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THESIS

OPTIMIZATION OF WAX ESTERS PRODUCTION FROM PALM FATTY ACID DISTILLATE AND OLEYL ALCOHOL OVER AMBERLYST 15 AS CATALYST BY RESPONSE SURFACE METHODOLOGY

MALLIKA TAPANWONG

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Mallika Tapanwong 2012: Optimization of Wax Esters Production from Palm Fatty Acid Distillate and Oleyl Alcohol over Amberlyst 15 as Catalyst by Response Surface Methodology. Master of Science (Chemistry), Major Field: Chemistry, Department of Chemistry. Thesis Advisor: Associate Professor Vittaya Punsuvon, Ph.D. 175 pages.

Wax esters were derived from long chain fatty acid and long chain alcohol with chain length of 12 carbons or more. These compounds had many potential applications in cosmetics, pharmaceuticals and food industries. The present work focuses on the synthesis of wax esters using palm fatty acid distillate and oleyl alcohol catalyzed by Amberlyst 15 catalyst. Response surface methodology (RSM) based on a five-level-four-factor-variable central composite design (CCD) was used to evaluate the interactive effects of synthesis, amount of Amberlyst 15 catalyst (10–40 %wt/wt), reaction time (0–4 hours), molar ratio of palm fatty acid distillate to oleyl alcohol (1–4 moles) and reaction temperature (40–80 ^{O}C) on the percentage conversion of palm fatty acid distillate and percentage yield of wax esters. The optimum conditions derived via RSM were 30 %wt/wt of Amberlyst 15, 3.5:1 of oleyl alcohol-to-PFAD molar ratio, 2.5 hours of reaction time and 70 ^{O}C of reaction temperature. The actual experimental conversion and yield were 93.89% and 83.93% under optimum condition which corresponded with the maximum predicted values of 93.98% and 83.02%, respectively. The experiment further studied on the efficiency of reusable Amberlyst 15 catalyst and wax esters synthesis without catalyst. The results showed the efficiency reduction of reusable catalyst about 15% on percentage conversion from the first cycle of experiment. The results on wax esters production without Amberlyst 15 catalyst showed that PFAD could be catalyzed itself to produce wax esters at high temperature. Wax esters obtained from this synthesis were further analyzed for physical properties and confirmed their functional groups by FT-IR.

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Mallika Tapanwong
April 2012

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LIST OF ABBREVIATIONS

ANOVA = Analysis of Variance
AOCS = American Oil Chemists' Society
ASTM = American Society for Testing and Material
BIB = Balanced Incompletely Block Design
CCD = Central Composite Design
CCRD = Central Composite Rotatable Design
CRD = Completely Randomized Design
CPO = Crude Palm Oil
cSt = Centistokes
DGA = Diacylglycerol
FAME = Fatty Acid Methyl Esters
FFA = Free Fatty Acid
FT-IR = Fourier Transform Infrared Sperctrometer
GC = Gas Chromatography
LOF = Lack of fit
mg = Milligram
µl = Microliter
µm = Micrometer
MT = Metric ton
N = Normality
PFAD = Palm Fatty Acid Distillate
ppm = Part Per Million
RBD = Randomized Complete Block Design
RBD = Refine Bleach Deodorize
RCBD = Randomized Complete Block Design
RCD = Randomized Complete Block Design
RF = Response Factor
rpm = Round Per Minute

LIST OF ABBREVIATIONS (Continued)

RSM = Response Surface Methodology
RT = Retention Time
Sig = Significant
TGA = Triacylglycerol

OPTIMIZATION OF WAX ESTERS PRODUCTION FROM PALM FATTY ACID DISTILLATE AND OLEYL ALCOHOL OVER AMBERLYST 15 AS CATALYST BY RESPONSE SURFACE METHODOLOGY

INTRODUCTION

Oil palm is one of economic plants which are cultivated in the southern and western part of Thailand. The plantation area is 4.4 billion square meters and its value is about 20 billion baht per year (Sitthiporn Kridakara Research Station, 2008). The rate of palm oil's production is 1.35 million ton per year which is 2.99% of the world's production (House Agricultural Magazine, 2011). The major product in palm refinery process is edible oil and also its by-product called palm fatty acid distillate (PFAD). The palm fatty acid distillate consists of free fatty acid as the main composition (about 80 – 90%) and the minor compositions are glyceride, squalene, vitamin E, sterols and other substances. The PFAD fraction is presented about 5 – 7% of crude palm oil. The process of palm fatty acid distillate production is demonstrated in Figure 1.

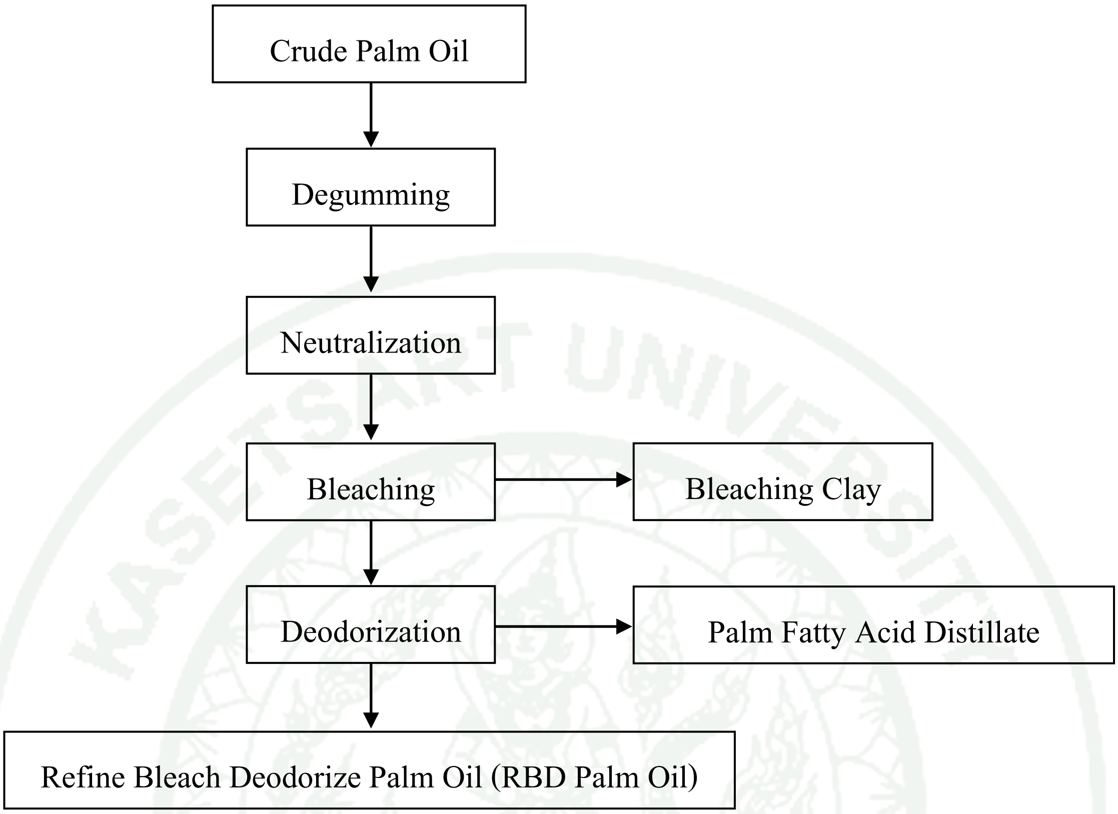


Figure 1 Process of palm fatty acid distillate (PFAD) production

Source: Gunstone (2004)

In fact, the cost of PFAD is cheaper than refine palm oil. The price of palm oil and its derivative are presented in Table 1.

Table 1 The price of palm oil and its derivatives

Year	Price (US$/MT)						
	RBD palm oil	RBD palm olein	RBD palm stearin	RBD palm kernel oil	RBD palm kernel olein	RBD palm kernel stearin	Palm fatty acid distillate
2006	433.50	441.71	407.08	567.46	516.95	865.71	352.21
2007	742.92	755.42	707.92	886.17	862.75	1179.36	552.38
2008	951.92	975.63	794.21	1146.29	1042.70	1430.57	552.33
2009	670.25	694.50	594.71	736.58	689.86	907.93	457.96
2010	876.17	886.08	847.63	1238.54	1163.77	1501.13	718.17
2011	1133.29	1147.21	1028.88	1692.50	1608.63	1882.79	829.88
2012	1071.33	1086.67	969.33	1417.83	1261.83	1775.50	805.00

Note: This data was last updated at March, 2012

Source: Malaysian Palm Oil Board (MPOB) (2012)

Table 1 indicates that PFAD has the lowest price as compared with the others. Thus, developing PFAD to value-added product is necessary. Fatty acid distillate can be generated to several value-added product products. These products are different depending on reaction sides of fatty acid which are alkane side and carboxylic side. The products of both functions are depicted in Figures 2 and 3.

