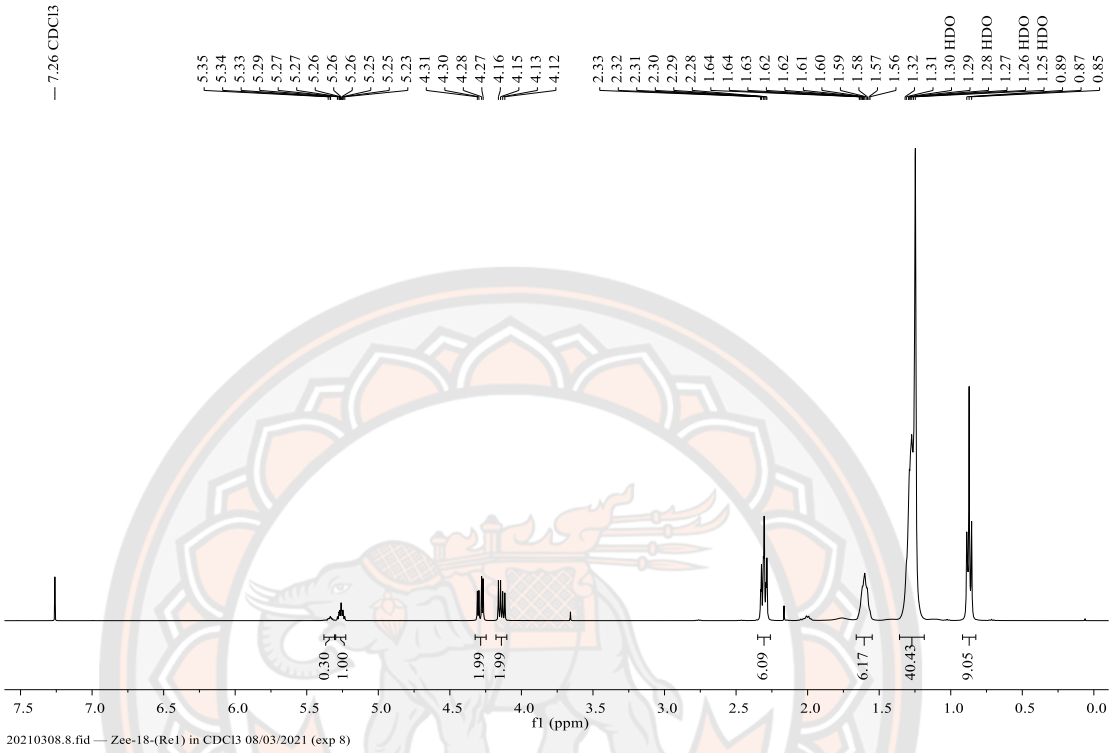


Figure 156 ^{1}H-NMR spectrum of the product of enzyme Lipozyme TL IM catalyzed interesterification of coconut oil by first-time recycling enzyme

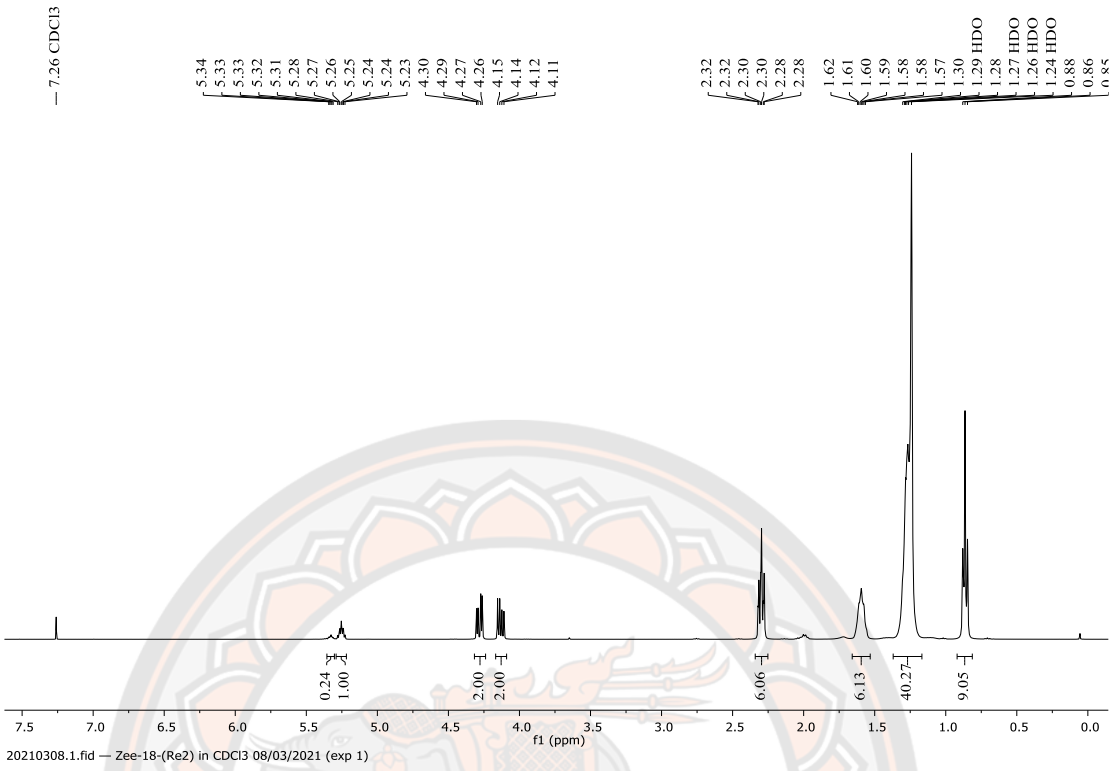


Figure 157 ^{1}H-NMR spectrum of the product of enzyme Lipozyme TL IM catalyzed interesterification of coconut oil by second-time recycling enzyme

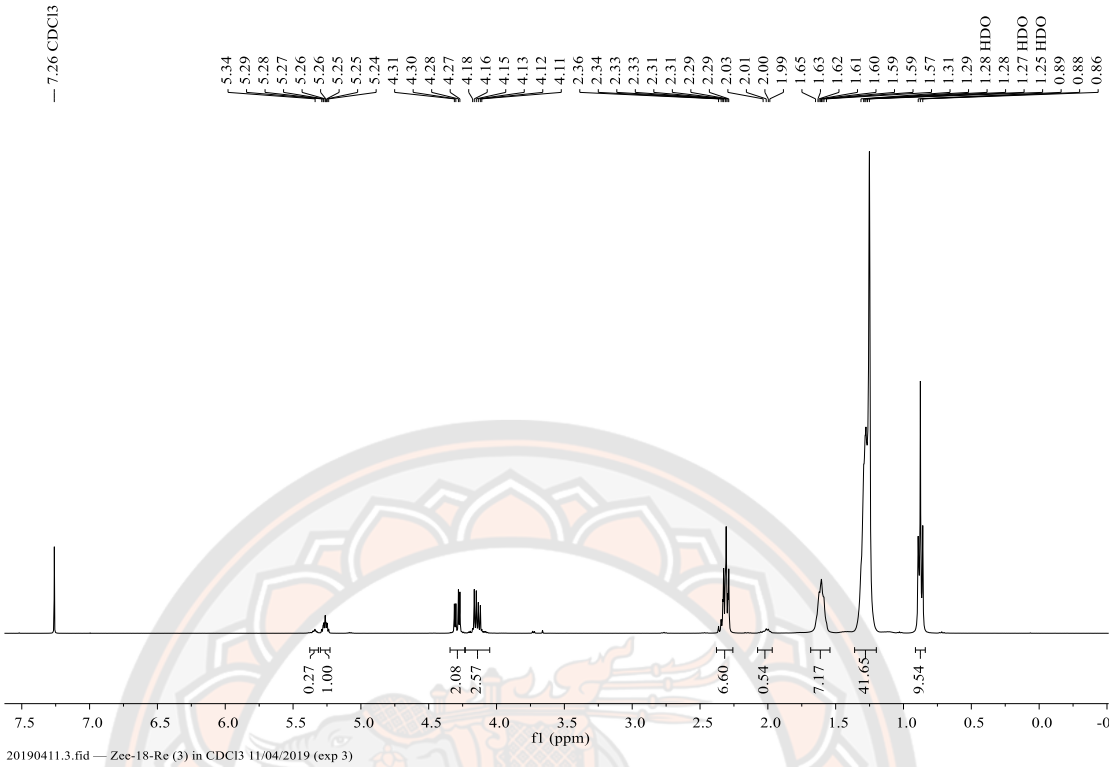


Figure 158 ^{1}H-NMR spectrum of the product of enzyme Lipozyme TL IM catalyzed interesterification of coconut oil by third-time recycling enzyme

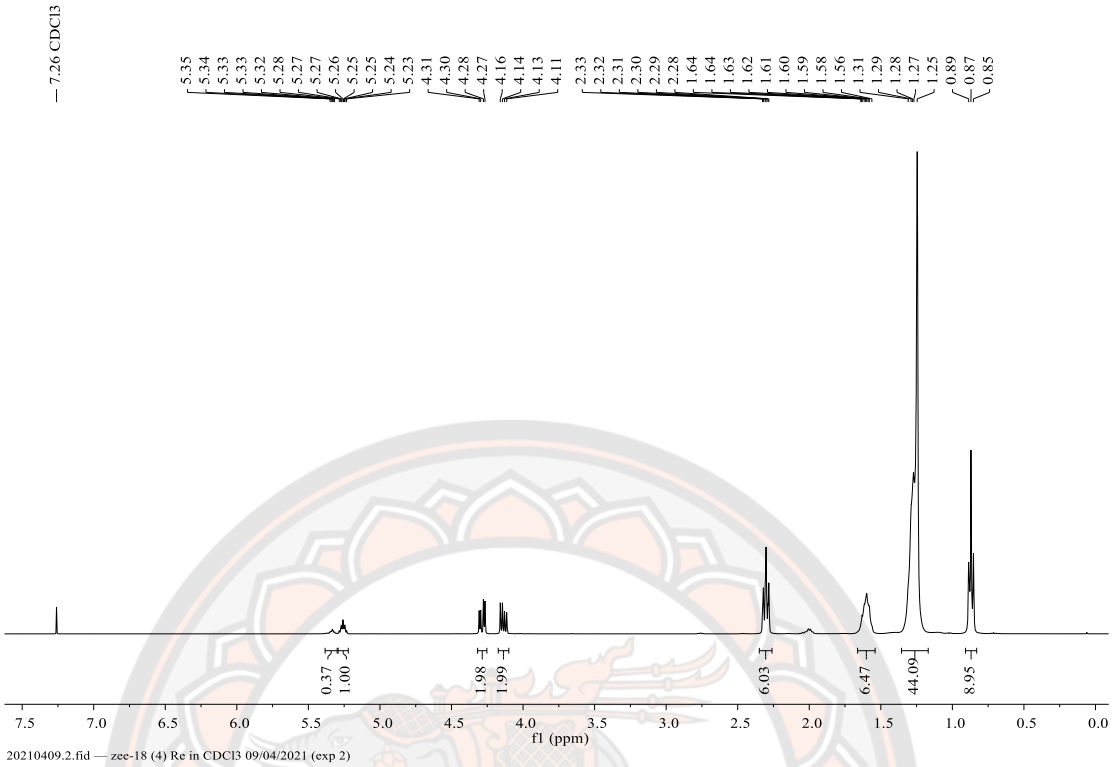


Figure 159 ^{1}H-NMR spectrum of the product of enzyme Lipozyme TL IM catalyzed interesterification of coconut oil by fourth-time recycling enzyme

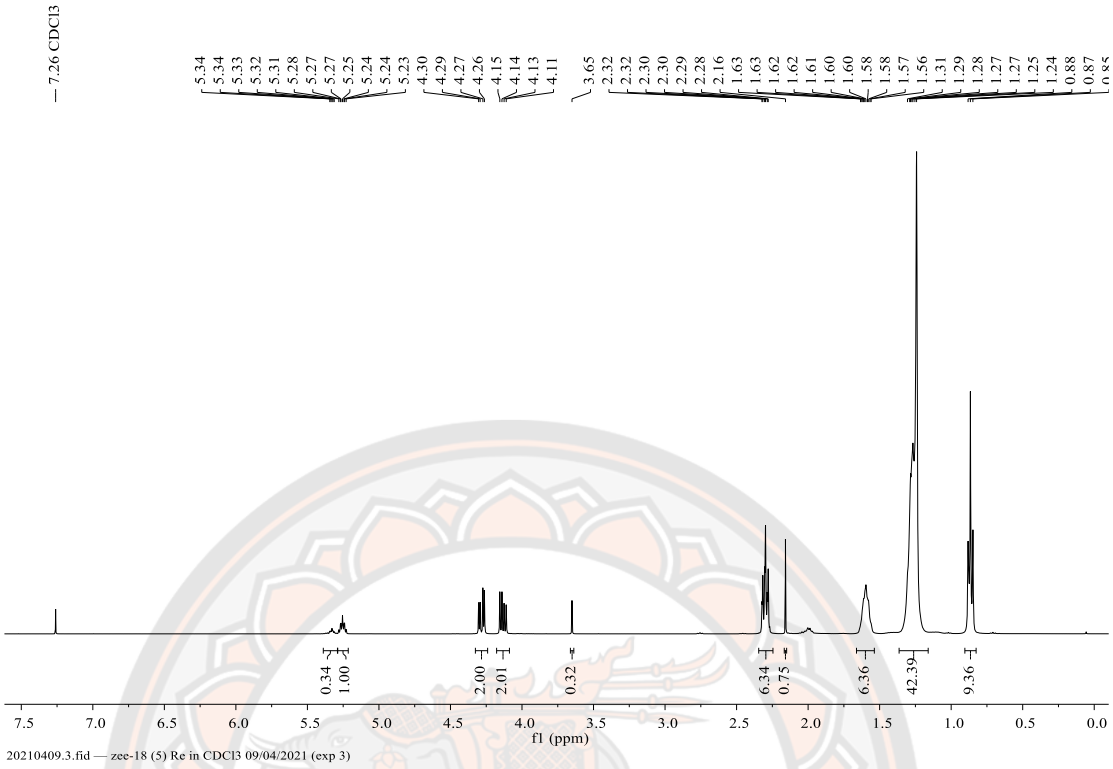


Figure 160 ^{1}H-NMR spectrum of the product of enzyme Lipozyme TL IM catalyzed interesterification of coconut oil by five-time recycling enzyme