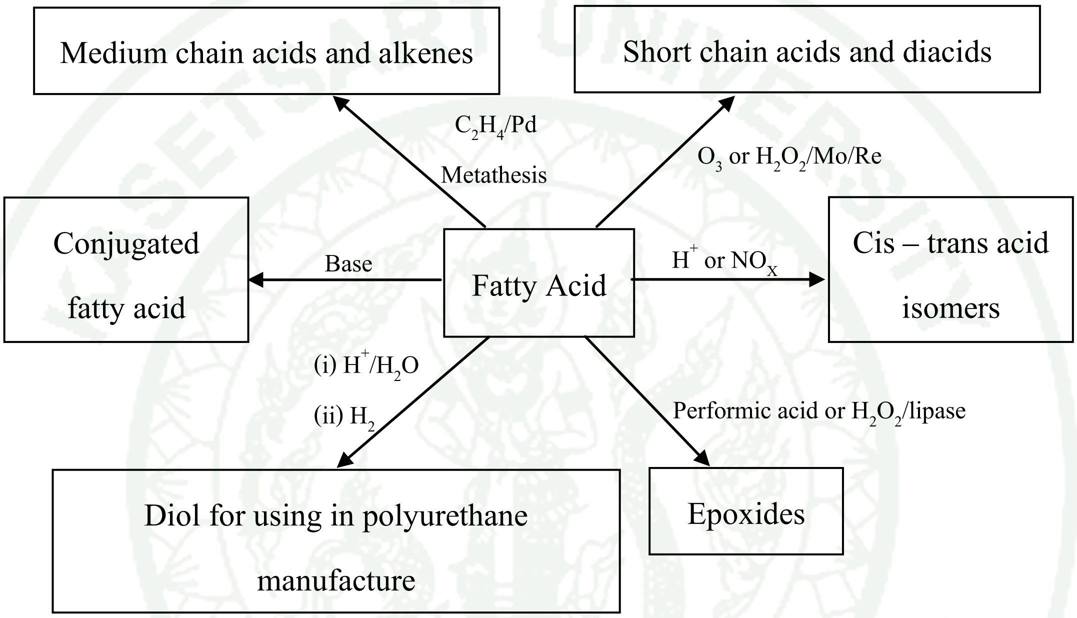


Figure 2 Value – added products from fatty acid (Reaction with alkane function)

Source: Lancaster (2002)

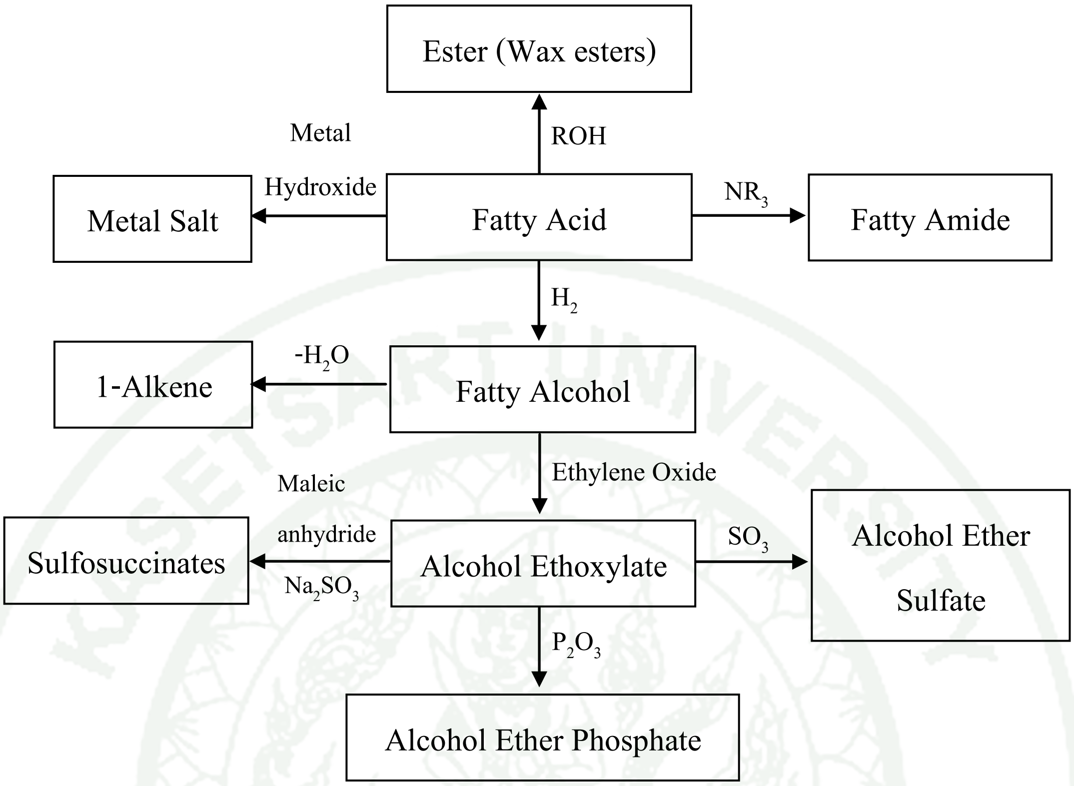


Figure 3 Value – added products from fatty acid (Reaction with acidic function)

Source: Lancaster (2002)

Figure 3 presents wax esters which are one form of value-added product from palm fatty acid distillate. They are synthesized by esterification reaction between long chain fatty acid and long chain alcohol with chain length of 12 or more than 12 carbon atoms (Gunawan, 2005). This reaction is illustrated in Figure 4.

$$\underset{\text{Fatty acid}}{R^{*}COOH} + \underset{\text{Alcohol}}{C_{18}H_{36}O} \overset{\text{Acid catalyst}}{\rightleftharpoons} \underset{\text{Wax esters}}{R^{*}COOC_{18}H_{35}} + H_2O$$

Figure 4 Production of wax esters by esterification reaction

Wax esters are useful in many applications because their excellent wetting behavior at interface and non greasy feeling when applied on skin surface (Hadzir *et al.*, 2001). These compounds are important ingredients in cosmetic formulation such as cleansers, conditioner and moisturizers, in pharmaceuticals such as anti-foaming agent, penicillin production and pharmaceutical tablet and in other chemical industries such as lubricants, plasticizers and polishes (Hallberg *et al.*, 1999).

Wax esters can replace natural waxes that originate from animals, vegetables and minerals such as beeswax. Beeswax which generates from animals is one of several commercial waxes containing high percentage of saturated and unsaturated wax esters. In addition, sperm whale and jojoba oil can be used to produce saturated and unsaturated wax esters (Hallberg *et al.*, 1999). As a result that natural wax esters are expensive and limited quality, the synthesized wax esters via chemical (Aracil *et al.*, 1992) and enzymatic reaction (Trani *et al.*, 1991) are necessary to be investigated.

There are three types of catalysts used for wax esters synthesis. The first type is homogeneous acid catalyst such as sulfuric acid. However, this catalyst may lead to several problems such as corrosion of equipment, high energy consumption and degradable wax esters (Knox and Cliffe, 1984; Yadav and Lathi, 2003). The second type reaction is catalyzed by lipase enzyme which is expensive and difficult to control. Finally, heterogeneous acid catalyst such as Amberlyst 15 is used to generate wax esters. The heterogeneous acid catalyst has more potential than homogeneous and enzyme catalyst because it is easily separated from liquid product, non-corrosive and can reduce environmental problem resulting from washing water for the eliminatation of the unreacted catalyst.

Generally, the determination of optimum condition is proceeded by changing one parameter value while another parameter value is kept constant. But this way cannot investigate the interaction between all parameters. So response surface methodology is a useful theory to solve this problem.

Response surface methodology (RSM) is a collection of statistical and mathematical techniques that are used for developing, improving and optimizing processes. It also has important applications in design and developing of existing product designs.

The most extensive applications of RSM are in industries, particularly in situations where several input variables potentially influence some performance measure or quality characteristic of the product or process. This performance measurement or quality characteristic is called the response. It is typically measured on a continuous scale, although attribute responses, ranks, and sensory responses are not unusual. Most real-world applications of RSM will involve more than one response. The input variables are sometimes called independent variables, and they are subject to the control of the engineer or scientist.

In this research, the synthesis of wax esters is investigated using palm fatty acid distillate with oleyl alcohol as a feedstock. Catalyst used in this reaction is Amberlyst 15 (acidic styrene-divinyl benzene sulfonated ion-exchange resin). A five-level-four-factor central composite design (CCD) is performed on RSM to reveal the optimum conditions for synthesis and analyze the interaction of various parameter values. The variables consist of the amount of Amberlyst 15, oleyl alcohol-to-PFAD molar ratio, reaction time and reaction temperature. The dependent variables of this RSM include two values. The first response value is percentage conversion which is calculated by comparing initial and final content of free fatty acid. The second response value is percentage conversion which is determined by gas chromatography. The FT-IR is used to confirm the wax esters product from its functional groups. The physicochemical properties of wax esters are also investigated. In addition, vitamin E content, reusability of Amberlyst 15 and esterification reaction without catalyst are also studied.

OBJECTIVE

The objective of this research is to investigate wax esters synthesis by using response surface methodology (RSM) to find the optimum condition and study the relationship between variable values. The four variables for RSM include the amount of Amberlyst 15, oleyl alcohol-to-PFAD molar ratio, reaction time and reaction temperature.

LITERATURE REVIEW

1. Palm Fatty Acid Distillate (PFAD)

The PFAD is a by-product from physical refining of crude palm oil (CPO). Normally, the physical refining processes of CPO include these steps: degumming, bleaching, deacidification and deodorization steps. This process gives refined bleached deodorized palm oil (RBD palm oil) product as presented in Figure 1. The propose of deacidification or deodorization step is to remove free fatty acid and odor of the oil in order to make the vegetable oil suitable for human consumption. The last step is performed by steam distillation under high vacuum pressure. The distilled fatty acid is known as palm fatty acid distillate which has highly odorant product and also contains some glycerides, squalene, vitamin E (tocopherols and tocotrienols) and sterols as a minor product. PFAD is a light brown solid at room temperature and converted to a brown liquid at high temperature (Top, 2010). The composition and physicochemical properties of PFAD are shown in Tables 2 and 3, respectively.

Table 2 The typical composition of palm fatty acid distillate

Composition	Amount (%wt)
Free fatty acid	81.7
Glycerides	14.4
Vitamin E	0.5
Squalene	0.8
Sterols	0.4
Other	2.2

Source: Top (2010)

Table 3 Physicochemical properties of PFAD

Properties	Value
Iodine value (I_2/100 g)	51.2 – 57.4
Free fatty acid (%) (as palmitic acid)	72.3 – 89.4
Moisture content (%)	0.05 – 0.15
Unsaponification matter (%)	1.5 – 3.4
Saponification matter (%)	190.7 – 203.5
Mass per volume (kg l^{-1})	0.8640 – 0.8880
Fatty acid composition (%wt)	
Lauric acid (C12:0)	0.1 – 0.3
Myristic acid (C14:0)	0.9 – 1.5
Palmitic acid (C16:0)	42.9 – 51.5
Steric acid (C18:0)	4.1 – 4.9
Oleic acid (C18:1)	32.8 – 39.8
Linoleic acid (C18:2)	8.6 – 11.3
Linolenic acid (C18:3)	0.2 – 0.6

Source: Top (2010)

The utilization of PFAD could be divided into two main categories. First, PFAD was used as itself such as animal feeds and laundry soap. Due to PFAD is the cheapest raw material comparing with other substances. In this case, the PFAD is reacted with calcium hydroxide to obtain calcium salt which is known in the name of “CaPFAD”. It is used for animal feed. This product is not digested in the first digestive compartment (rumen) of ruminants (e.g. cows) but it is broken down to fatty acid in the abomasums. The fatty acids are absorbed by small intestine and animal utilizes it for milk production or energy source. The other advantages are low linolenic acid content which is promoted rancidity. PFAD contained about 10% of linoleic acid which was essential fatty acid (EFA) and also included vitamin E as an antioxidant. In addition, PFAD could also be estertified to generate product for oleochemicals industry and synthesized to biofuel (Top, 2010).

On the other hand, PFAD is used as a source of vitamin E, squalene and phytosterols. Vitamin E consist of tocopherols which has saturated side chain and tocotrienols which has unsaturated side chain. Both are important for the antioxidant activities in food and biological system. Palm vitamin E is a tocotrienols rich product which is a beneficial effect such as anti-cancer (e.g. breast, prostate), hypocholesterolemic and neuron protection activities (Top, 2010). The chemical structures of tocopherols and tocotrienols is presented in Figures 5 and 6.

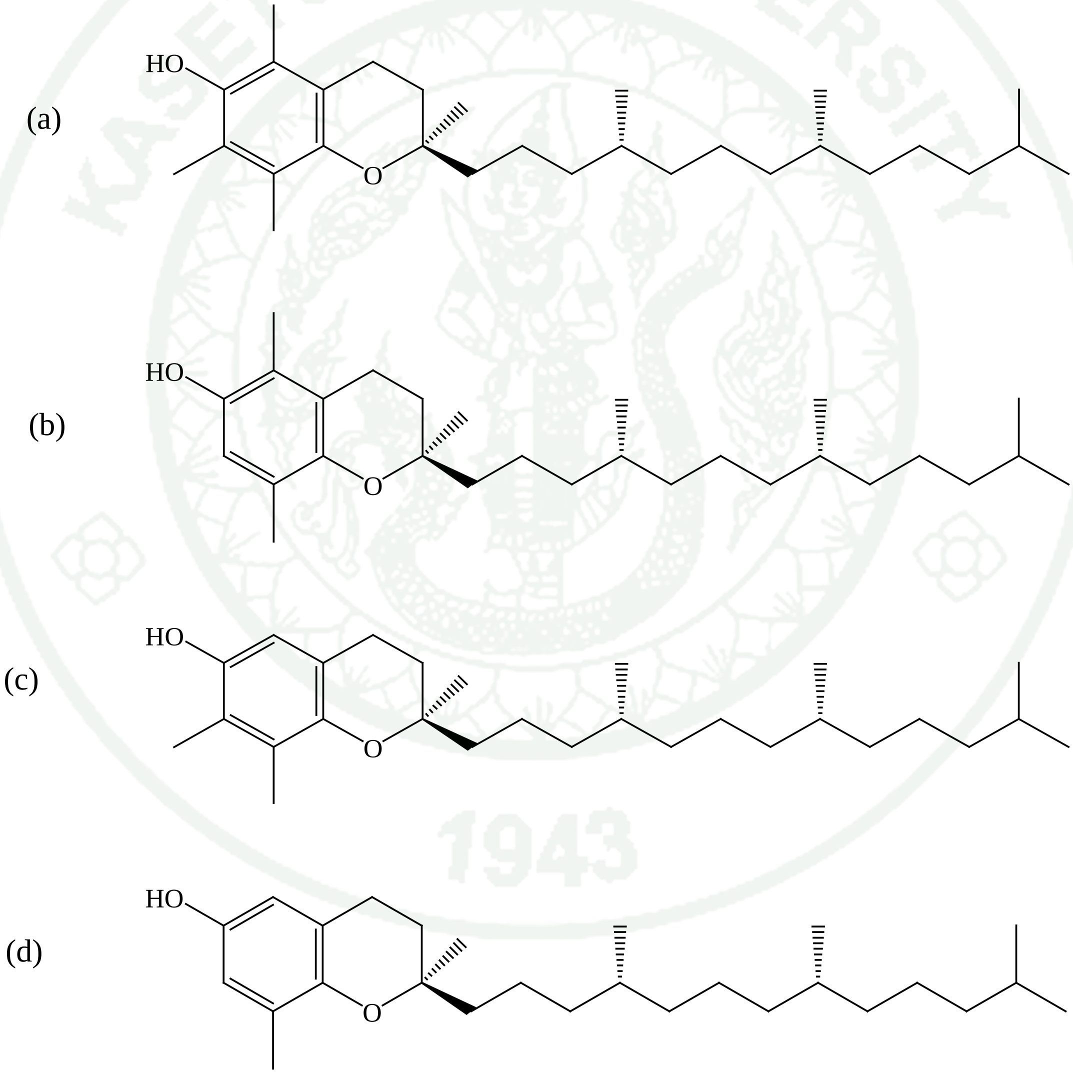


Figure 5 Chemical structures of tocopherols; (a) α-tocopherol; (b) β-tocopherol; (c) γ-tocopherol; (d) δ-tocopherol

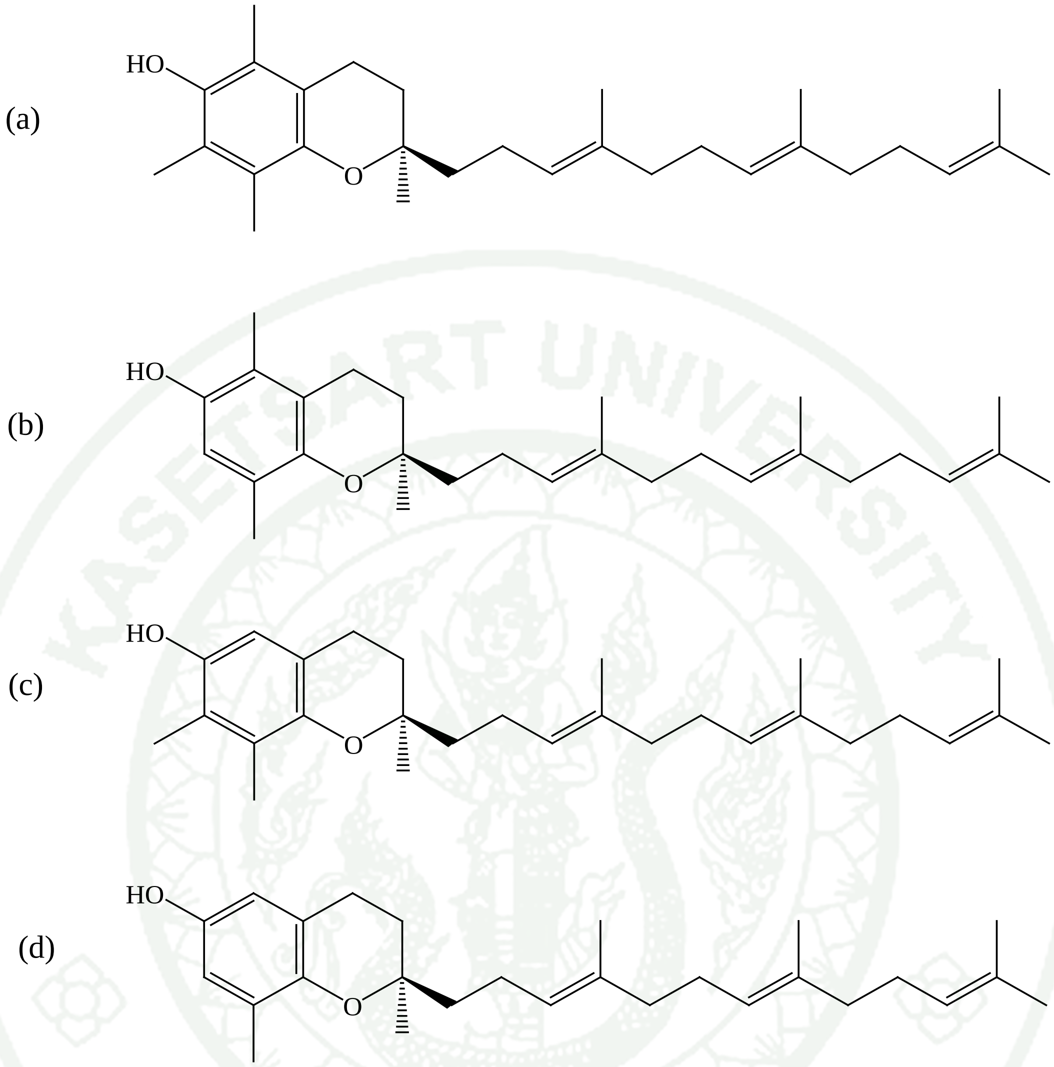


Figure 6 Chemical structures of tocotrienols; (a) α-tocotrienol; (b) β-tocotrienol; (c) γ-tocotrienol; (d) δ-tocotrienol

Squalene is a natural emollient and used as a cosmetic’s ingredient. The other utilizations of squalene are in health food industry and pharmaceutical industry. Normally, the major sources of squalene come from olive oil industry and shark liver oil industries which are limited sources, right now. Hence, PFAD is one choice of squalene source (Top, 2010). The chemical structure of squalene is illustrated in Figure 7.