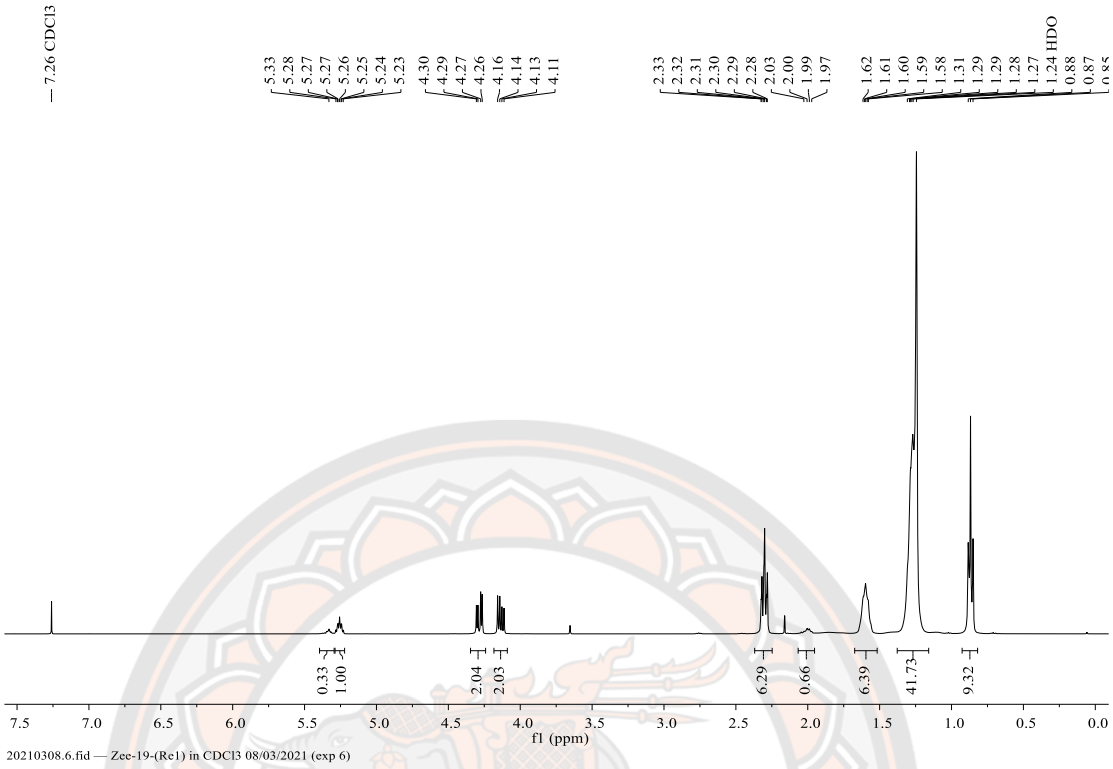


Figure 161 ^{1}H-NMR spectrum of the product of enzyme lipase B from *Candida antarctica* immobilized on acrylic resin catalyzed interesterification of coconut oil by first-time recycling enzyme

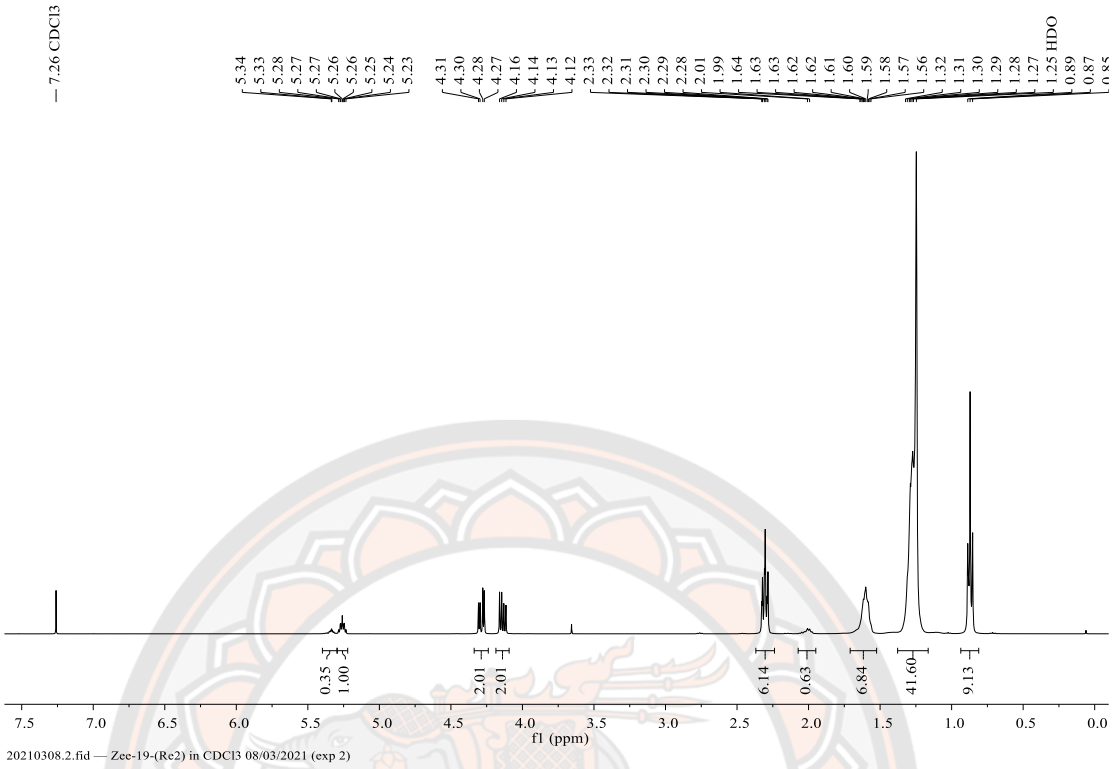


Figure 162 ^{1}H-NMR spectrum of the product of enzyme lipase B from *Candida antarctica* immobilized on acrylic resin catalyzed interesterification of coconut oil by second-time recycling enzyme

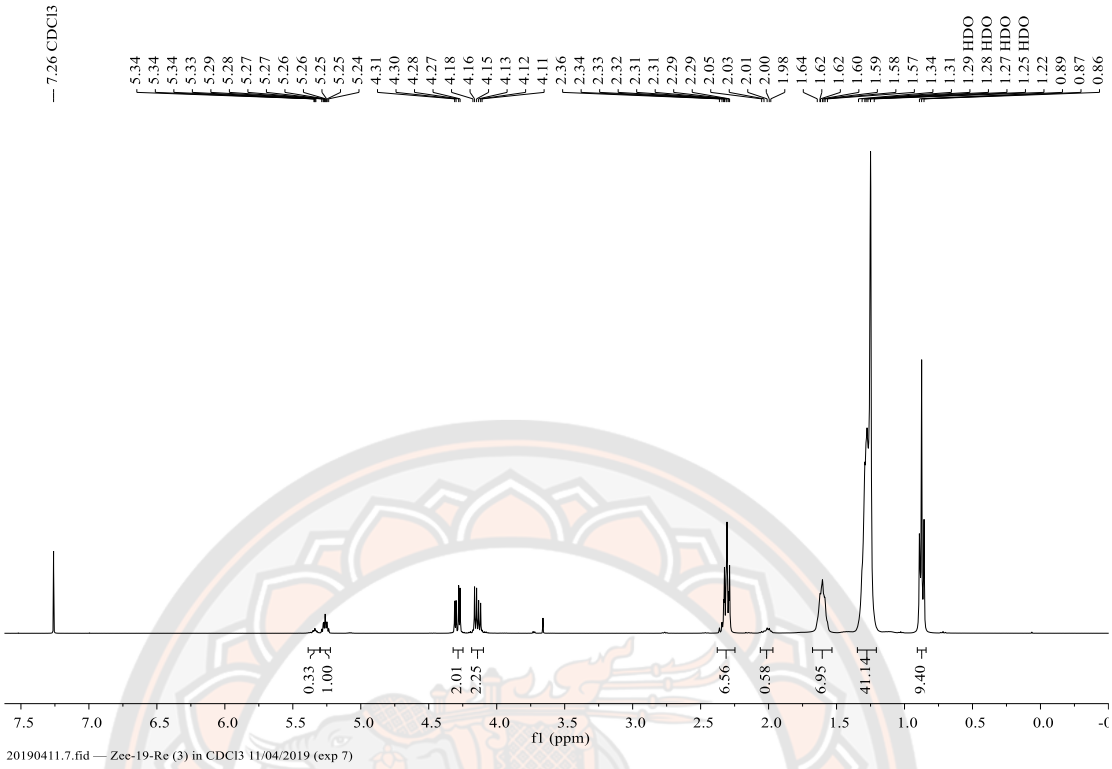


Figure 163 ^{1}H-NMR spectrum of the product of enzyme lipase B from *Candida antarctica* immobilized on acrylic resin catalyzed interesterification of coconut oil by third-time recycling enzyme

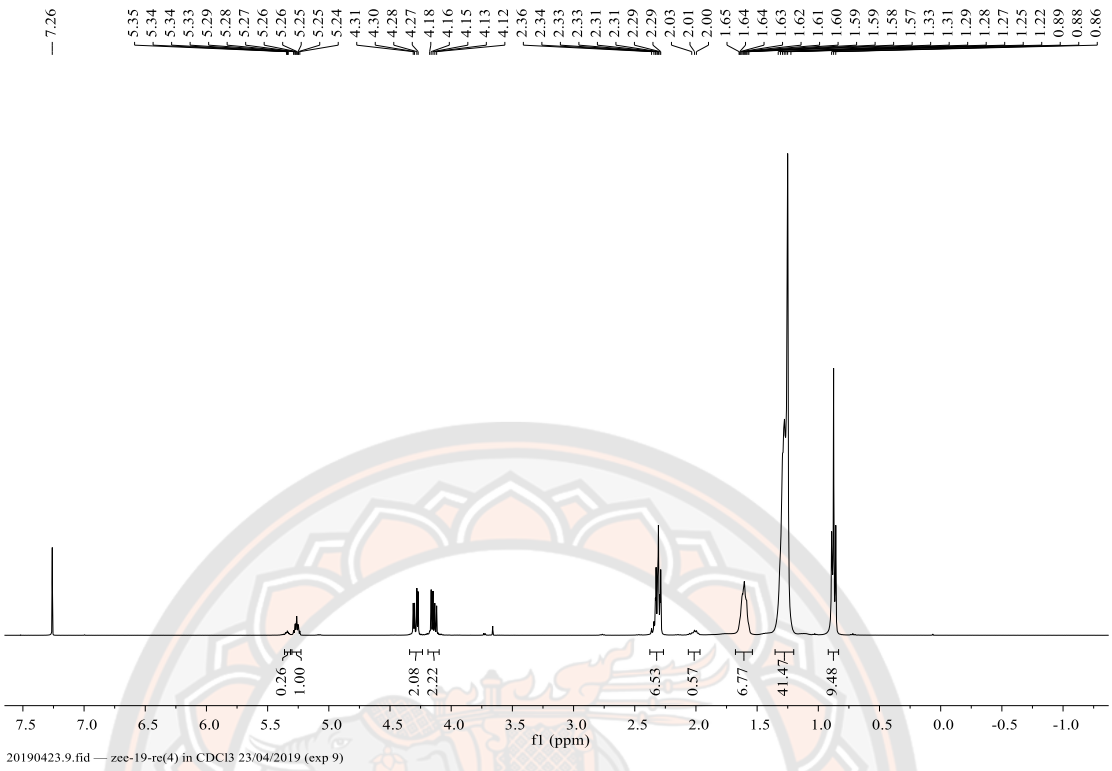


Figure 164 ^{1}H-NMR spectrum of the product of enzyme lipase B from *Candida antarctica* immobilized on acrylic resin catalyzed interesterification of coconut oil by fourth-time recycling enzyme

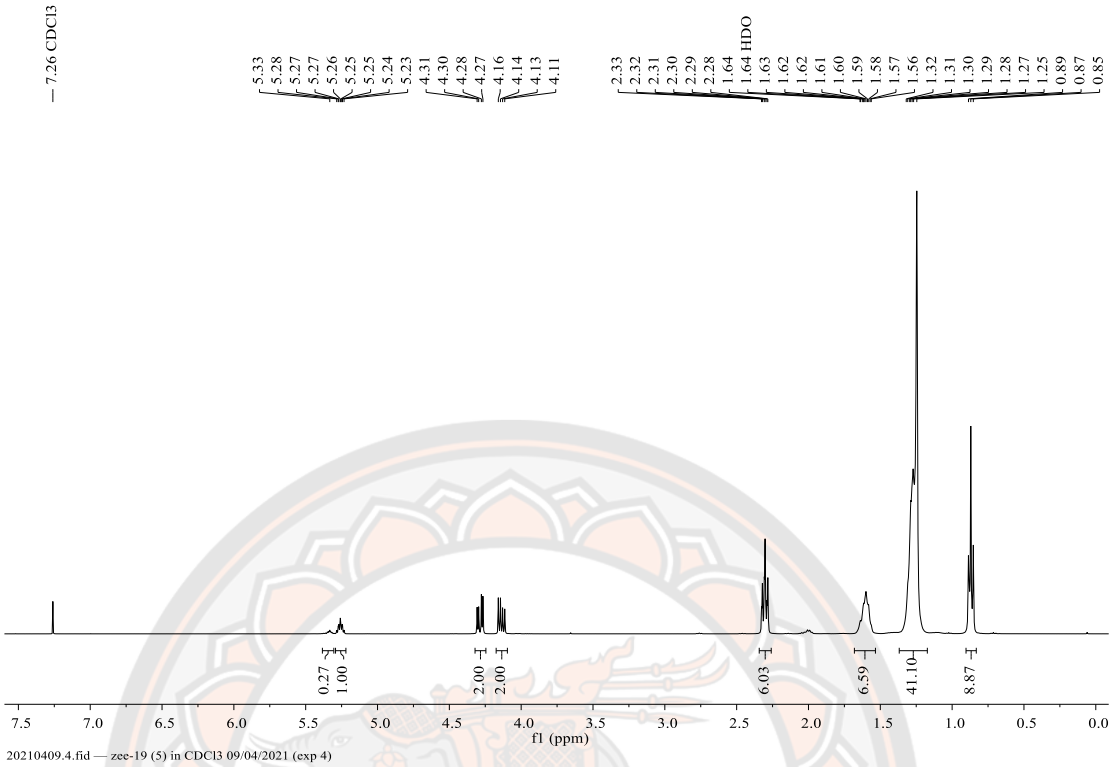


Figure 165 ^{1}H-NMR spectrum of the product of enzyme lipase B from *Candida antarctica* immobilized on acrylic resin catalyzed interesterification of coconut oil by five-time recycling enzyme

The appendix relates to Part II: Preparation of fatty acids from the hydrolysis of capsaicinoids