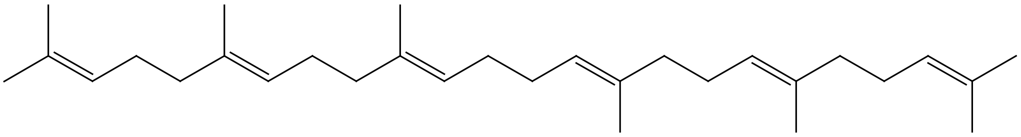
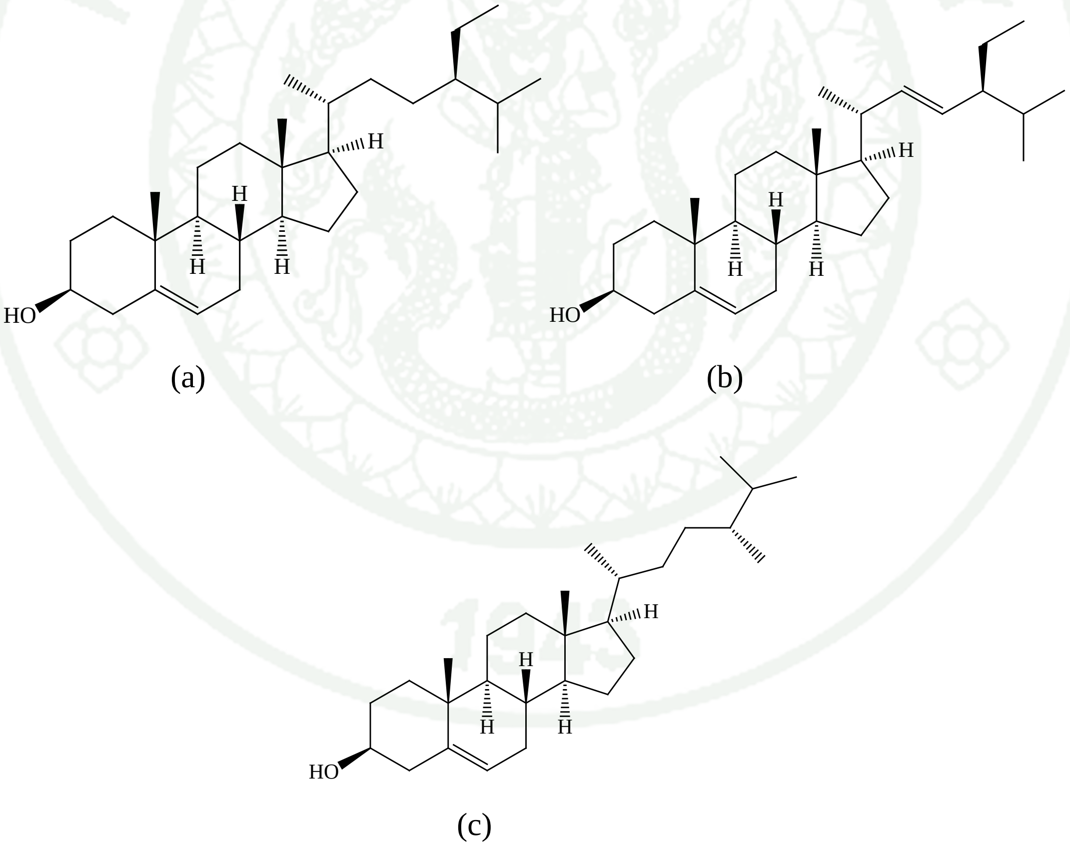


Figure 7 Chemical structure of squalene

The major components of palm phytosterols are β-sitosterol, stigmasterol and campesterol. The phytosterols could reduce cholesterol absorption, both of total serum and LDL cholesterol levels in animals and humans. In addition, phytosterols also showed the anti-cancer properties (Top, 2010). The structures of β-sitosterol, campesterol and stigmasterol are presented in Figure 8.



(a)

(b)

(c)

Figure 8 Chemical structures of; (a) β-sitosterol; (b) stigmasterol; (c) campesterol

2. Amberlyst 15 catalyst

Amberlyst 15 catalyst is a strongly acidic ion exchange resin developed particularly for heterogeneous acid catalysis in a variety of organic reactions. It is also used in non aqueous ion exchange system to remove of cationic impurities. In aqueous system, the catalyst activity decreased by the effect of water. The applications of Amberlyst 15 catalyst are alkylation, esterification, etherification, condensation and hydrolysis reactions (Rohm and Haas, 2005).

Amberlyst 15 catalyst is styrene divinyl benzene sulphonate. In this research, it performs the same sulfuric acid as a homogeneous catalyst to catalyze the esterification reaction for wax esters synthesis. The advantages of Amberlyst 15 catalyst are that it is easily separation from mixture and it can be reused with negligible loss of activity. The properties of Amberlyst 15 catalyst are presented in Table 4.

Table 4 Properties of Amberlyst 15 catalyst

Properties	Value
Physical form	Opaque beads
Ionic form	H^+
Concentration of acid site (eq/kg)	≥ 4.7
Water content (%)	≤ 1.6
Nitrogen BET	
Surface area (m^2/g)	53
Average pore diameter ($\mathring{A}$)	300
Total pore volume (cc/g)	0.4
Maximum temperature (^0C)	120

Source: Rohm&Haas (2005)

This catalyst is synthesized by the copolymerization of styrene with divinylbenzene which acts as a cross-linking agent. The number of cross-links has an effect on the catalyst’s ability to swell when immersed in solution (Lattikainen *et al*., 2002). The reaction is presented in Figure 9.

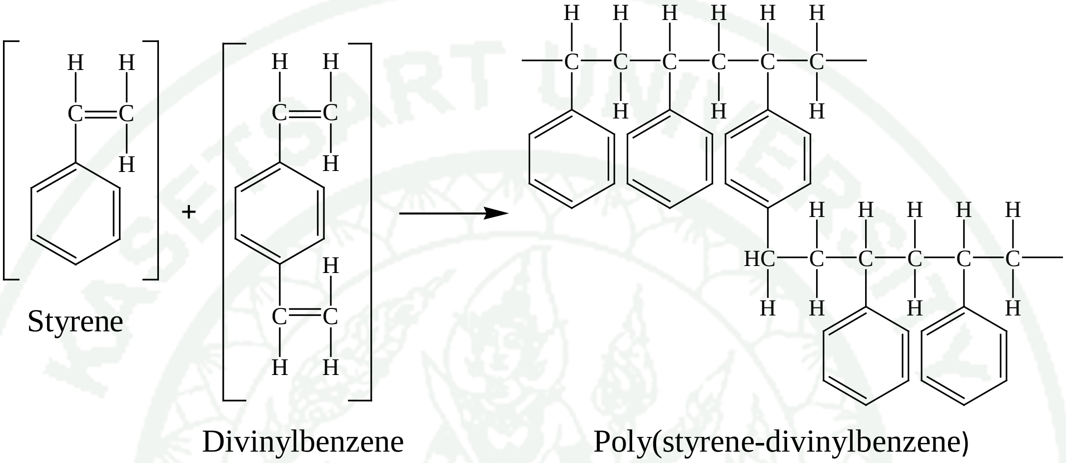


Figure 9 The copolymerization of styrene and divinylbenzene

Source: Helfferich (1962)

After that, the active site is needed to be placed on the resin matrix. It is treated with a strong acid to deposit on acid sites of polymer matrix. For the formation of sulphonated cation exchange resin, the polymer matrix is treated with concentrated sulfuric acid. The acid loading on a resin is limited catalyst activity and it is role as an important part for catalytic reaction (Chakrabarti and Sharma, 1993). The formation of sulphonated cation exchange resin is illustrated in Figure 10.

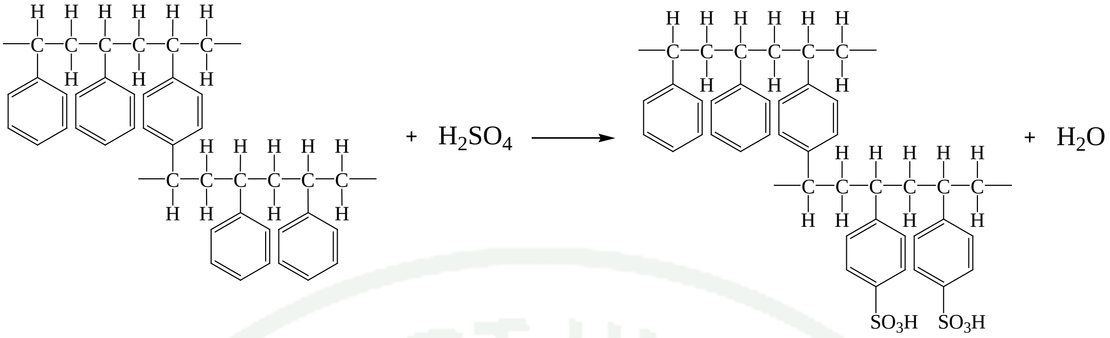


Figure 10 The sulphonation reaction of polymer matrix

Source: Schwarzer (2006)

The heterogeneous catalyst has more advantage than homogeneous catalyst as follows (Harmer and Sun, 2001):

1. It reduces equipment corrosion.

2. It is easily separated, thus decreasing the cost of separation.

3. It reduces the possibility for the contamination of recycle and product stream.

4. The selectivity of heterogeneous catalyst is better than homogeneous catalyst.

5. It could result in many process options available for engineer.

3. Wax esters

Wax esters have high-molecular weight and they usually occur from the combination of long chain aliphatic alcohol and long chain fatty acid. Besides aliphatic alcohols, the cyclic alcohols such as sterols can exist in wax esters molecule too. In the metabolism of natural organism, there is a relationship of equilibrium between acid, alcohol, esters and hydrocarbon together. Thus, all four components are encountered in several of waxes. Naturally, these esters have even number of carbon atoms (Warth, 1947). Wax esters could be used in several applications such as a raw material for cosmetic industry, coating material for pharmaceutical and food industry, lubricants, polishes, plasticizers and other chemical industry (Hallberg *et al*., 1999). The unique properties of wax esters are excellent wetting behavior at interface, non-oily feeling when applied on skin, non-toxic and fat soluble properties (Hadzir *et al*., 2001). The example of chemical structure of wax esters is presented in Figure 11.

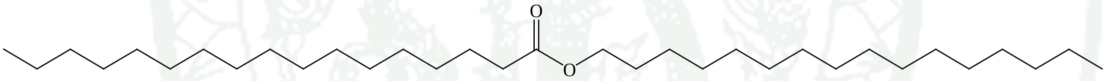


Figure 11 The chemical structure of wax ester

Wax esters can be divided into two main groups. The first group is natural wax esters such as beeswax, spermaceti, lanolin, carnauba wax and jojoba oil. Beeswax is secreated by bees (*Apis mellifera*). Spermaceti is extracted from the head of a cachalot and also known as sperm whale. Lanolin is secreted by sheep sebaceous gland. Carnauba wax is presented on the leave of Brazilian palm trees. It protects the water losing on palm’s leave. Jojoba oil is extracted from jojoba plant (*Simmondsia chinensis*). The second group is synthesized wax esters. These wax esters can be synthesized by the following detail.

3.1 Synthesized wax esters

The synthesized wax esters are prepared by esterification or transesterification reaction. Chemical or enzyme catalysts can be used in both reaction as follow:

3.1.1 Chemical catalyst

The chemical catalysts can be homogeneous and heterogeneous catalysts. For the homogeneous catalyst, the wax esters are synthesized by fatty acid or triglyceride as starting materials. The fatty acid can generate the wax esters through esterification reaction. A catalyst in this reaction is usually acid such as sulfuric acid. The reaction is presented in Figure 12.

Figure 12 The esterification reaction

The mechanism of esterification reaction contains two mechanism parts which are acid-catalyzed addition of the alcohol to the carbonyl group and acid-catalyzed dehydration. For the first part, hydronium ion is protonated on oxygen of carbonyl group. Then, the alcohol is added at carbon of carbonyl group. The last step of the first part is deprotonation and gives esters hydrate as a product. For the second mechanism part, oxygen atom is protonated. Then, the molecule is rearrangement to lose water molecule. Finally, intermediated molecule is deprotonated to generate an esters product. Both mechanism parts of esterification reaction are presented in Figure 13.

Part 1: Acid-catalyzed addition of the alcohol to the carbonyl group.

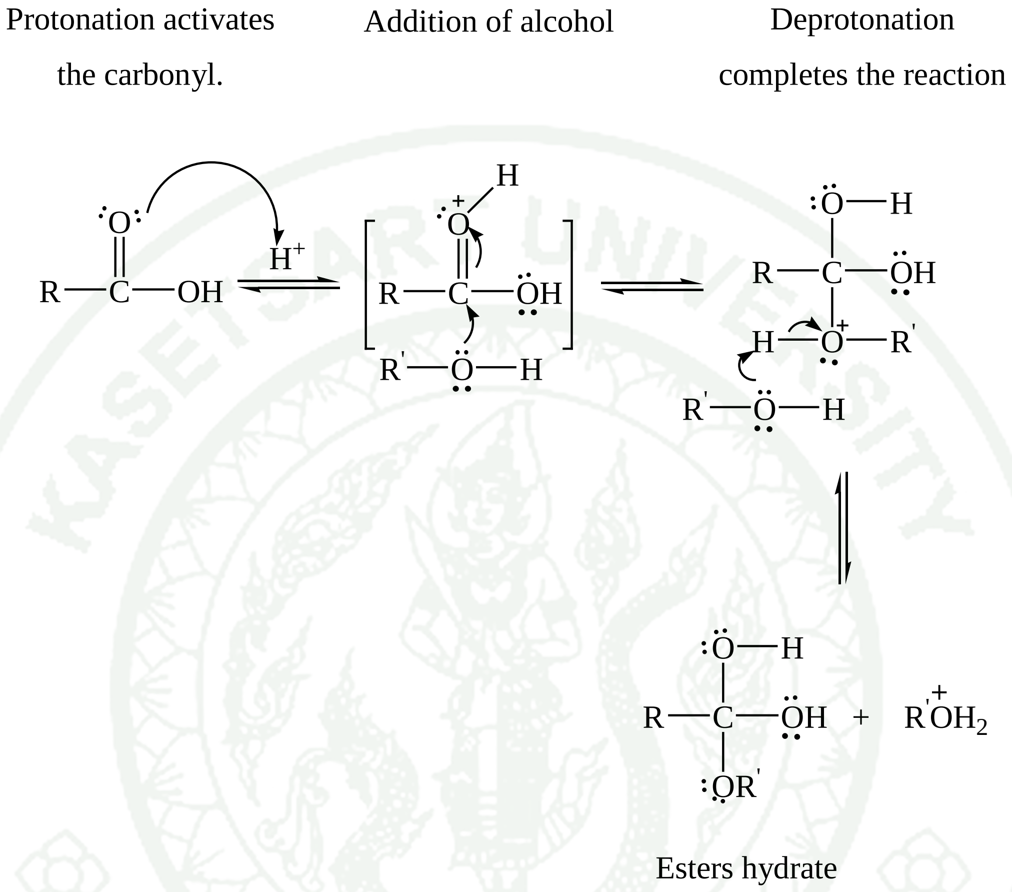


Figure 13 The mechanism of esterification reaction

Part 2: Acid-catalyzed dehydration.

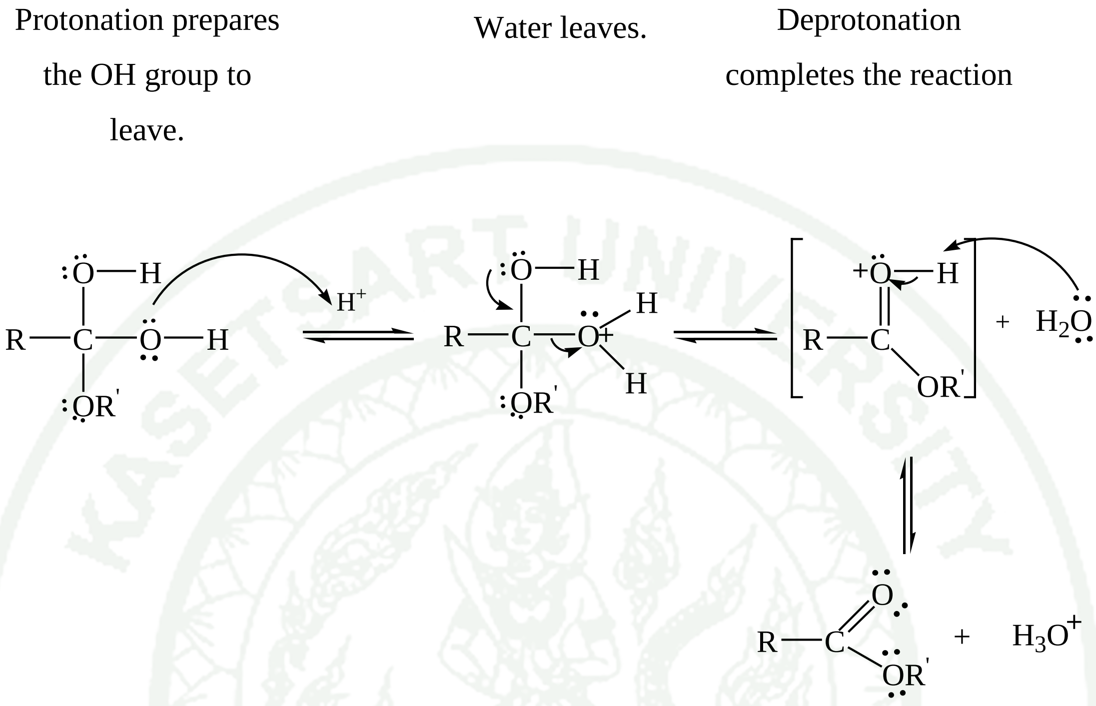


Figure 13 (Continued)

Source: Wade (2006)

For the triglyceride raw material, the transesterification reaction is usually used to synthesize wax esters. Both types of catalyst can be used in this reaction. They include acid-catalyzed such as sulfurous acid or sulfuric acid and basic catalyst such as sodium hydroxide or potassium hydroxide are normally used. The transesterification reaction is illustrated in Figure 14.