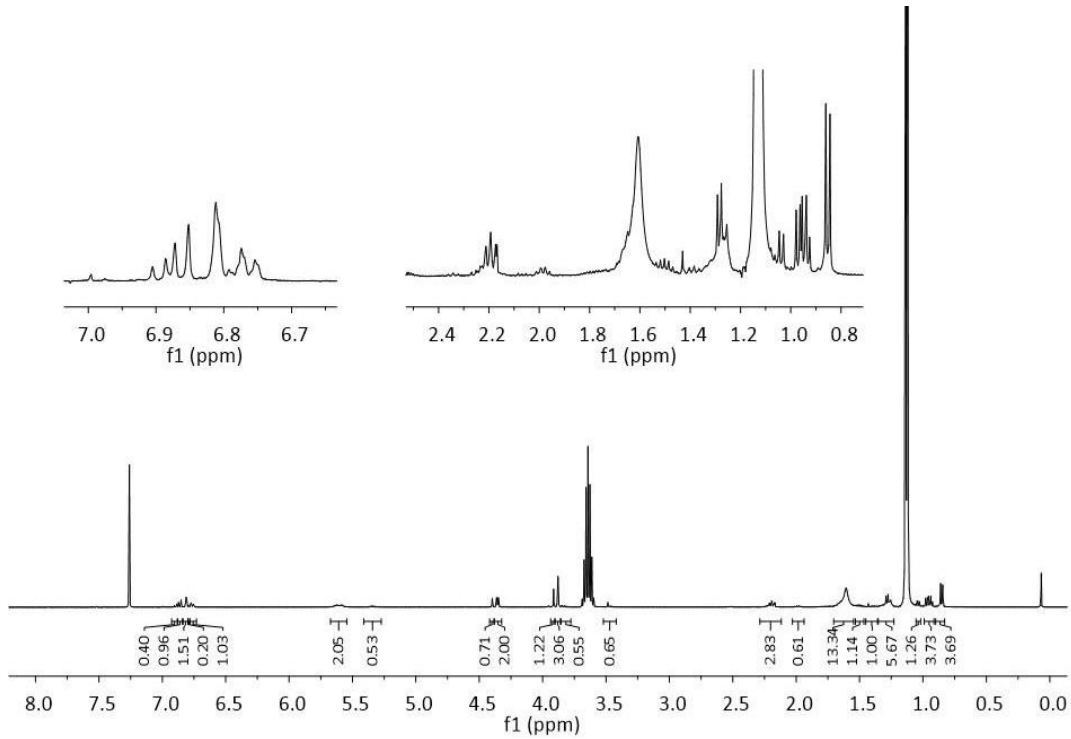


Figure 166 Example of ^{1}H-NMR spectrum from initial test of enzymatic hydrolysis reactions (lipase B from *Candida antarctica* immobilized on acrylic resin)

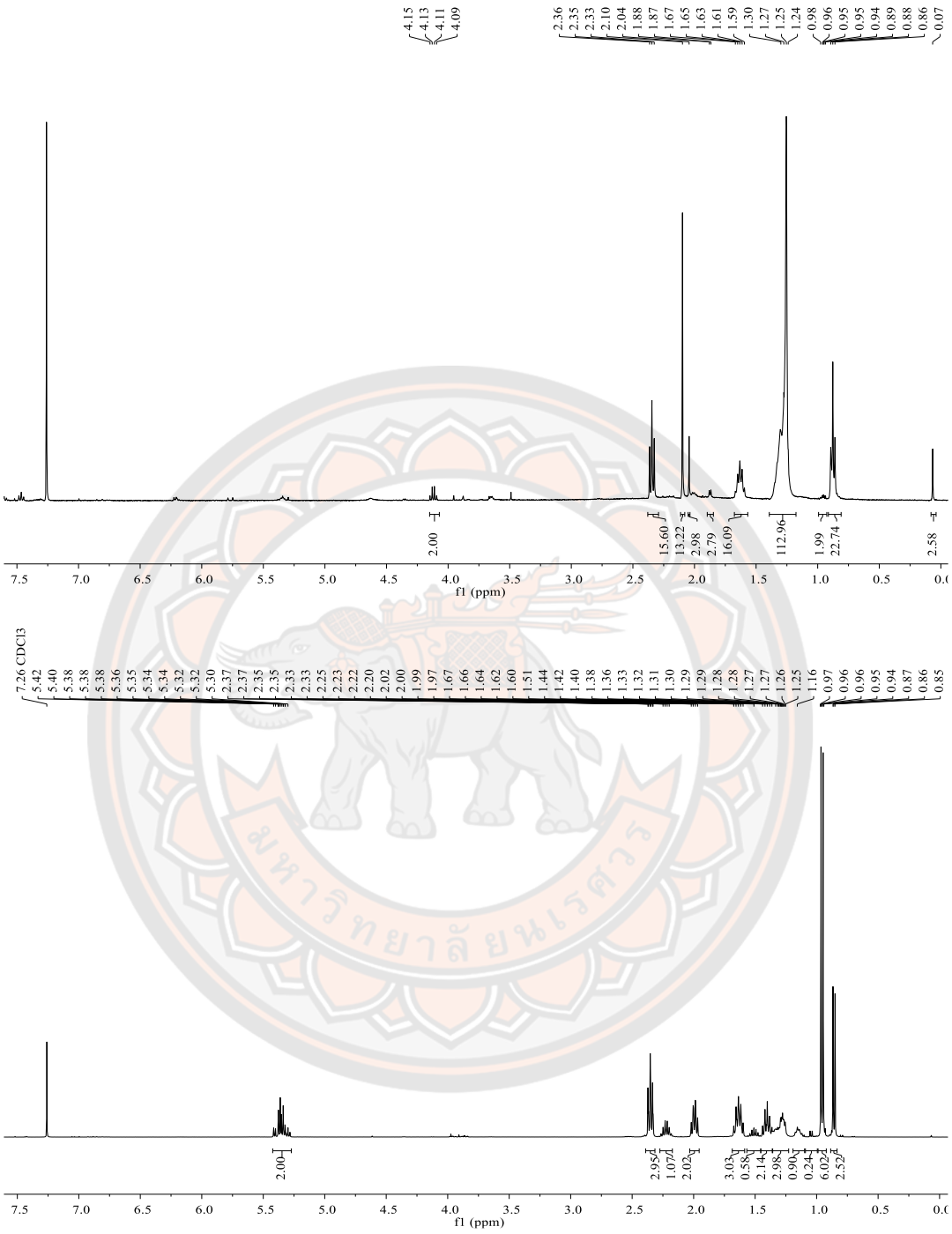


Figure 167 ^{1}H-NMR spectrum of the product after hydrolysis with the enzymes Lipozyme TL IM (above) and Novozyme 435 (below)

Investigation of the effect of enzyme loading for modification of coconut oil

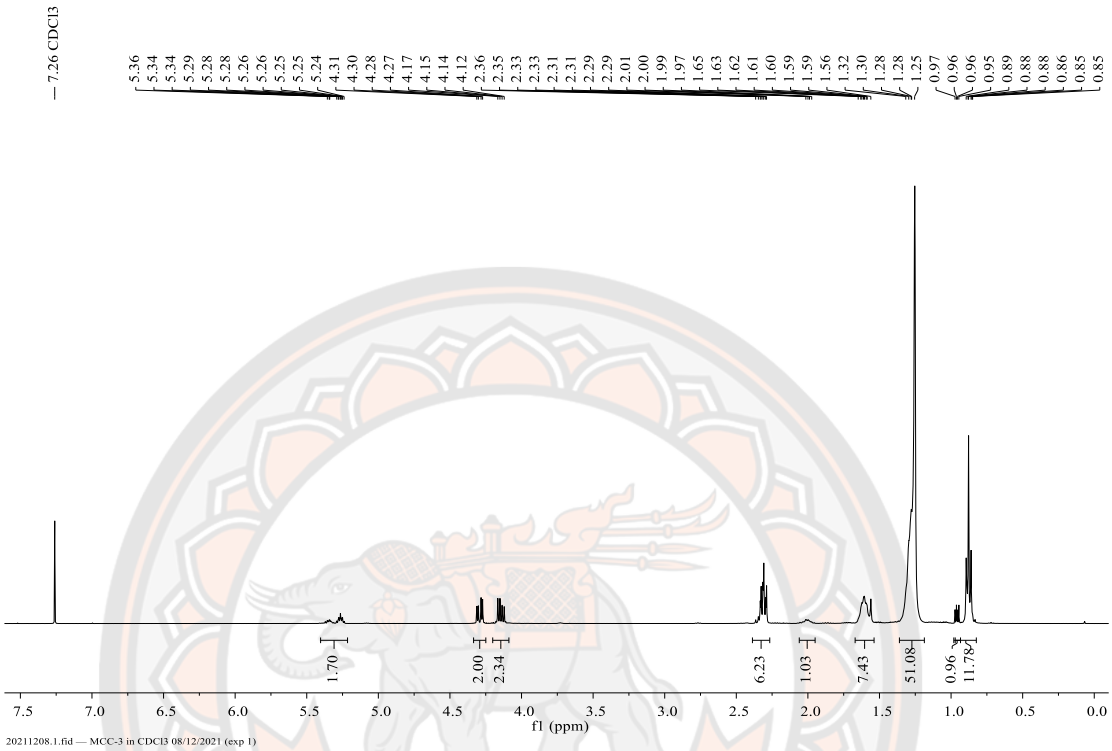


Figure 168 ^{1}H-NMR spectrum of product from modification of coconut oil with enzyme loading 1%

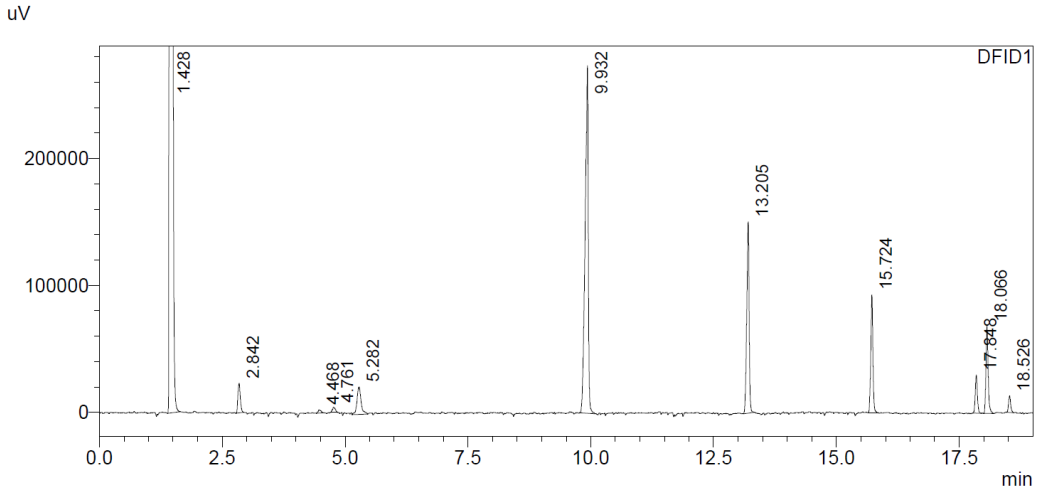


Figure 169 Gas chromatogram of product from modification of coconut oil with enzyme loading 1%

Table 36 Fatty acid composition of modification of coconut oil with enzyme loading 1% determined by gas chromatography

Fatty acid symbol	Fatty acid trivial name	Retention time (min)	Specifications (%)
C8	Caprylic acid	2.842	3.07
	8-methylnonanoic acid	4.468	0.40
	8-methyl-6-nonenoic acid	4.761	0.68
C10	Capric acid	5.282	4.84
C12	Lauric acid	9.932	47.26
C14	Myristic acid	13.205	19.60
C16:0	Palmitic acid	15.724	10.79
C18:2	Linoleic acid	17.848	3.50
C18:1	Oleic acid	18.066	8.32
C18:0	Stearic acid	18.526	1.54

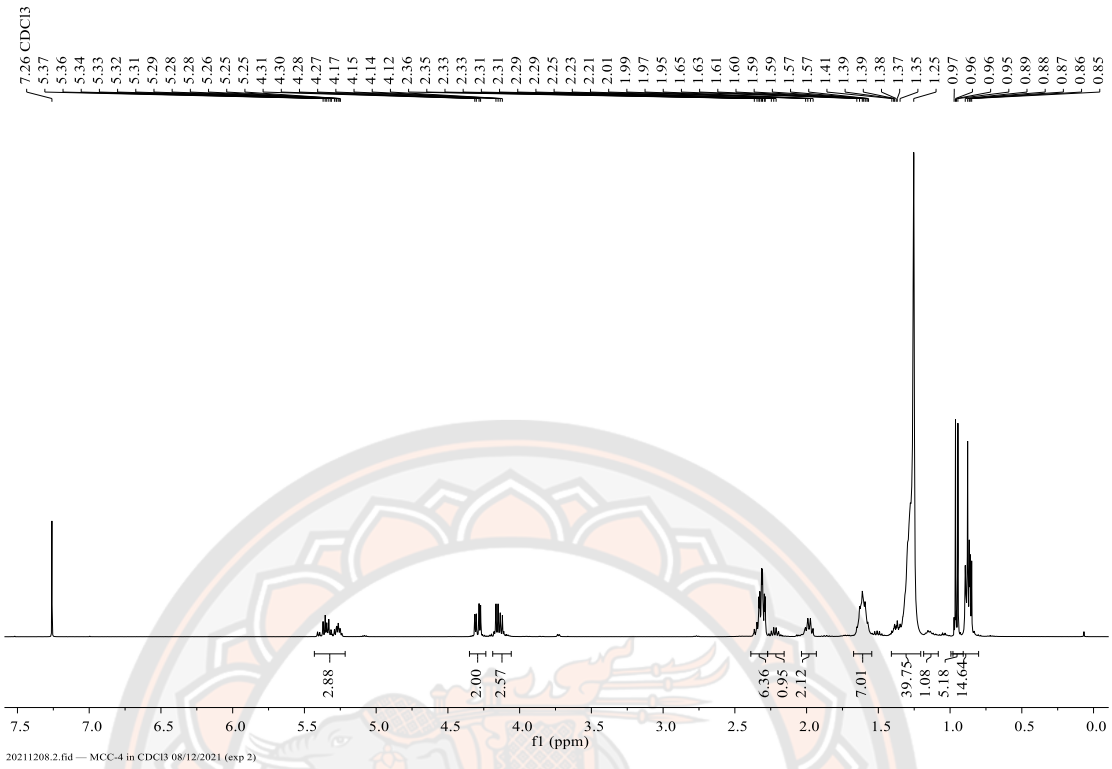


Figure 170 ^{1}H-NMR spectrum of product from modification of coconut oil with enzyme 5%

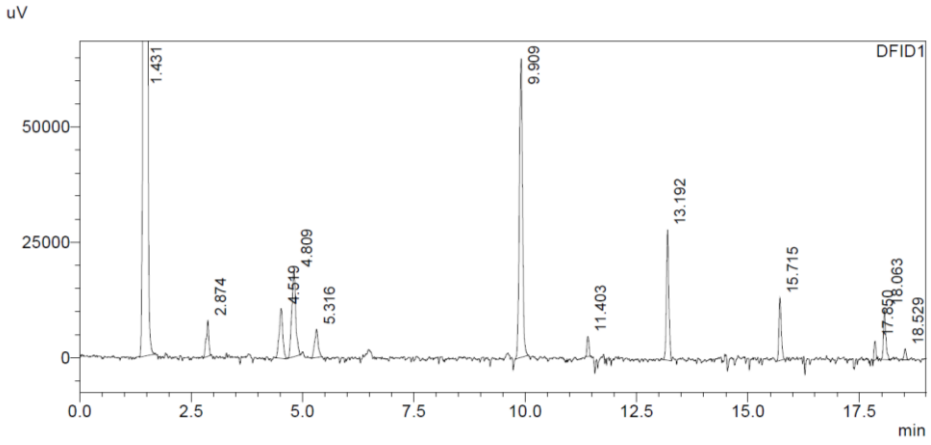


Figure 171 Gas chromatogram of product from modification of coconut oil with enzyme loading 5%

Table 37 Fatty acid composition of modification of coconut oil with enzyme loading enzyme 5% determined by gas chromatography

Fatty acid symbol	Fatty acid trivial name	Retention time (min)	Specifications (%)
C8	Caprylic acid	2.874	3.94
	8-methylnonanoic acid	4.519	7.56
	8-methyl-6-nonenoic acid	4.809	14.59
C10	Capric acid	5.316	4.30
C12	Lauric acid	9.909	40.78
	-	11.403	1.71
C14	Myristic acid	13.192	13.38
C16:0	Palmitic acid	15.715	6.26
C18:2	Linoleic acid	17.850	1.43
C18:1	Oleic acid	18.063	5.04
C18:0	Stearic acid	18.529	1.01