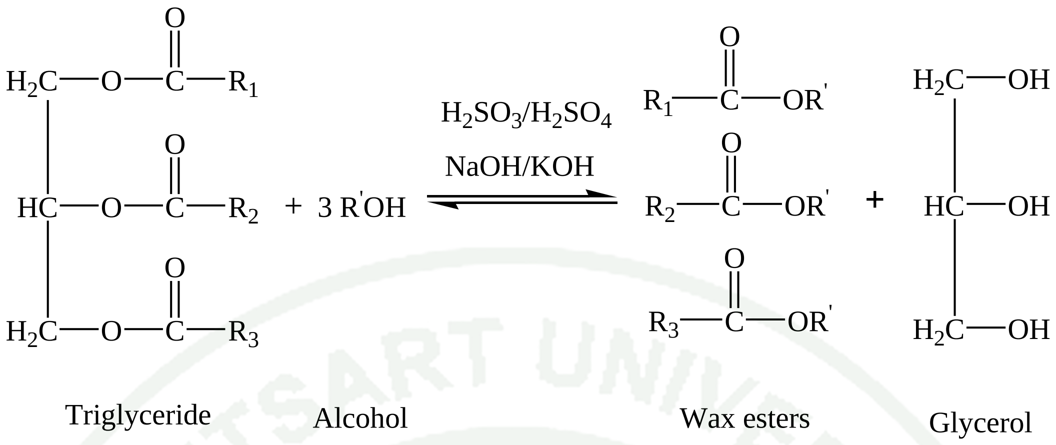


Figure 14 The transesterification reaction

The acid-catalyzed transesterification reaction gives a high yield of alkyl esters but the reaction is slow and performs at high temperature. Normally, the temperature must be above 100 ^{0}C and more than 3 h of reaction time is required to complete the reaction. The starting mechanism is the protonation of carbonyl group of triglyceride and leads to carbocation (II). Then, the nucleophilic of alcohol attacks a carbocation to produce the tetrahedral intermediate (III). Finally, molecule forms esters (IV) and eliminates a glycerol. According to this mechanism, carboxylic acid can be generated by the reaction of carbocation (II) with water in reaction mixture resulting in decreasing of esters yield. Thus, the acid-catalyzed transesterification should be performed under the absence of water to reduce the competitive reaction (Schuchardt *et al*., 1998). This mechanism is shown in Figure 15.

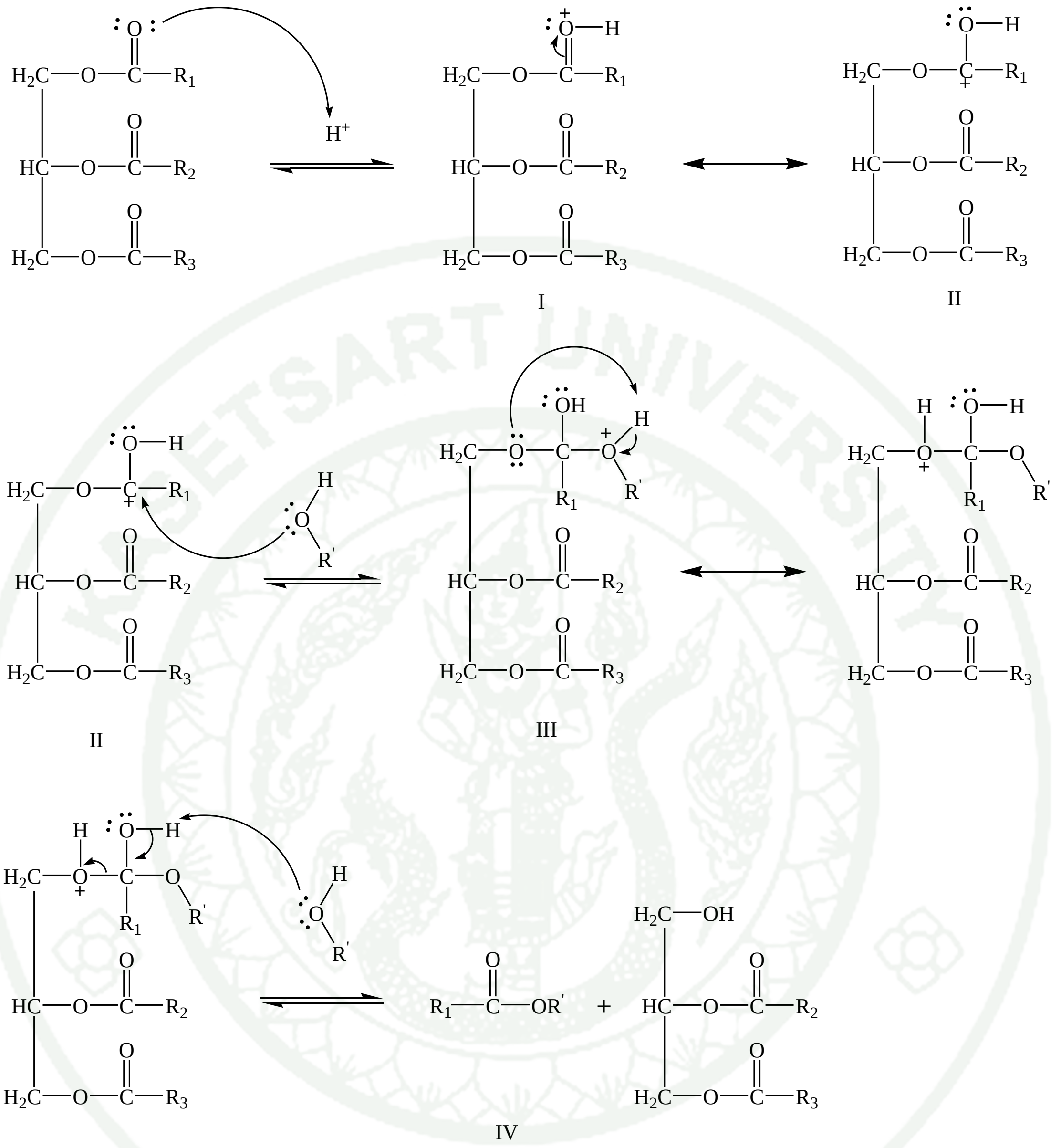


Figure 15 The acid-catalyzed mechanism of transesterification reaction

Source: Schuchardt *et al*. (1998)

The basic-catalyzed transesterification is faster than acid-catalyzed transesterification. The first step of basic-catalyzed transesterification is the reaction between base and alcohol to produce an alkoxide as (1). This alkoxide attacks at the carbon atom on the carbonyl group of triglyceride and gives tetrahedral intermediate as (2). This intermediate can form alkyl esters and anions of the diglyceride as (3). Lastly, anion of the diglyceride is reacted with water to produce diglyceride and hydroxide ion for using as a catalyst again. The diglyceride and monoglyceride are converted by the same mechanism to give esters and glycerol as a final product (Schuchardt *et al*., 1998). This mechanism is presented in Figure 16.

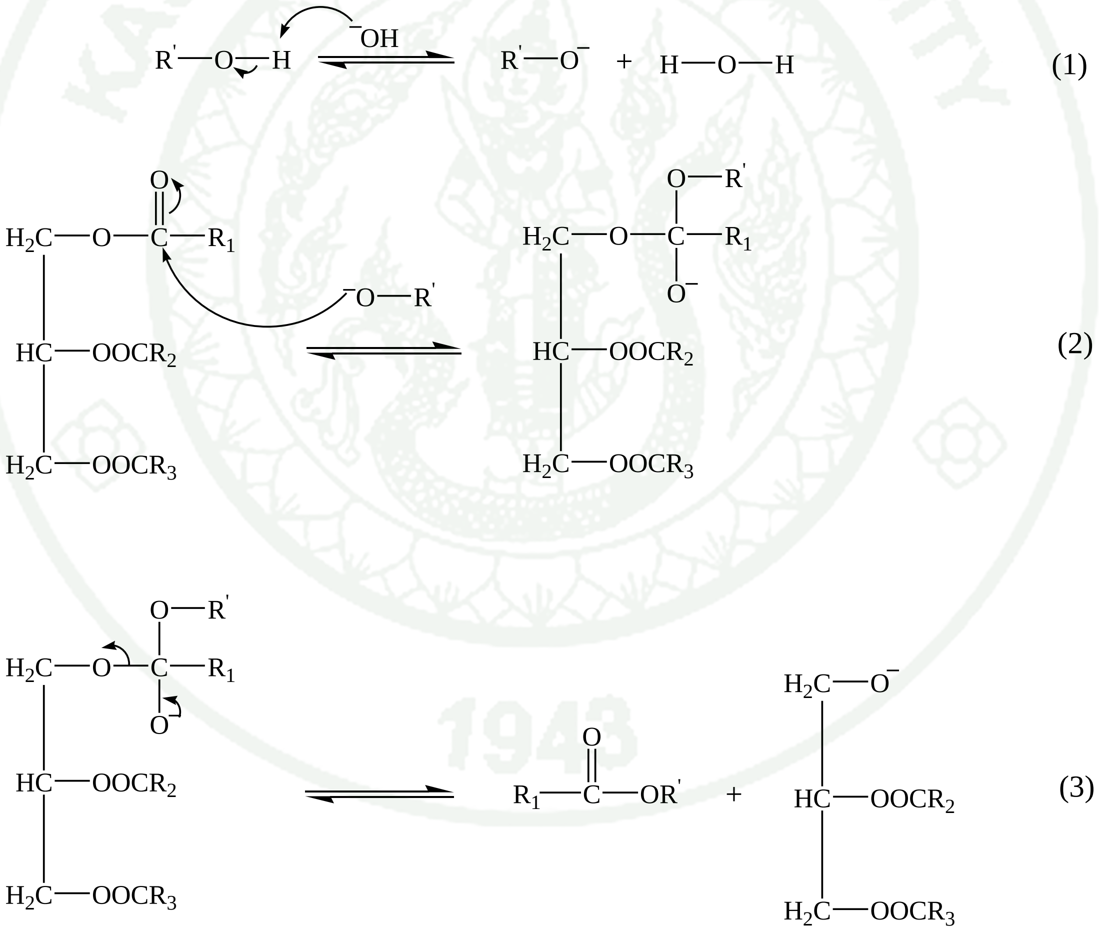
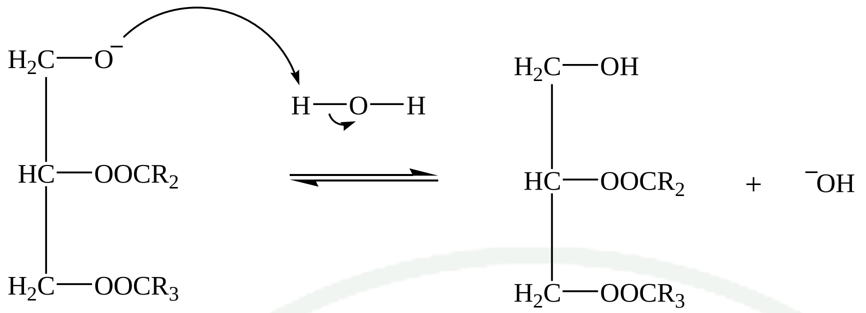


Figure 16 The basic-catalyzed mechanism of transesterification reaction

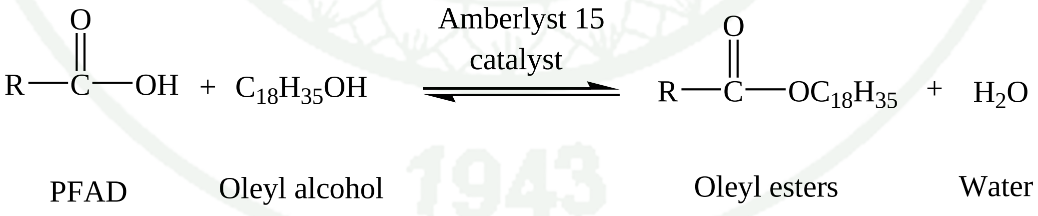


(4)

Figure 16 (Continued)

Source: Schuchardt *et al*. (1998)

The heterogeneous catalyst is acid coating on solid support. When comparing between both catalysts, it finds that homogeneous catalyst is cheaper than heterogeneous catalyst but the heterogeneous catalyst can be recovered to use again. In addition, there is no need to wash the product with water. Thus, the heterogeneous catalyst through esterification reaction is studied in this work. The mechanism of the reaction is similar to the homogeneous-acid-catalyzed esterification reaction, except that this reaction is performed on the surface of hetergoneous catalyst. The chemical reaction and its mechanism are shown in Figures 17 and 18, respectively.

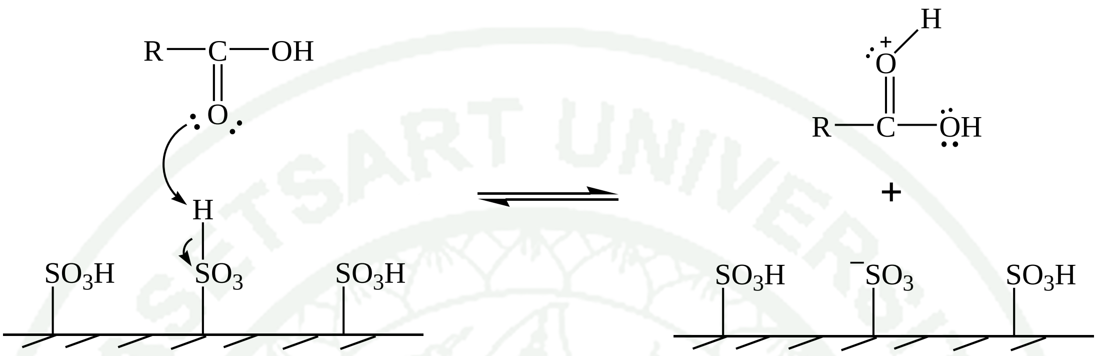


$$R-\overset{\overset{\displaystyle O}{\|}}{C}-OH + C_{18}H_{35}OH \xrightleftharpoons[]{\text{Amberlyst 15 catalyst}} R-\overset{\overset{\displaystyle O}{\|}}{C}-OC_{18}H_{35} + H_2O$$

Figure 17 The esterification reaction using heterogeneous acid catalyst

Part 1: Acid-catalyzed addition of the alcohol to the carbonyl group.

Protonation activates the carbonyl.



The alcohol is adds.

Deprotonation completes the reaction.

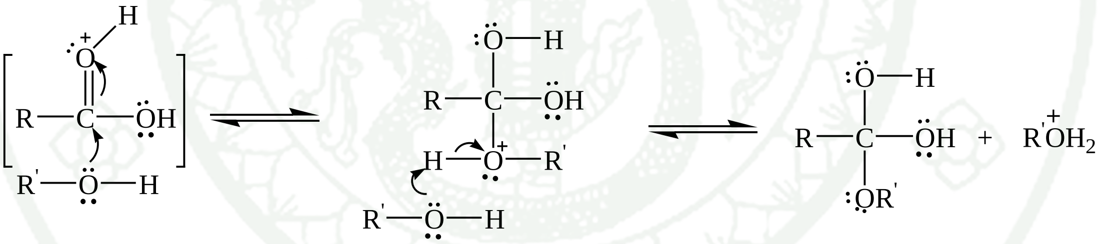


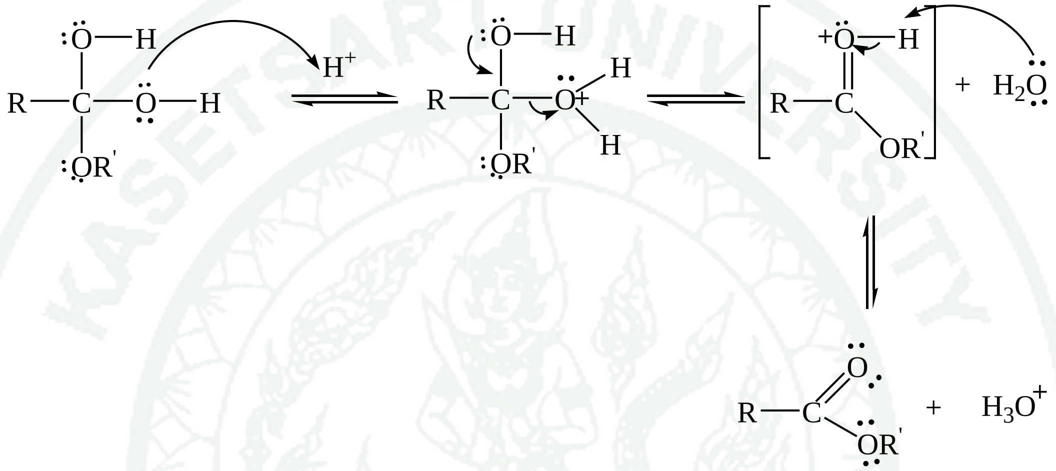
Figure 18 The mechanism of heterogeneous acid catalyst through esterification reaction

Part 2: Acid-catalyzed dehydration.

Protonation prepares the OH group to leave.

Water leaves.

Deprotonation completes the reaction



Part 3: The regenerated of catalyst

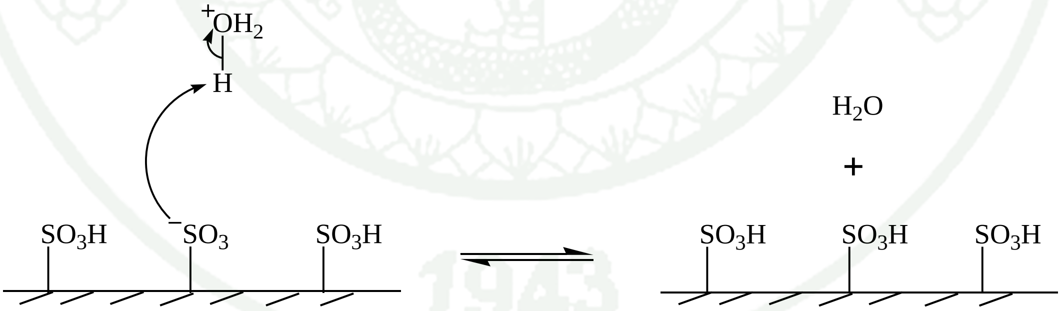


Figure 18 (Continued)

3.1.2 Enzymatic catalyst

This catalyst is usually lipase enzyme. The advantage of enzymatic reaction is performed under mild condition but the disadvantage is high cost. Moreover, enzyme is in good performance under specific pH and suitable temperature because unspecific pH and unsuitable temperature might promote the denature of enzyme. Lipases enzyme are a subclass of the esterase enzyme. Typically, the enzyme contains several amino acids linking together through peptide bond. Thus, lipases could be different depending on the amino acid content. The wax esters can be synthesized from triglyceride as raw material using enzymatic alcoholysis reaction which is the same as transesterification reaction. The product is generated through acyl-enzyme complex. The reaction and mechanism of enzyme are presented in Figures 19 and 20, respectively.