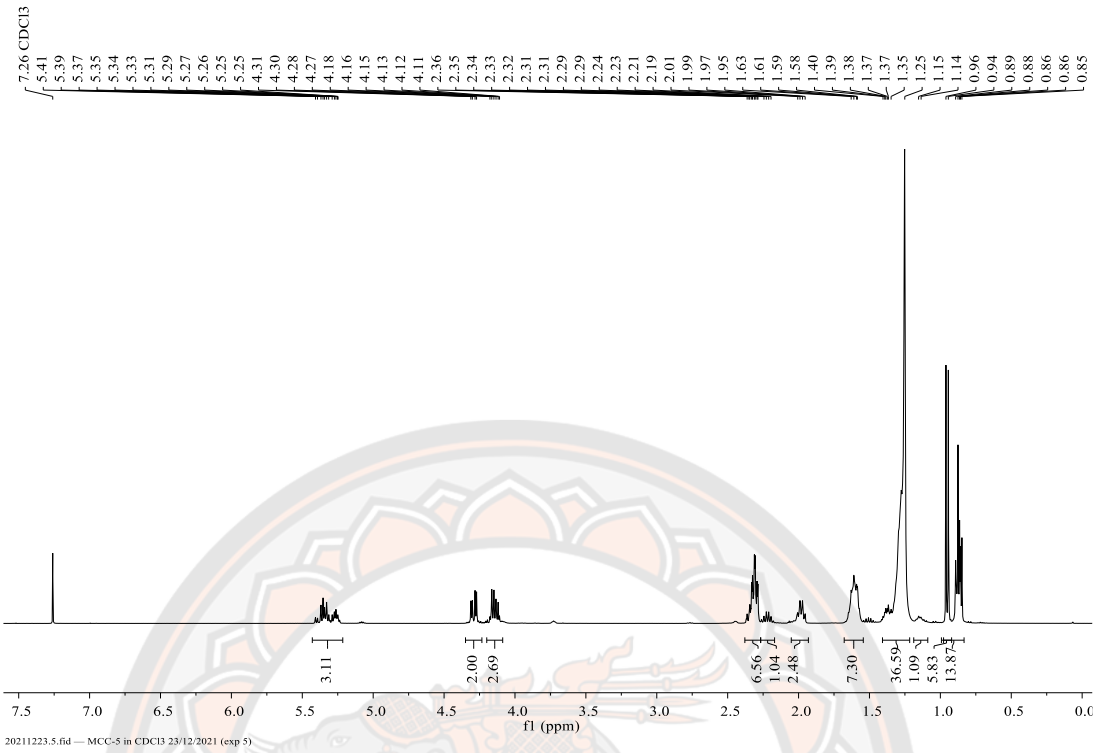


Figure 172 ^{1}H-NMR spectrum of product from modification of coconut oil with enzyme loading 7.5%

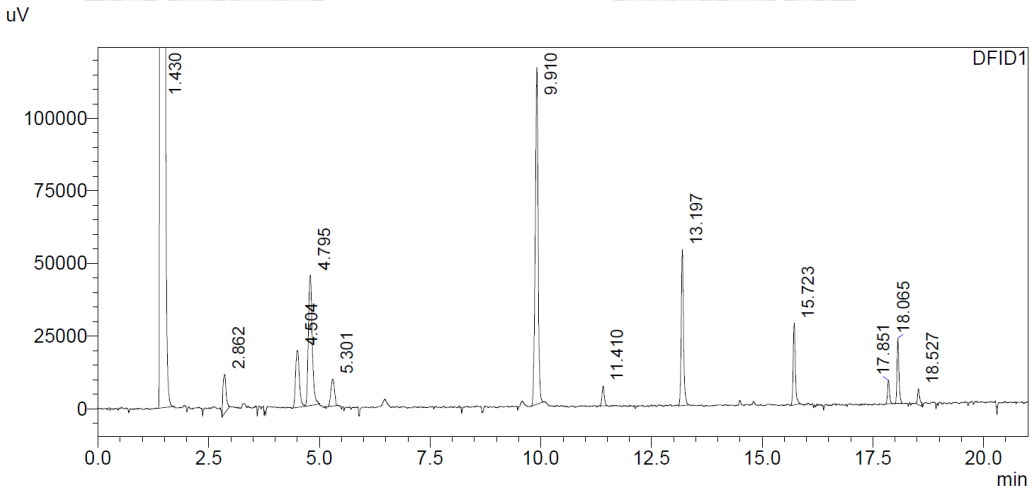


Figure 173 Gas chromatogram of product from modification of coconut oil with enzyme loading 7.5%

Table 38 Fatty acid composition of modification of coconut oil with enzyme loading enzyme 7.5% at 60ºC determined by gas chromatography

Fatty acid symbol	Fatty acid trivial name	Retention time (min)	Specifications (%)
C8	Caprylic acid	2.862	4.52
	8-methylnonanoic acid	4.504	8.05
	8-methyl-6-nonenoic acid	4.795	18.71
C10	Capric acid	5.301	3.62
C12	Lauric acid	9.910	35.61
	-	11.410	1.67
C14	Myristic acid	13.197	13.40
C16:0	Palmitic acid	15.723	6.58
C18:2	Linoleic acid	17.851	1.73
C18:1	Oleic acid	18.065	4.76
C18:0	Stearic acid	18.527	1.35

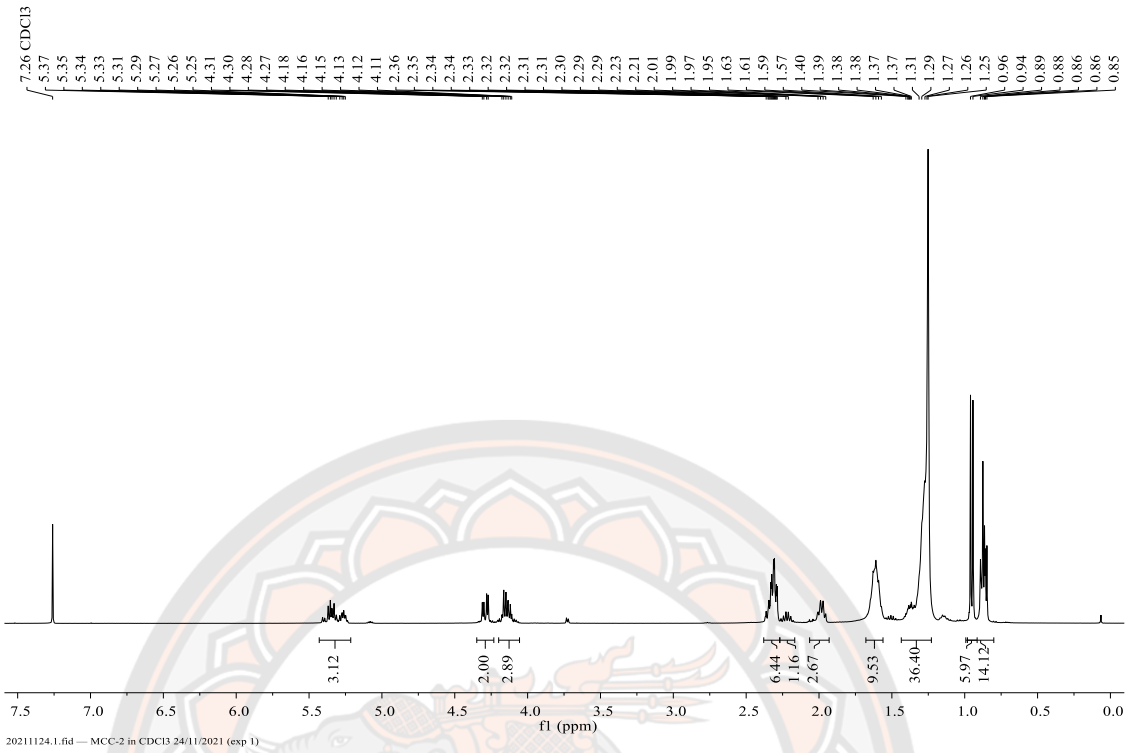


Figure 174 ^{1}H-NMR spectrum of product from initial modification of coconut oil with Lipozyme TL IM 10% and fatty acid from the hydrolysis of capsaicin at 60^{o}C

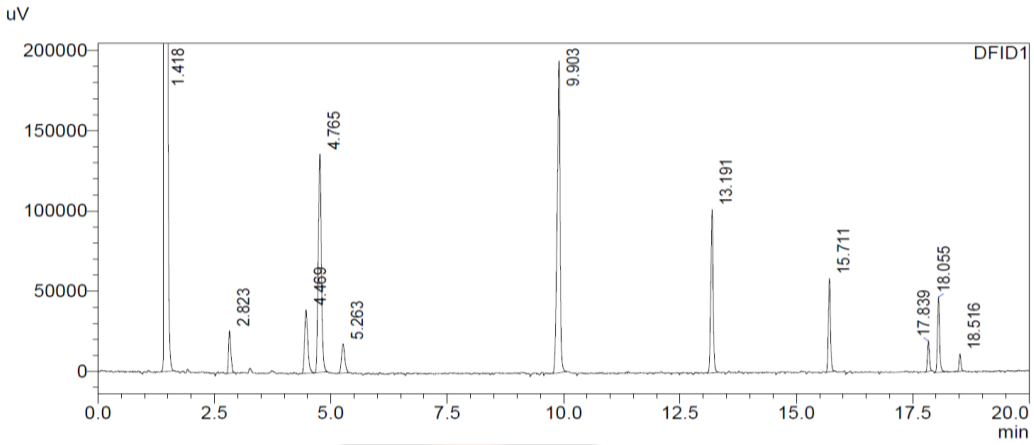


Figure 175 Gas chromatogram of product from initial modification of coconut oil with Lipozyme TL IM 10% and fatty acid from the hydrolysis of capsaicin at 60°C

Table 39 Fatty acid composition of product from initial modification of coconut oil with Lipozyme TL IM 10% and fatty acid from the hydrolysis of capsaicin at 60°C determined by gas chromatography

Fatty acid symbol	Fatty acid trivial name	Retention time (min)	Specifications (%)
C8	Caprylic acid	2.823	3.50
	8-methylnonanoic acid	4.469	7.11
	8-methyl-6-nonenoic acid	4.765	24.06
C10	Capric acid	5.263	3.37
C12	Lauric acid	9.903	32.80
C14	Myristic acid	13.191	13.37
C16:0	Palmitic acid	15.711	6.95
C18:2	Linoleic acid	17.839	2.07
C18:1	Oleic acid	18.055	5.62
C18:0	Stearic acid	18.516	1.15

APPENDIX B (RESULTS OF ANIMAL TESTS)

Animal Testing

The modified virgin coconut oil rich in medium chain fatty acids (MCFAs), which has been synthesized as described above has been utilized for testing of its biological properties in mice (female C57BL/6 mice) by our collaborators from the Department of Physiology, Faculty of Medical Science, Naresuan University. The goal of this work was to investigate the ability of various type of oils to counter the effects of high fat diet by evaluating their effects on food intake, body weight (BW), and lipid profiles in blood and liver.

The modified coconut oil produced in this work was labeled low lauric acid (LLA) oil and was compared against virgin coconut oil (VCO) and medium-chain triglyceride (MCT) oil. VCO contains saturated fatty acids and approximately 50% of these is lauric acid (C12), for which its status as an MCFA is disputed. On the other hand, MCT oils are synthetic and are composed purely of C8 and C10 fatty acids (Table 22). In this experiment the C57BL/6 mice were split into six groups, which were fed the following six diets: 1) control (normal diet), 2) high-fat diet (HF), 3) high-fat diet (HF) + virgin coconut oil (VCO), 4) high-fat diet (HF) + medium chain triglycerides (MCT), 5) high-fat diet (HF) + low lauric acid coconut oil (LLA), and 6) normal diet + LLA (C+LLA), for three months.

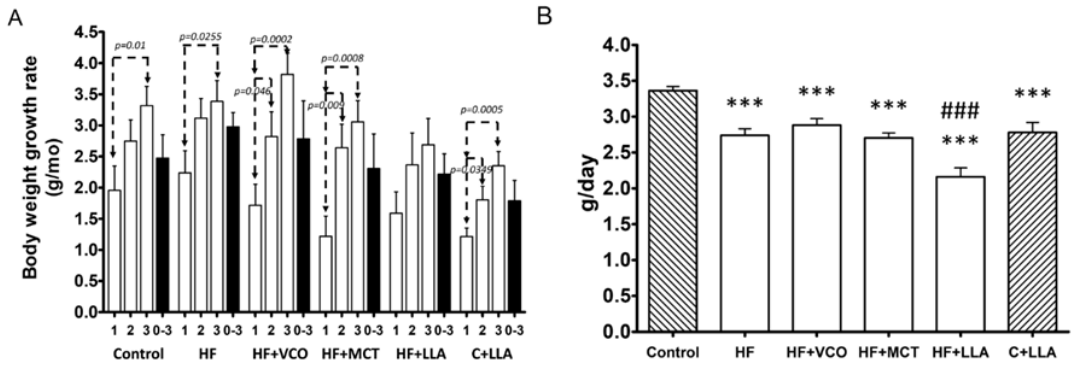


Figure 176 (A) Body weight gain in different treatment groups. The grey bars represent averaged values for the 3-month period for each treatment. The error bars are the standard error of the mean (SEM), and the P values are for unpaired, two-tailed comparisons denoted by the dashed lines. (B) Food intake averaged over 12 weeks. n =10-16 mice. Control, normal diet; HF, high-fat diet; HF +VCO, high-fat diet + virgin coconut oil; HF+MCT, high-fat diet + medium chain triglyceride; HF+LLA, high-fat diet + low lauric acid coconut oil; C + LLA, normal diet+ low lauric acid coconut oil. Values are means ± SEM (n =10-16). *** P < 0.001 vs. control. ### P < 0.001 vs. high fat diet

The graphs in Appendix B Figure 176 show effects of the diets and treatments in terms of body weight gain and food intake. Figure Appendix B Figure 176a shows that neither the high fat diet alone or in combination with VCO or MCT supplementation resulted in increased body weight gains in comparison to the control diet group. On the other hand, body weight gain was lower in both groups, fed normal and HF diet, which received the LLA oil. Appendix B Figure 176b then shows that mice in all groups fed high fat diet had lower food intake, which may be due to the fact that the high-fat diet has higher caloric density or lower palatability leading to a lower food intake. This may also be the reason for the mice in these groups not showing weight gain exceeding that of the weight gain in the control group. Mice fed the HF diet exhibited no difference in food intake compared to the group fed the HF diet with VCO or MCT. The group fed the HF+LLA diet, had lower food intake than the control

and the HF diet group. These results show that LLA can control body weight gain and by reducing food intake in the animals.

Appendix B Figure 177 shows the results observed in terms of plasma lipid profiles for the different treatment groups. Comparison of total cholesterol levels (TC) between the various treatment groups can be seen in Appendix B Figure 177a. Increased TC level, in comparison to the control group, was observed in all animal groups receiving the HF diet. The TC level in the animal group receiving the C+LLA regiment was also higher than in the control group but also lower than in the HF fed groups. Appendix B Figure 177b shows the results of the investigation of blood triglyceride levels (TG). A decrease in TG levels compared to the control group was observed for the HF treatment. The HF+VCO and HF+MCT groups did not show significant differences in their TG levels in comparison to the control group. The TG levels in the HF+LLA-fed mice and C+LLA-fed mice were higher than in the HF group but were not significantly different from the control group. The results for the HDL blood profiles can be seen in Appendix B Figure 177c. The HF and HF+VCO treatments did not result in any changes in the HDL levels, while the HF+MCT treatment led to a its decrease. On the other hand, both C+LLA and HF+LLA treatments led to increased LDL levels in comparison to both the control ant the HF groups. The effects of the various treatments on blood LDL can be seen in Appendix B Figure 177d. The HF group, as well as all of the other groups where the mice were on high fat diet, show increased LDL in comparison to the control group. The C+LLA group shows LDL levels comparable to the control group and significantly lower than in the HF group.

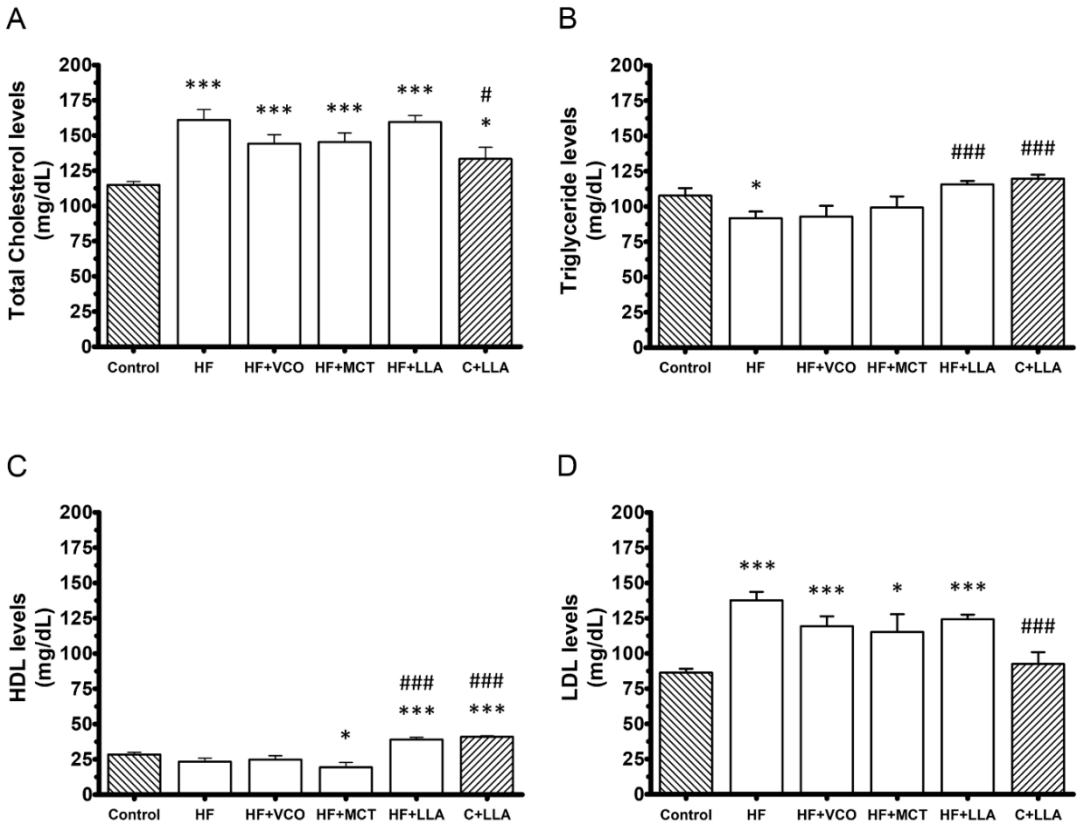


Figure 177 Plasma lipid profiles at 3 months. Control, normal diet; HF, high-fat diet; HF +VCO, high-fat diet + virgin coconut oil; HF+MCT, high-fat diet + medium chain triglyceride; HF+LLA, high-fat diet + low lauric acid coconut oil; C + LLA, normal diet+ low lauric acid coconut oil. HDL, high density lipoprotein; LDL, low density lipoprotein. Values are means ± SEM (n =10-16). *P <0.05, ***P < 0.001 vs. control. #P <0.05, ###P < 0.001 vs. high fat diet

The hepatic lipid profile was investigated in addition to the blood lipid profile and the results are shown in Appendix B Figure 178. Appendix B Figure 178a shows the liver TC content. It can be seen that the HF diet resulted in increased liver TC relative to the control group. This was efficiently countered by both the VCO and MCT treatments, which resulted in decreases in hepatic TC relative to HF group. This is especially true in the HF+MCT group where the liver TC level is not significantly different from the control group. The HF+LLA group exhibits liver TC levels, which

are higher than the control and comparable to the HF group. Finally, the effects of the LLA oil in the C+LLA group led to liver TC levels lower than in both the HF and control groups. Appendix B Figure 178b shows the liver triglyceride levels. These were not significantly affected by the HF treatment relative to the control group. A significant decrease in the triglyceride levels in comparison to the control group is observed for the HF+VCO treatment. The triglyceride levels observed in the HF+LLA group appear higher than in the control group, but the difference is not significant. The hepatic HDL levels for the different treatment groups can be seen in Appendix B Figure 178c. Only the HF+MCT group shows significantly increased level of hepatic HDL in comparison to the control group. The changes in the other groups are not significant. Finally, Figure 64d shows the effects of the different treatments on hepatic LDL levels. The HF treatment has led to significant increase in the liver LDL content, which was effectively countered by the treatments with VCO and MCT. On the other hand, the group receiving the HF+LLA treatment also showed increased liver LDL levels. Finally, addition of LLA oil to the control diet resulted in decrease of liver LDL levels in comparison to the control group.

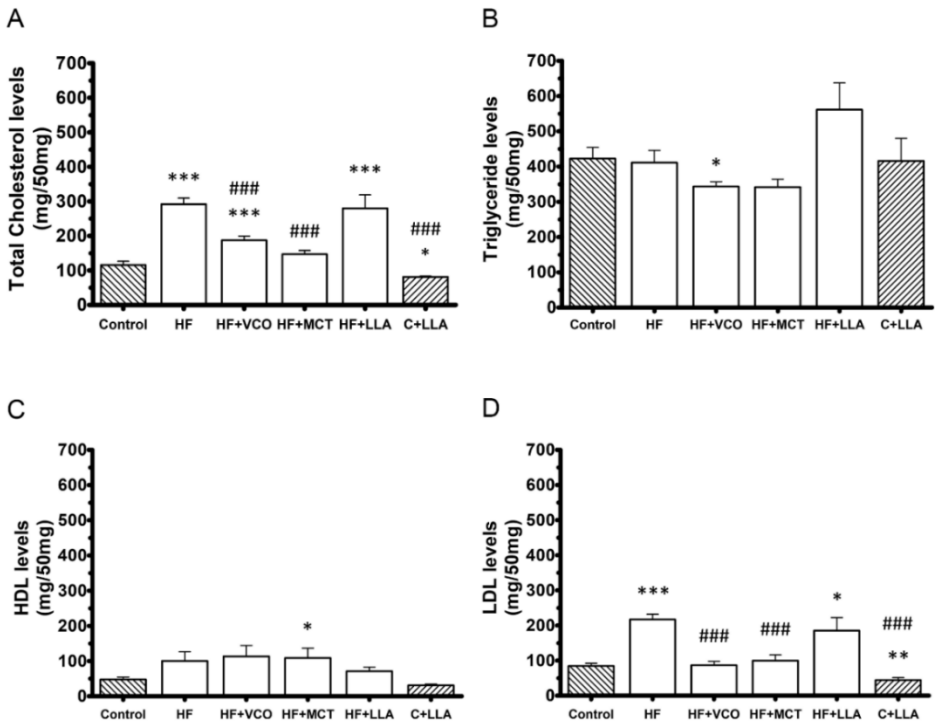


Figure 178 Hepatic lipid profiles at 3 months. Control, normal diet; HF, high-fat diet; HF +VCO, high-fat diet + virgin coconut oil; HF+MCT, high-fat diet + medium chain triglyceride; HF+LLA, high-fat diet + low lauric acid coconut oil; C + LLA, normal diet+ low lauric acid coconut oil. HDL, high density lipoprotein; LDL, low density lipoprotein. Values are means ± SEM (n =10-16). *P < 0.05, ***P < 0.001 vs. control. ###P < 0.001 vs. high fat diet

Furthermore, the results of liver sections stained by oil red O and photographed at 20X magnification by light microscopy to detect lipid accumulation in the liver tissue are shown in Appendix B Figure 179. The fat droplets were small with sparse staining in the control and C+LLA groups, but were large and intensely stained in the HF group (Appendix BFigure 179 A). The HF+VCO, HF+MCT, and HF+LLA groups displayed less strong staining and smaller-sized fat droplets than the HF mice (Appendix B Figure 179A). Fat accumulation in the liver was greatest in the HF group, and this was reduced by all treatments, of which the VCO supplementation was the most effective (Appendix B Figure 179B).

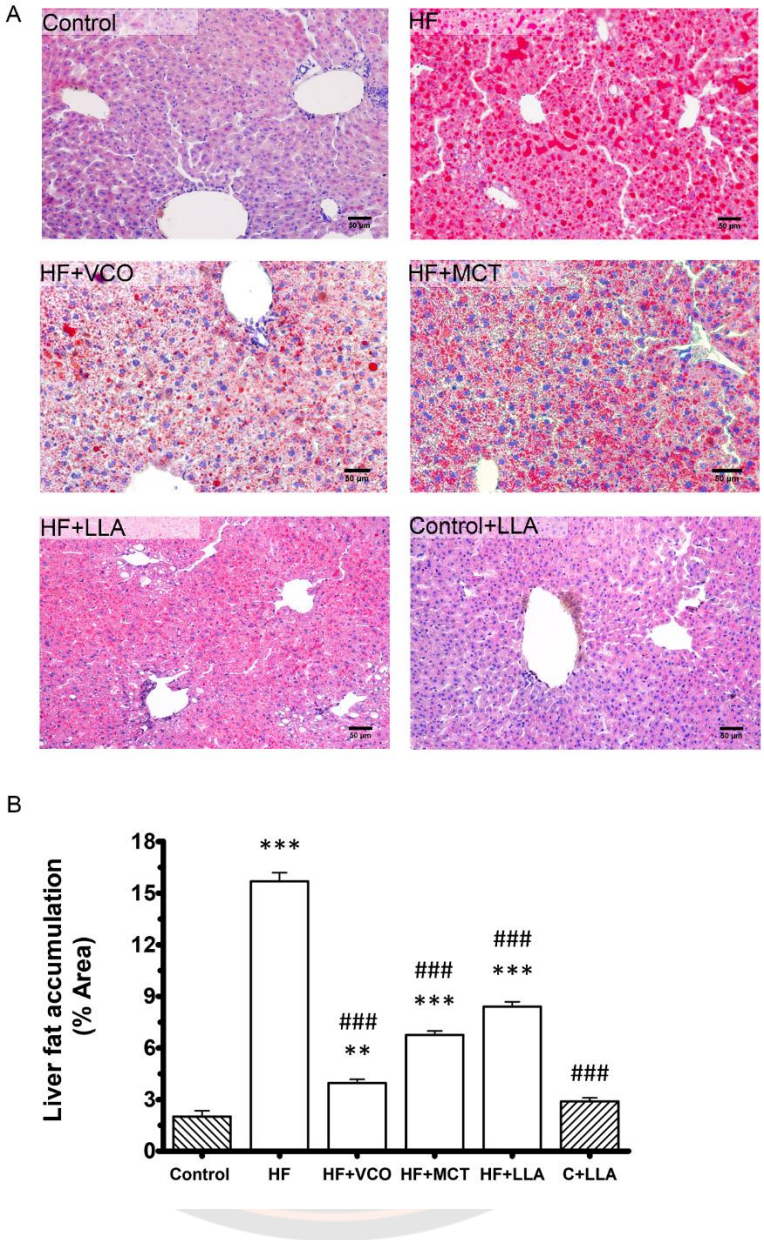


Figure 179 (A) Oil-Red-O partition into hepatic fat droplets in frozen sections of livers from mice fed diets as indicated. (B) Proportion of hepatic lipid (%). Control, normal diet; HF, high-fat diet; HF +VCO, high-fat diet + virgin coconut oil; HF+MCT, high-fat diet + medium chain triglyceride; HF+LLA, high-fat diet + low lauric acid coconut oil; C + LLA, normal diet+ low lauric acid coconut oil. Values are means ± SEM (n =10-16). *P < 0.05, **P< 0.01 ,*P < 0.001 vs. control. ###P < 0.001 vs. high fat diet.**

Overall, the study of the effects of the various diets on lipid profiles in blood and liver of the tested animals provides a mixed set of results. The LLA oil has shown beneficial properties on plasma lipids, especially by improving the TC:HDL ratio. This was not mirrored by the VCO and MC oils. On the other hand, the LLA, together with the VCO and MCT, was not able to counter the plasma TG and LDL increases induced by the HF diet. LLA supplemented to the control diet did show small increases in plasma TC, TG, and LDL. However, these effects were smaller than those of the HF diet. The effects of the LLA on hepatic lipid accumulation induced by the HF diet was positive, but not to the same extent as that of the VCO and MCT supplements. In addition, unlike the MCT and VCO, the LLA was not able to counter the increases in liver TC, TG, and LDL induced by the HF diet. With the apparent increase of the liver TG levels in the HF+LLA group exceeding that of HF diet alone. On the other hand, supplementation of the LLA oil to the control diet does not appear to lead to marked deterioration of the liver lipid profile. Thus, these results are interesting and could stimulate further work.

The above results are a short summary of a manuscript, which has been prepared in collaboration with researchers at the faculties of Medical Science and Pharmaceutical science and which is currently in the review process. The whole manuscript is shown below.

Reduction of lauric acid content in virgin coconut oil improved plasma lipid profile in high-fat diet-induced hypercholesterolemic mice

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Short version of title (running head)

Low lauric acid virgin coconut oil

ABSTRACT

Virgin coconut oil (VCO) is claimed to have various health benefits, but favorable effects of its major component (~50%), lauric acid, are controversial. Therefore, we aimed to reduce lauric acid content (~30%) in VCO and evaluate its effect compared to VCO and medium chain triglycerides (MCT), on food intake, bodyweight (BW), lipid profiles and hepatic histology. Female C57BL/6 mice were treated with different diets for 3 months: control (normal diet), high-fat diet (HF), HF+VCO, HF+MCT, HF+low lauric acid coconut oil (LLA), and normal diet+LLA (C+LLA). LLA was prepared by enzymatic interesterification of VCO with methyl octanoate (methyl caprylate) and methyl decanoate (methyl caprate). Plasma and liver lipids including total cholesterol (TC), high-density lipoprotein (HDL), and triglyceride (TG) were measured by colorimetric assay and hepatic fat accumulation was examined by oil-red-O staining. HF mice exhibited high plasma and liver TC and low-density lipoprotein (LDL). VCO or MCT treatment lowered liver TC and LDL, while LLA increased plasma HDL and markedly improved TC:HDL ratio. The HF-induced hepatic fat accumulation was attenuated by all treatments, of which VCO was the most effective. Control mice administered with LLA demonstrated lower liver TC and LDL, but higher plasma TC and HDL compared to controls. Lowest BW gain and food intake was found in mice treated with LLA. In conclusion, VCO, MCT and LLA ameliorated hepatic histopathology caused by HF. VCO and MCT improved liver lipid profiles, while LLA has more beneficial effect on plasma lipids via a better TC:HDL ratio and showed promise for BW control.

Keywords: Virgin coconut oil, Lauric acid, High fat diet, Lipid profiles, Hepatic fat accumulation

1. Introduction

Virgin coconut oil (VCO) has become a popular dietary supplement due to its beneficial effects on many diseases and general health problems. These include: lowering the risk of cardiovascular diseases (CVDs) (Babu, Veluswamy, Arena, Guazzi, & Lavie, 2014; Nevin & Rajamohan, 2004) and Alzheimer's disease (Ramesh, Krishnan, Praveen, & Hebbar, 2021), being an antioxidant, showing anti- inflammatory activity (Illam, Narayanankutty, & Raghavamenon, 2017; Karunasiri, Senanayake, Hapugaswatta, Jayathilaka, & Seneviratne, 2020), decreasing blood sugar levels in diabetic patients (Malaeb & Spoke, 2020; Zicker et al., 2019), and reducing body weight (Assunção, Ferreira, dos Santos, Cabral, & Florêncio, 2009; Cardoso, Moreira, de Oliveira, Luiz, & Rosa, 2015). However, the use of coconut oil in the diet remains controversial owing to the possible detrimental effects of the oil's high saturated fatty acid (SFA) content, which is associated with dyslipidemia and CVDs (Sacks et al., 2017). This elevates the total cholesterol (TC) and low density lipoprotein cholesterol (LDL) circulating in the blood (Mensink, 2016), interacting with blood vessel walls, triggering immune cells, and activating inflammation, resulting in endothelial dysfunction and plaque formation (Mensink, 2016; Nayor, Brown, & Vasan, 2021). Thus, the lipid-lowering effect of coconut oil is still debated. Some evidence points to the benefits of VCO on lipid profiles (Nevin & Rajamohan, 2004; Vogel, Crovesy, Rosado, & Soares-Mota, 2020), but others show possible detrimental effects (Eyres, Eyres, Chisholm, & Brown, 2016; Mensink, 2016). In addition, many recent studies recommend limiting saturated fat consumption, including advice from the American Heart Association Presidential Advisory Board on dietary fats, which recommended lowering dietary saturated fat (Sacks et al., 2017). Despite being composed of about 90% SFA, it has been claimed that the beneficial health effects of VCO come from the medium-chain fatty acids (MCFA, 6-12 carbon atoms), which represents about 70% of the fatty acids in coconut oil (Marina, Che Man, & Amin, 2009; Orsavova, Misurcova, Ambrozova, Vicha, & Mlcek, 2015). The most abundant MCFA present in coconut oil is lauric acid (C-12, 30-47.7%), which is accompanied by small amounts of caprylic acid (C-8, 7.19-8.81%) and capric acid (C-10, 5.65-6.59%). In addition, the VCO contains long-chain fatty acid (LCFA, > 12 carbon atoms) such as myristic acid (C-14, 18.1%) and palmitic acid (C-

16, 8.8%) (Jayathilaka & Seneviratne, 2022; Lima & Block, 2019; Marina et al., 2009; Orsavova et al., 2015).

It is believed that lauric acid accounts for most of the physiological and metabolic properties of VCO. However, there is an ongoing argument over the categorization of lauric acid as an MCFA or LCFA (Dayrit, 2015; Sacks, 2020) because the commercial medium-chain triglycerides (MCT) are produced mainly from caprylic acid (C-8) and capric acid (C-10) (Dayrit, 2015), which are absorbed directly into the portal circulation (McCarty & DiNicolantonio, 2016; Sacks, 2020). The metabolism of lauric acid has been shown to behave in 2 different ways once ingested: it is transported via the portal vein to be utilized by the liver, and via the lymphatic system into the circulatory system (McDonald, Saunders, Margaret Weidman, & Fisher, 1980; McDonald & Weidman, 1987; Sigalet & Martin, 1999; Sigalet, Winkelaar, & Smith, 1997). Lauric acid has also been shown to raise total serum LDL and high density lipoprotein cholesterol (HDL) concentrations (Denke & Grundy, 1992; Mensink, Zock, Kester, & Katan, 2003; Santos, Howell, Earnest, & Teixeira, 2019). Previous studies of lauric acid have shown inconsistent results, as some studies reported lipid-lowering effects (Lekshmi Sheela, Nazeem, Narayanankutty, Manalil, & Raghavamenon, 2016; Roos, Schouten, & Katan, 2001; Xia et al., 2021), while other studies show the opposite effects (Denke & Grundy, 1992; Mensink et al., 2003; Saraswathi et al., 2020; Tsai, Park, Kovacic, & Snook, 1999). The inconsistent recommendations regarding the consumption of SFA have raised the concern about the consumption of VCO together with a high-fat diet (HF) (Eyres et al., 2016). Therefore, in this study, our objective was to reduce the lauric acid content in virgin coconut oil and use the resulting formula to investigate the effects of low lauric acid coconut oil (LLA) compared to VCO and commercial MCT on plasma and liver lipid profiles and hepatic lipid accumulation. Their effects on food intake and bodyweight (BW) were also evaluated in HF-induced hypercholesterolemia and nonalcoholic fatty liver in C57BL/6J mice.

2. Materials and Methods

2.1. Oil samples

Virgin coconut oil (Thai Pure Brand) and MCT oil (Healtholicious Brand) were purchased from local store and online, respectively. The low lauric acid (LLA) oil has been prepared from the virgin coconut oil as described below.

2.2. Preparation of low lauric acid coconut oil

Thai pure virgin coconut oil (100 g), methyl octanoate (72 g), methyl decanoate (48 g), and lipase enzyme (11 g) were mixed, and the mixture was stirred at 45°C for 96 h. The enzyme catalyst was removed by filtration using filter paper. Hexane was removed from the reaction mixture using a rotary evaporator and the fatty acid esters were removed by vacuum distillation using an oil pump. The residue was further purified by column chromatography on silica using hexane as the mobile phase. The solvents were removed by rotary evaporation to give a clear oil (51.3 g). The fatty acid composition of the product was analyzed by gas chromatography (Table 1).

2.3. Animals and diets

Experiments were ethically approved by Naresuan University Animal Care and Use Committee (NUACUC, Naresuan University, Phitsanulok, Thailand, Animal ethics approval number: NU-AE610515) for the care and use of animals for scientific purposes. Female C57BL/6J mice (8 weeks old) were purchased from Nomura Siam International Co. Ltd., Bangkok, Thailand. The mice were acclimatized for 1 week in their polycarbonate cages under controlled conditions (22°C ± 1°C), 12-12 h light/dark cycle environment and allowed access to food and water *ad libitum* at the Center for Animal Research, Naresuan University. They were randomly divided into 6 groups and fed with different diets for 3 months as follow (Chaiwong et al., 2021).

i) Control group: Mice were fed with normal diet (2018, Teklad irradiated global 18% protein rodent diet; Harlan Teklad Laboratory, Madison, Wisconsin, USA, Energy 3.1 Kcal/g containing 6.2% fat, 44.2% carbohydrate and 18.6% protein) and gavaged with water.

ii) High-fat diet (HF): Mice were fed with HF (TD02028, Teklad atherogenic rodent diet; Harlan Teklad Laboratory, Madison, Wisconsin, USA, Energy 4.5 Kcal/g containing 21.2% fat, cholesterol

1.3%, cholic acid 0.5%, 46.9% carbohydrate and 17.3% protein) and gavaged with water.

iii) HF+VCO: Mice were fed with HF and VCO (6.15 ml/kg BW).

iv) HF+ MCT: Mice were fed with HF and MCT (6.15 ml/kg BW).

v) HF+LLA: Mice were fed with HF and LLA (6.15 ml/kg BW).

vi) Control+LLA: Mice were fed with a normal diet and LLA (6.15 ml/kg BW).

Food intake and BW were recorded daily throughout the 3-month experimental period. At the end of the experiment, following overnight fasting, the animals were anesthetized using sodium thiopental 50 mg/kg BW by intraperitoneal injection and then were sacrificed by exsanguination. The whole blood was collected by cardiac puncture and kept at -80° C for evaluation of the plasma lipid profiles. The livers were collected for lipid profiles and histological studies.

2.4. Determination of plasma and liver lipid profiles

The blood was centrifuged at 3000 rpm at 4°C for 10 min and the plasma was collected from the supernatant. The livers were collected, weighed, and placed in the chloroform and methanol mixture (2:1) for lipid extraction following Folch's method (Folch, Lees, & Sloane Stanley, 1957). Then the plasma and liver samples were subjected to analysis to determine TC, triglyceride (TG), and HDL contents using an enzymatic colorimetric test according to manufacturer instruction (Human diagnostic company, Wiesbaden, Germany). LDL content was calculated by the formula: LDL = TC – HDL (Chaiwong et al., 2021).

2.5. Determination of hepatic lipid accumulation

The collected livers were washed with normal saline and then preserved in 30% sucrose in paraformaldehyde at 4ºC. The samples were embedded in paraffin and trimmed to 5 μm-thickness by frozen sections and then were stained with hematoxylin and oil red O and permounted with aqueous mounting solution (Chaiwong et al., 2021). They were subsequently photographed at 20x magnification by light microscopy. Lipid accumulation in the liver tissue was analyzed by Image J software (version 1.51j8, National Institutes of Health, USA). Liver lipid accumulation was calculated as a percentage of the total hepatocyte area.

2.6. Statistical analysis

All data were presented as mean ± standard error of the mean (SEM) of *n* animals. The statistical significance between groups was analyzed using Student's t-test and/or ANOVA followed by a post hoc Tukey-Kramer test. Values of $p<0.05$ were considered statistically significant.

3. Results

3.1 BW and food intake

All animal groups, except HF+LLA, showed a significant increase in the BW growth rate from month-1 to month-3 **(Figure 1A)**, while food intake was significantly lower in all groups compared to the control group **(Figure 1B)**. The lowest food intake was observed in HF mice treated with LLA.

3.2 Plasma and liver lipid profiles

Mice fed with HF exhibited higher TC and LDL levels in both plasma (~1.4-1.6 fold, **Figure 2A and 2D**, $p<0.001$ vs control) and liver (~2.5 fold, **Figure 3A and 3D,** $p<0.001$ vs control), but slightly lower plasma TG (**Figure 2B**, $p<0.05$ vs control). An increase in liver HDL was observed in all animal groups, except C+LLA compared to controls (**Figure 3C**). VCO or MCT treatment had no significant effect on plasma lipid profiles compared to HF mice (**Figure 2**), but remarkably lowered liver TC and LDL (**Figure 3A and 3D**, $p<0.001$ vs HF). LLA administration increased plasma HDL ~1.7 fold (**Figure 2C**, $p<0.0001$ vs HF and control), thus improving the TC:HDL ratio from 7:1 for HF to 4:1 for HF+LLA. Control diet fed mice administered with LLA (C+LLA) demonstrated higher plasma TC ($p<0.05$) and HDL ($p<0.001$) compared to controls (**Figure 2A and 2C**) and the TC:HDL ratio was improved (4:1 for control and 3.3:1 for C+LLA). A slight increase in plasma TG was observed in HF mice and control mice treated with LLA compared to HF mice, however such TG level was not different from the controls **(Fig. 2B)**.

3.3 Hepatic fat accumulation

The changes in lipid accumulation in liver tissue are illustrated in **Figure 4**. The density of oil red O in stained tissues was directly proportional to lipid accumulation. The fat droplets were small with sparse staining in the control and C+LLA groups, but were large and intensely stained in the HF group (**Figure 4A**). The HF+VCO, HF+MCT, and

HF+LLA groups exhibited smaller size of fat droplets with less intense staining compared to the HF mice (**Figure 4A**). The percentage area of the hepatic fat accumulation was highest in the HF group, and this was reduced by all treatments, of which the VCO supplementation was the most effective (**Fig. 4B**).

4. Discussion

The current study demonstrates the effect of LLA consumption in combination with HF or normal diet in mice and is compared to the effects of VCO and MCT. In the present study, VCO naturally contains mostly MCFAs of which the majority component is lauric acid (C12, 47.5%). The LLA was prepared by enzymatic interesterification of VCO resulting in the reduction of lauric acid content to ~30%, while caprylic (C8, 27%) and capric (C10, 25%) acids were increased compared to VCO (C8, 7.8%; C10, 6.7%). The MCT used is commercial synthetic product mainly composed of caprylic (50-80%) and capric (20-50%) acids. Here, we clearly demonstrated that reducing lauric acid content in VCO showed promising body weight lowering effect and produced more favorable results on plasma lipid profiles compared to the effects of VCO and MCT.

In our study, mice fed with HF exhibited similar growth rate, but less food intake as compared to the control. Their plasma and liver TC and LDL and hepatic fat accumulation were dramatically increased indicating that HF-induced hypercholesterolemia and fatty liver in the C57BL/6J mice model was successfully achieved. This result is consistent with previous study using similar formulations of HF diet to induce non-obese nonalcoholic fatty liver disease in mice (Tu et al., 2017). The lower food intake due to HF or higher caloric diet consumption was also similarly observed in other studies (Hambly et al., 2005; Li, Wu, Liu, & Yang, 2020; Townsend, Lorenzi, & Widmaier, 2008). Treatment with LLA caused lower BW growth rate and lowest food intake as compared to VCO and MCT. The BW growth lowering effect of LLA could be due to the action of all three fatty acids i.e., lauric acid, caprylic acid and capric acid as consumption of caprylic triglyceride, capric triglyceride and lauric triglyceride have been shown to reduce the BW and fat coefficient in obese rats and the plasma and liver lipid profiles were also improved (Xia et al., 2022). Interestingly, the studies on the effects of caprylic triglyceride, capric triglyceride and lauric triglyceride show differential effects of these oils on food intake, plasma lipid profiles,

triacylglycerol metabolism and cholesterol metabolism (Xia et al., 2022, Xia et al. 2022 Food and Function, *13*, 8068-8080). This suggests that the different fatty acid compositions of VCO, MCT and LLA could indeed lead to different outcomes as observed in our work. In addition, LLA favorably affected plasma lipids via increasing HDL thus, improving the plasma TC:HDL ratio, while VCO and MCT produced significantly reduced hepatic TC and LDL. It has been reported that high-dose lauric acid or coconut oil down regulated the LDL receptor in the liver, thus leading to the impairment of the LDL transport to the liver, resulting in high levels of plasma TC and LDL (Stucchi, Terpstra, & Nicolosi, 1995; Xia et al., 2022). These observations were consistent with several previous studies which also reported that consumption of either coconut oil or lauric acid raised plasma TC and LDL (Denke & Grundy, 1992; Sankararaman & Sferra, 2018; Santos et al., 2019; Temme, Mensink, & Hornstra, 1996). Indeed, prolonged exposure to high dose of lauric triglyceride has been linked to increased PCSK9 expression, which leads to LDLR degradation and increased plasma and LDL levels. In addition, this intervention can also increase uptake of sterols in the intestine leading to further increases in plasma TC and LDL levels (Xia et al., 2021, Journal of Agricultural and Food Chemistry, *69*, 4453-4463). Thus it is sensible that reduction of lauric acid content in VCO produced favorable effect on blood lipids. Fat intake promotes hepatic fat accumulation, thus inducing fatty liver as demonstrated in our study. All treatments could ameliorate HF-induced hepatic histopathology. This is consistent with the previous studies showing that treatment with lauric acid or oil containing lauric acid or MCT reduced pro-inflammatory cytokines, liver inflammation, and downregulated the expression of TLR4/NF-κB mediating proteins in liver and adipose tissues (Du et al., 2020; Khan et al., 2021). In one instance, the beneficial effects of caprylic and capric acids present in a structured lipid on liver fat profiles were shown to increase with their content in the structured lipid products (Zhou *et al*., 2018, Journal of Food Science, *8*, 1968-1977). Another study has shown that, while MCT supplementation can have beneficial effects, further understanding of effects of MCTs on liver lipids and their metabolism needs to be developed as high doses of MCT led to alteration of lipid metabolism that might be harmful in the long-term (Chamma *et al.*, 2017, Food and Function, *8*, 778-787).

5. Conclusion

The present study demonstrated that VCO and MCT improved liver lipid profiles, while LLA has more beneficial effect on plasma lipids via a better TC:HDL ratio and showed promise for BW control. The reduction of lauric acid in VCO produced favorable effect towards plasma lipid profiles, thus further in-depth mechanistic evaluation of the composition of saturated fatty acids has yet to be justified.

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Conflicts of Interest

The authors declare that there are no conflicts of interest.

References

Assunção, M. L., Ferreira, H. S., dos Santos, A. F., Cabral, C. R., & Florêncio, T. M. M. T. (2009). Effects of dietary coconut oil on the biochemical and anthropometric profiles of women presenting abdominal obesity. *Lipids, 44*(7), 593-601. doi:10.1007/s11745-009-3306-6

Babu, A. S., Veluswamy, S. K., Arena, R., Guazzi, M., & Lavie, C. J. (2014). Virgin coconut oil and its potential cardioprotective effects. *Postgraduate Medicine, 126*(7), 76-83. doi:10.3810/pgm.2014.11.2835

Cardoso, D. A., Moreira, A. S., de Oliveira, G. M., Luiz, R. R., & Rosa, G. (2015). A coconut extra virgin oil-rich diet increases HDL cholesterol and decreases waist circumference and body mass in coronary artery disease patients. *Nutricion Hospitalaria, 32*(5), 2144-2152. doi:DOI:10.3305/nh.2015.32.5.9642

Chaiwong, S., Chatturong, U., Chanasong, R., Deetud, W., To-on, K., Puntheeranurak, S., Chootip, K. (2021). Dried mulberry fruit ameliorates cardiovascular and liver histopathological changes in high-fat diet-induced hyperlipidemic mice. *Journal of Traditional and Complementary Medicine, 11*(4), 356-368. doi:https://doi.org/10.1016/j.jtcme.2021.02.006

Dayrit, F. M. (2015). The properties of lauric acid and their significance in coconut oil. *Journal of the American Oil Chemists' Society, 92*(1), 1-15. doi:10.1007/s11746-014-2562-7

Denke, M. A., & Grundy, S. M. (1992). Comparison of effects of lauric acid and palmitic acid on plasma lipids and lipoproteins. *The American Journal of Clinical Nutrition, 56*(5), 895-898. doi:10.1093/ajcn/56.5.895

Du, Y.-X., Chen, S.-N., Zhu, H.-L., Niu, X., Li, J., Fan, Y.-W., & Deng, Z.-Y. (2020). Consumption of interesterified medium- and long-chain triacylglycerols improves lipid metabolism and reduces inflammation in high-fat diet-induced obese rats. *Journal of Agricultural and Food Chemistry, 68*(31), 8255-8262. doi:10.1021/acs.jafc.0c03103

Eyres, L., Eyres, M. F., Chisholm, A., & Brown, R. C. (2016). Coconut oil consumption and cardiovascular risk factors in humans. *Nutrition Reviews, 74*(4), 267-280. doi:10.1093/nutrit/nuw002

Folch, J., Lees, M., & Sloane Stanley, G. H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *J biol Chem, 226*(1), 497-509.

Hambly, C., Adams, A., Fustin, J.-M., Rance, K. A., Bünger, L., & Speakman, J. R. (2005). Mice with Low Metabolic Rates Are Not Susceptible to Weight Gain When Fed a High-Fat Diet. *Obesity Research, 13*(3), 556-566. doi:https://doi.org/10.1038/oby.2005.59

Illam, S. P., Narayanankutty, A., & Raghavamenon, A. C. (2017). Polyphenols of virgin coconut oil prevent pro-oxidant mediated cell death. *Toxicology Mechanisms and Methods, 27*(6), 442-450. doi:10.1080/15376516.2017.1320458

Jayathilaka, N., & Seneviratne, K. N. (2022). Phenolic antioxidants in coconut oil: Factors affecting the quantity and quality. A review. *Grasas y Aceites, 73*(3), e466. doi:10.3989/gya.0674211

Karunasiri, A. N., Senanayake, C. M., Hapugaswatta, H., Jayathilaka, N., & Seneviratne, K. N. (2020). Protective effect of coconut oil meal phenolic antioxidants against macromolecular damage: *In vitro* and *In vivo* study. *Journal of Chemistry, 2020*, 3503165. doi:10.1155/2020/3503165

Khan, H. U., Aamir, K., Jusuf, P. R., Sethi, G., Sisinthy, S. P., Ghildyal, R., & Arya, A. (2021). Lauric acid ameliorates lipopolysaccharide (LPS)-induced liver

inflammation by mediating TLR4/MyD88 pathway in Sprague Dawley (SD) rats. *Life Sciences, 265*, 118750. doi:https://doi.org/10.1016/j.lfs.2020.118750

Lekshmi Sheela, D., Nazeem, P. A., Narayanankutty, A., Manalil, J. J., & Raghavamenon, A. C. (2016). In silico and wet lab studies reveal the cholesterol lowering efficacy of lauric acid, a medium chain fat of coconut oil. *Plant Foods for Human Nutrition, 71*(4), 410-415. doi:10.1007/s11130-016-0577-y

Li, J., Wu, H., Liu, Y., & Yang, L. (2020). High fat diet induced obesity model using four strains of mice: Kunming, C57BL/6, BALB/c and ICR. *Experimental Animals, 69*(3), 326-335. doi:10.1538/expanim.19-0148

Lima, R. d. S., & Block, J. M. (2019). Coconut oil: what do we really know about it so far? *Food Quality and Safety, 3*(2), 61-72. doi:10.1093/fqsafe/fyz004

Malaeb, S., & Spoke, C. (2020). The glucose-lowering effects of coconut oil: A case report and review of the literature. *Case Reports in Endocrinology, 2020*, 8841781. doi:10.1155/2020/8841781

Marina, A. M., Che Man, Y. B., & Amin, I. (2009). Virgin coconut oil: emerging functional food oil. *Trends in Food Science & Technology, 20*(10), 481-487. doi:https://doi.org/10.1016/j.tifs.2009.06.003

McCarty, M. F., & DiNicolantonio, J. J. (2016). Lauric acid-rich medium-chain triglycerides can substitute for other oils in cooking applications and may have limited pathogenicity. *Open Heart, 3*(2), e000467. doi:10.1136/openhrt-2016-000467

McDonald, G. B., Saunders, D. R., Margaret Weidman, & Fisher, L. (1980). Portal venous transport of long-chain fatty acids absorbed from rat intestine. *American Journal of Physiology-Gastrointestinal and Liver Physiology, 239*(3), G141-G150. doi:10.1152/ajpgi.1980.239.3.G141

McDonald, G. B., & Weidman, M. (1987). Partitioning of polar fatty acids into lymph and portal vein after intestinal absorption in the rat. *Quarterly Journal of Experimental Physiology, 72*(2), 153-159. doi:https://doi.org/10.1113/expphysiol.1987.sp003059

Mensink, R. P. (2016). Effects of saturated fatty acids on serum lipids and lipoproteins: a systematic review and regression analysis. *World Health Organization*.

Mensink, R. P., Zock, P. L., Kester, A. D., & Katan, M. B. (2003). Effects of dietary fatty acids and carbohydrates on the ratio of serum total to HDL cholesterol and on

serum lipids and apolipoproteins: a meta-analysis of 60 controlled trials. *The American Journal of Clinical Nutrition, 77*(5), 1146-1155. doi:10.1093/ajcn/77.5.1146

Nayor, M., Brown, K. J., & Vasan, R. S. (2021). The Molecular Basis of Predicting Atherosclerotic Cardiovascular Disease Risk. *Circulation Research, 128*(2), 287-303. doi:doi:10.1161/CIRCRESAHA.120.315890

Nevin, K. G., & Rajamohan, T. (2004). Beneficial effects of virgin coconut oil on lipid parameters and in vitro LDL oxidation. *Clinical Biochemistry, 37*(9), 830-835. doi:https://doi.org/10.1016/j.clinbiochem.2004.04.010

Orsavova, J., Misurcova, L., Ambrozova, J. V., Vicha, R., & Mlcek, J. (2015). Fatty acids composition of vegetable oils and its contribution to dietary energy intake and dependence of cardiovascular mortality on dietary intake of fatty acids. *International Journal of Molecular Sciences, 16*(6), 12871-12890. Retrieved from https://www.mdpi.com/1422-0067/16/6/12871

Ramesh, S. V., Krishnan, V., Praveen, S., & Hebbar, K. B. (2021). Dietary prospects of coconut oil for the prevention and treatment of Alzheimer's disease (AD): A review of recent evidences. *Trends in Food Science & Technology, 112*, 201-211. doi:https://doi.org/10.1016/j.tifs.2021.03.046

Roos, N. M. d., Schouten, E. G., & Katan, M. B. (2001). Consumption of a solid fat rich in lauric acid results in a more favorable serum lipid profile in healthy men and women than consumption of a solid fat rich in trans-fatty acids. *The Journal of Nutrition, 131*(2), 242-245. doi:10.1093/jn/131.2.242

Sacks, F. M. (2020). Coconut oil and heart health. *Circulation, 141*(10), 815-817. doi:doi:10.1161/CIRCULATIONAHA.119.044687

Sacks, F. M., Lichtenstein, A. H., Wu, J. H. Y., Appel, L. J., Creager, M. A., Kris-Etherton, P. M., Horn, L. V. V. (2017). Dietary fats and cardiovascular disease: a presidential advisory from the american heart association. *Circulation, 136*(3), e1-e23. doi:doi:10.1161/CIR.0000000000000510

Sankararaman, S., & Sferra, T. J. (2018). Are we going nuts on coconut oil? *Current Nutrition Reports, 7*(3), 107-115. doi:10.1007/s13668-018-0230-5

Santos, H. O., Howell, S., Earnest, C. P., & Teixeira, F. J. (2019). Coconut oil intake and its effects on the cardiometabolic profile – A structured literature review. *Progress*

in Cardiovascular Diseases, 62(5), 436-443. doi:https://doi.org/10.1016/j.pcad.2019.11.001

Saraswathi, V., Kumar, N., Gopal, T., Bhatt, S., Ai, W., Ma, C., Desouza, C. (2020). Lauric acid versus palmitic acid: effects on adipose tissue inflammation, insulin resistance, and non-alcoholic fatty liver disease in obesity. *Biology, 9*(11), 346. Retrieved from https://www.mdpi.com/2079-7737/9/11/346

Sigalet, D. L., & Martin, G. (1999). Lymphatic absorption of glucose and fatty acids as determined by direct measurement. *Journal of Pediatric Surgery, 34*(1), 39-43. doi:https://doi.org/10.1016/S0022-3468(99)90225-7

Sigalet, D. L., Winkelaar, G. B., & Smith, L. J. (1997). Determination of the route of medium-chain and long-chain fatty acid absorption by direct measurement in the rat. *Journal of Parenteral and Enteral Nutrition, 21*(5), 275-278. doi:https://doi.org/10.1177/0148607197021005275

Stucchi, A. F., Terpstra, A. H. M., & Nicolosi, R. J. (1995). LDL receptor activity is down-regulated similarly by a cholesterol-containing diet high in palmitic acid or high in lauric and myristic acids in cynomolgus monkeys. *The Journal of Nutrition, 125*(8), 2055-2063. doi:10.1093/jn/125.8.2055

Temme, E. H., Mensink, R. P., & Hornstra, G. (1996). Comparison of the effects of diets enriched in lauric, palmitic, or oleic acids on serum lipids and lipoproteins in healthy women and men. *The American Journal of Clinical Nutrition, 63*(6), 897-903. doi:10.1093/ajcn/63.6.897

Townsend, K. L., Lorenzi, M. M., & Widmaier, E. P. (2008). High-fat diet-induced changes in body mass and hypothalamic gene expression in wild-type and leptin-deficient mice. *Endocrine, 33*(2), 176-188. doi:10.1007/s12020-008-9070-1

Tsai, Y.-H., Park, S., Kovacic, J., & Snook, J. T. (1999). Mechanisms mediating lipoprotein responses to diets with medium-chain triglyceride and lauric acid. *Lipids, 34*(9), 895-905. doi:10.1007/s11745-999-0438-9

Tu, L. N., Showalter, M. R., Cajka, T., Fan, S., Pillai, V. V., Fiehn, O., & Selvaraj, V. (2017). Metabolomic characteristics of cholesterol-induced non-obese nonalcoholic fatty liver disease in mice. *Scientific reports, 7*(1), 1-14.

Vogel, C. É., Crovesy, L., Rosado, E. L., & Soares-Mota, M. (2020). Effect of coconut oil on weight loss and metabolic parameters in men with obesity: a randomized

controlled clinical trial. *Food & Function, 11*(7), 6588-6594. doi:10.1039/D0FO00872A

Xia, J., Yu, P., Zeng, Z., Ma, M., Yan, X., Zhao, J., Wang, J. (2022). Effects of medium chain triglycerides on lipid metabolism in high-fat diet induced obese rats. *Food & Function, 13*(17), 8998-9009. doi:10.1039/D2FO01711C

Xia, J., Yu, P., Zeng, Z., Ma, M., Zhang, G., Wan, D., Wang, J. (2021). Lauric triglyceride ameliorates high-fat-diet-induced obesity in rats by reducing lipogenesis and increasing lipolysis and β-oxidation. *Journal of Agricultural and Food Chemistry, 69*(32), 9157-9166. doi:10.1021/acs.jafc.0c07342

Zicker, M. C., Silveira, A. L. M., Lacerda, D. R., Rodrigues, D. F., Oliveira, C. T., de Souza Cordeiro, L. M., Ferreira, A. V. M. (2019). Virgin coconut oil is effective to treat metabolic and inflammatory dysfunction induced by high refined carbohydrate-containing diet in mice. *The Journal of Nutritional Biochemistry, 63*, 117-128. doi:https://doi.org/10.1016/j.jnutbio.2018.08.013

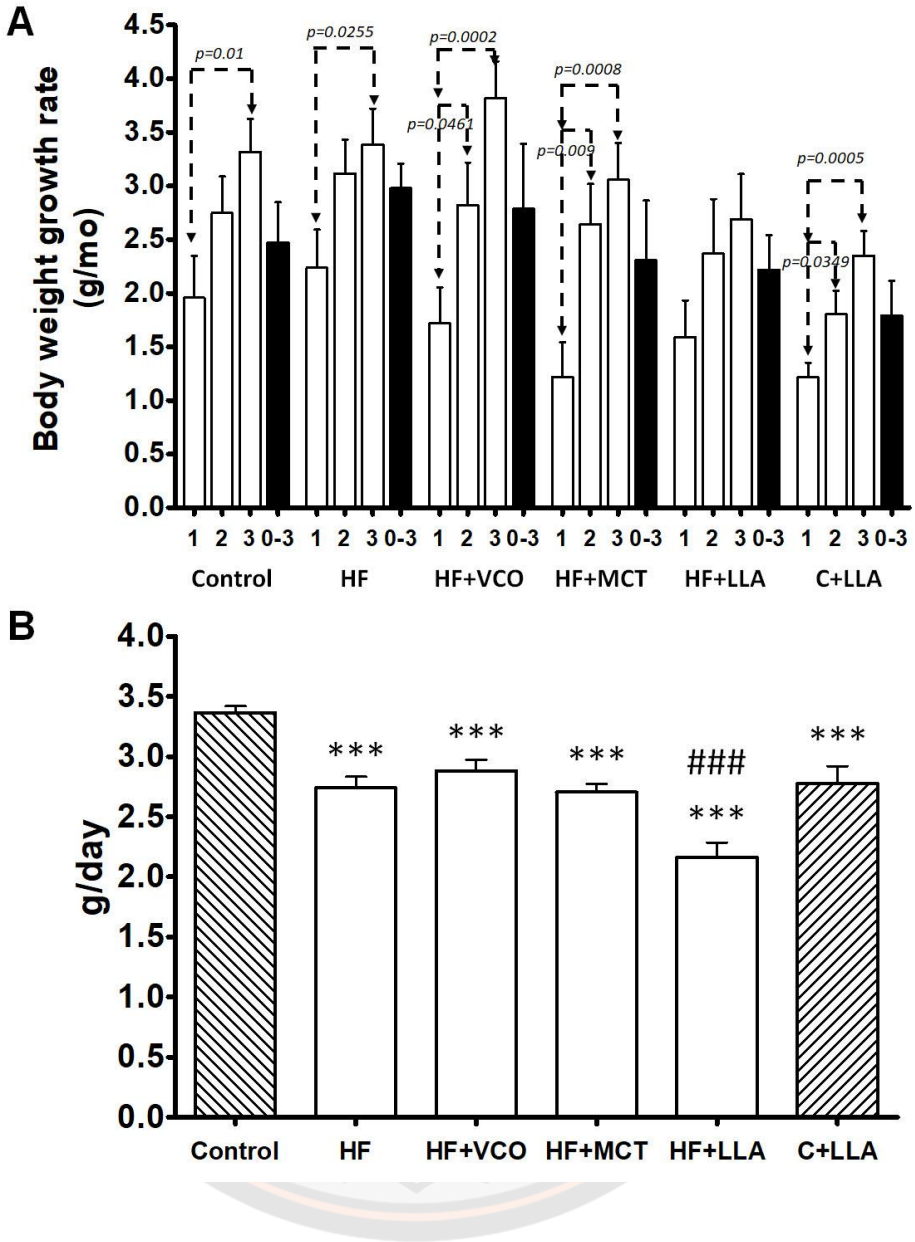

Figure 1. (A) Body weight gain in different groups. The grey bars are averaged values for the 3-month period for each treatment. Error bars are SEM, and the *p*-values are for unpaired, 2-tailed comparisons denoted by the dotted lines. (B) Food intake averaged over 12 weeks. Control, normal diet; HF, high-fat diet; HF+VCO, HF + virgin coconut oil; HF+MCT, HF + medium chain triglyceride; HF+LLA, HF + low lauric acid coconut oil; C+LLA, normal diet + low lauric acid coconut oil. Values are means ± SEM (*n* =10-16). *** $p<0.001$ *vs* control and $^{###}$ $p<0.001$ *vs* HF.

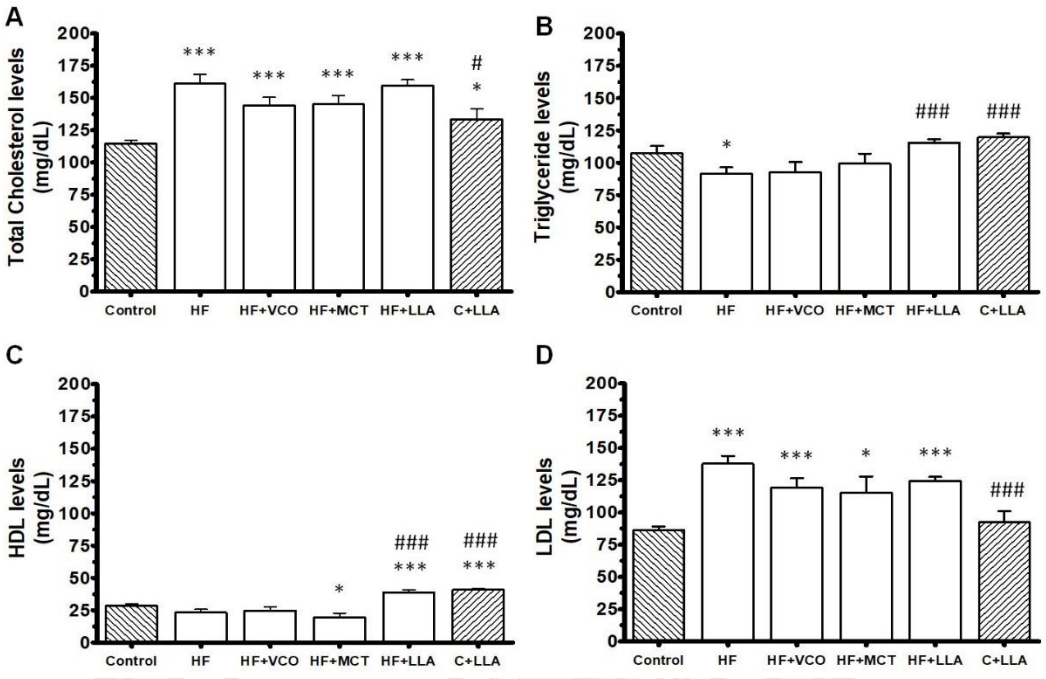

Figure 2. Plasma lipid profiles at 3 months. Control, normal diet; HF, high-fat diet; HF+VCO, HF + virgin coconut oil; HF+MCT, HF + medium chain triglyceride; HF+LLA, HF + low lauric acid coconut oil; C+LLA, normal diet + low lauric acid coconut oil. HDL, high-density lipoprotein; LDL, low-density lipoprotein. Values are means ± SEM (n =10-16). *p<0.05, ***p<0.001 *vs* control. $^{\#}p$<0.05, $^{\#\#\#}p$<0.001 *vs* HF.

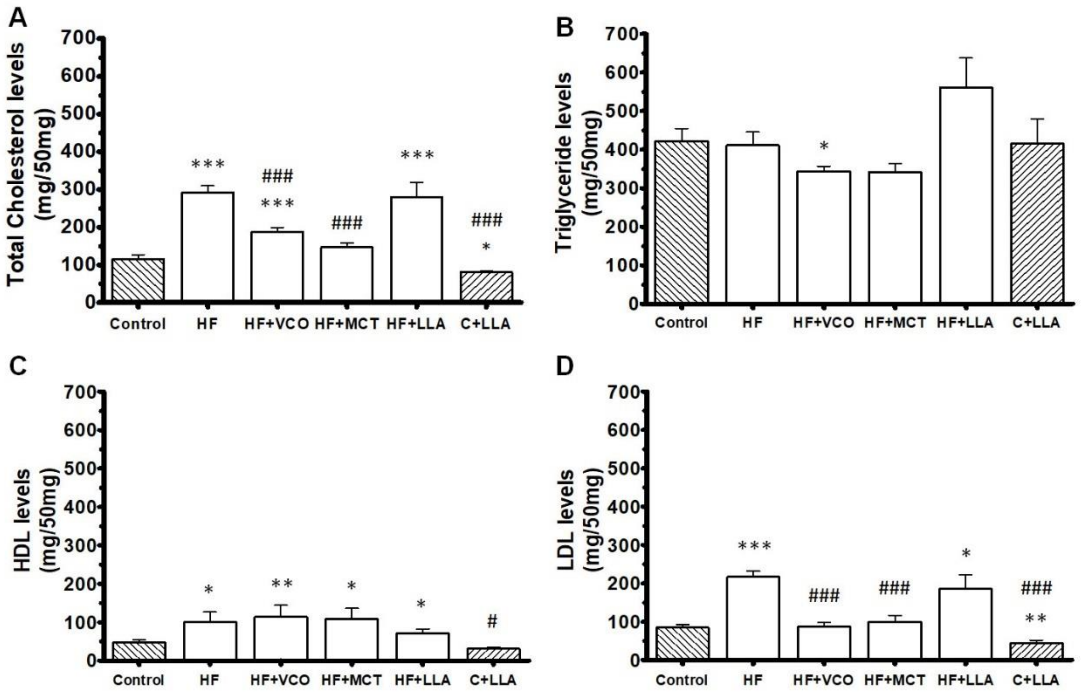

Figure 3. Hepatic lipid profiles at 3 months. Control, normal diet; HF, high-fat diet; HF+VCO, HF + virgin coconut oil; HF+MCT, HF + medium chain triglyceride; HF+LLA, HF + low lauric acid coconut oil; C+LLA, normal diet + low lauric acid coconut oil. HDL, high-density lipoprotein; LDL, low-density lipoprotein. Values are means ± SEM (n =10-16). *p<0.05, ***p<0.001 *vs* control. $^{###}p$<0.001 *vs* HF.

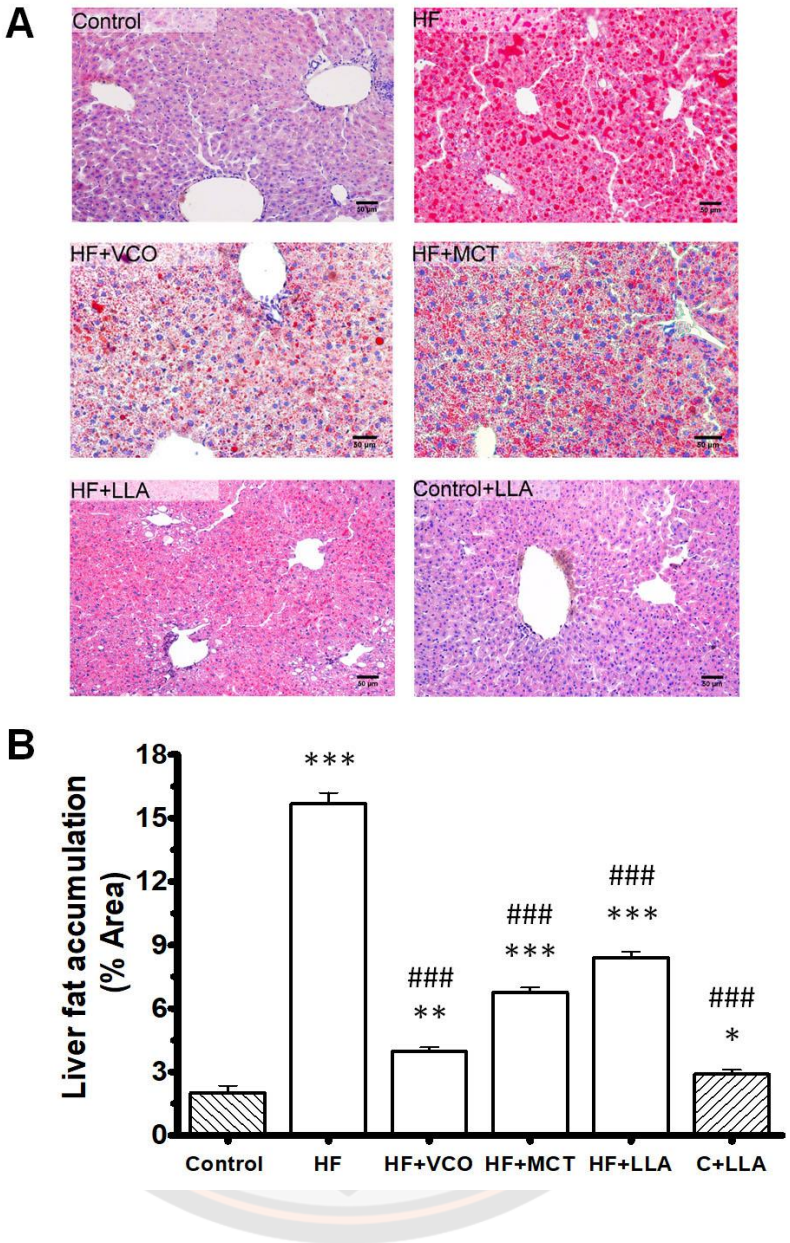

Figure 4. (A) Oil-Red-O partition into hepatic fat droplets in frozen sections of livers from mice fed diets as indicated. (B) Proportion of hepatic lipid (%). Control, normal diet; HF, high-fat diet; HF+VCO, HF + virgin coconut oil; HF+MCT, HF + medium chain triglyceride; HF+LLA, HF + low lauric acid coconut oil; C+LLA, normal diet + low lauric acid coconut oil. Values are means ± SEM (n =10-16). *p<0.05, **p<0.01, ***p<0.001 *vs* control. $^{\#}p$<0.05, $^{\#\#\#}p$< 0.001 *vs* HF.

Table 1 Composition of virgin coconut oil (VCO), low lauric acid coconut oil (LLA) and medium-chain triglyceride (MCT)

No.	Characteristics/ Fatty acid composition		VCO	LLA	MCT
1	Tocopherol content (ppm) Beta - Tocopherol		6	-	-
2	Tocotrienol content (ppm) Alpha - Tocotrienol		49	-	-
3	Fatty acid composition (%)				
	3.1	Caproic (C-6:0)	0.5	-	<2
	3.2	Caprylic (C-8:0)	7.8	27.0	50-80
	3.3	Capric (C-10:0)	6.7	25.6	20-50
	3.4	Lauric (C-12:0)	47.5	30.3	< 3
	3.5	Myristic (C-14:0)	18.1	8.7	< 1
	3.6	Palmitic (C-16:0)	8.8	3.9	0
	3.7	Stearic (C-18:0)	2.6	1.5	0
	3.8	Oleic (C-18:1)	6.2	1.6	0
	3.9	Linoleic (C-18:2)	1.6	1.4	0
	3.10	Arachidic (C-20:0)	0.1	-	0
	3.11	Gadoloic (C-20:1)	Trace	-	0
4	Triglyceride composition (%)				
	Triglyceride carbon number range		C28-C52	C24-C52	C24-C32
	Mean MW[a] of triglycerides		638	570	512
	C24-C30 content		< 4%	34%	95%
	Physical characteristic		Solid at ambient temp.	Liquid at ambient temp.	Liquid at all temp.
	4.1	Trisaturated (GS3)	84.0	92	100
	4.2	Disaturated (GS20)	12.0	8	0

[a]MW, Molecular weight