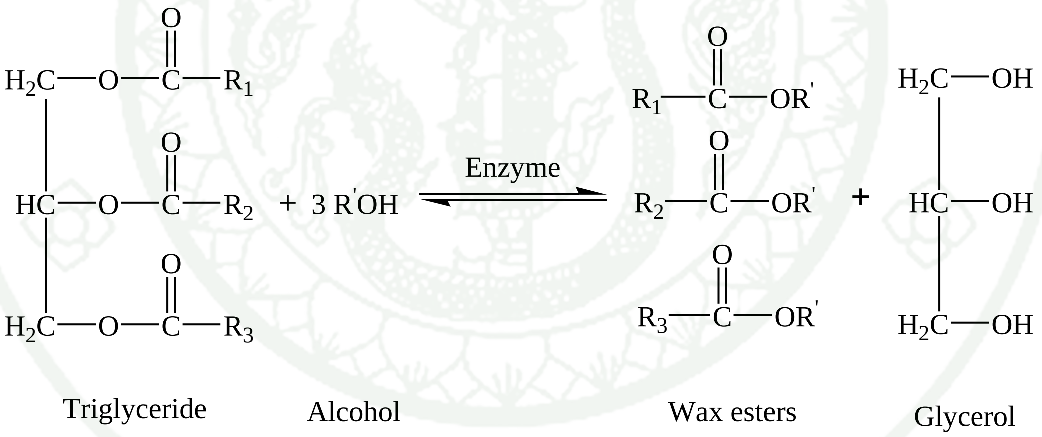


Figure 19 The alcoholysis reaction

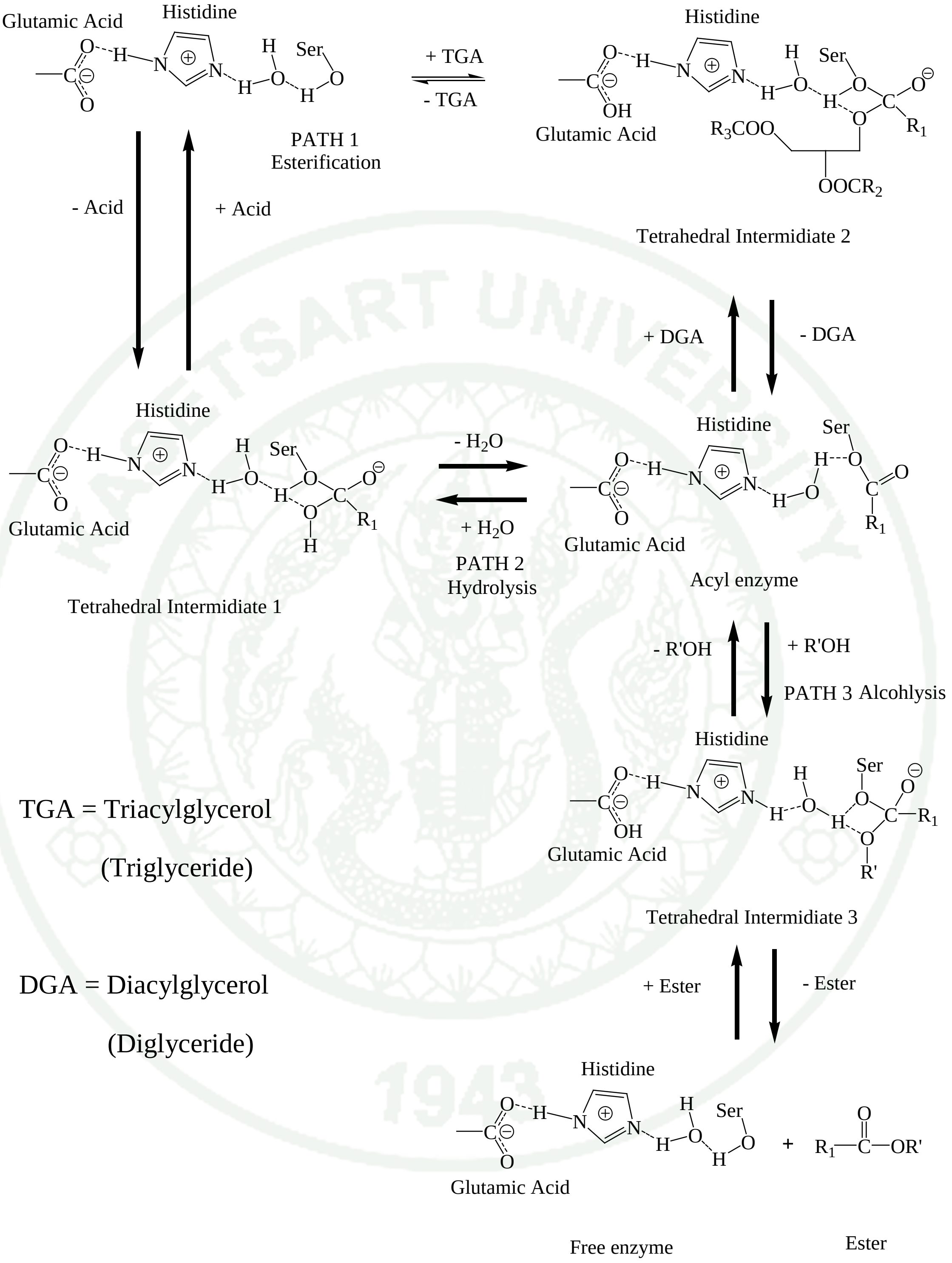


Figure 20 The mechanism of enzymatic reaction

Source: Gudiño *et al*. (2012)

3.2 The effect of factors on synthesis of wax esters

Several researches have reported the factors affecting was esters synthesis, including are catalyst amount, molar ratio of substrate, reaction time, reaction temperature and agitation speed.

3.2.1 Effect of catalyst amount

The function of catalyst is to reduce the activation energy and drives the reaction faster than the reaction without the catalyst.

3.2.2 Effect of molar ratio of substrate

Esterification and transesterification are reversible reactions. Both starting materials are alcohol and fatty acid that are huge molecules. So, they are difficult to react with each others. From the Le Chatelier’s principle, increasing of substrate molar ratio will drive the equilibrium to forward reaction.

3.2.3 Effect of reaction time

The longer reaction time increases percentage yield of wax esters product until the optimum condition is met. After that, reaction time does not affect wax esters synthesis.

3.2.4 Effect of reaction temperature

Increasing the reaction temperature will increase the kinetic energy of substrate molecules. The molecules move faster than the molecule at low temperature. The collision between substrate molecules at high temperature is more often than low temperature. Thus, the reaction at higher temperature generates more products than the one at lower temperature.

3.2.5 Effect of agitation speed

The main purpose of agitator is to homogenize substrate and catalyst. Higher agitation speed can increase molecular movement resulting in better combination of substrate and catalyst.

3.3 The properties of synthesized wax esters

After complete reaction, the composition and quantity of the synthesized wax esters yield are usually analyzed by using gas chromatography (GC). The first step of GC method is performing the calibration curve of each ester which depends on fatty acid compositions containing in raw material. Methyl laurate is used as internal standard for this study. After that, the properties wax esters are analyzed such as reflective index, density, surface tension, slip melting point, saponification value, iodine value and acid value. Moreover, the wax esters might be performed the specific test for those applications such as the irritancy test and moisturizing test for wax esters using in cosmetic’s ingredient. Every test must be performed under standard method. The physicochemical characteristics of wax esters derived from various palm oil raw material are presented in Table 5.

Table 5 Physicochemical characteristic of palm oil and palm-based esters

Characteristic	Palm oil	Palm oil esters	Palm olein esters	Palm sterin esters	Palm kernel oil esters	Palm kernel olein esters
Reflective index	1.465[a]	1.456[a]	1.457[a]	1.453[a]	1.453[a]	1.456[a]
Density (g/cm^3)	0.910[a]	0.839[a]	0.838[a]	0.841[a]	0.810[a]	0.824[a]
Surface tension (mN/m)	31.0[a]	27.0[a]	27.2[a]	27.7[a]	26.7[a]	28.3[a]
Slip melting point (^{O}C)	34.2	23.9	16.4	27.1	9.8	12.1
Saponification value (mg KOH/g)	195.7	93.1	84.0	96.5	102.1	105.0
Iodine value (g I_2/100g)	52.9	69.7	71.6	62.1	58.8	61.3
Acid value (mg KOH/g)	<1.0	0.6	0.8	0.7	1.2	1.2

Note: [a] The analyses were conducted at 25 ^{O}C

Source: Keng *et al*. (2009)

Wax esters synthesis has been studied by several researchers which could be summarized as following:

Decagny *et al.* (1998) studied the wax esters synthesis between triolein and stearyl alcohol through enzymatic reaction. The various types of lipase enzyme were also investigated. The result showed that lipase from *Alcaligenes* sp. and *Chromobacterium viscosum* gave the best yield which was about 53%.

Poisson *et al*. (1999) studied the synthesis of wax esters through enzymatic reaction by comparing five types of lipases. Milk fat and oleyl alcohol were used as substrates. *Alcaligenes* sp. and *Chromobacterium viscosum* showed the highest yield of synthesized wax esters. It gave about 60% of yield within 2 and 48 hours of reaction time.

Steinke *et al.* (2000) reported transesterification of ceambe oil and camelina oil with n-octanol or isopropanol using Novozyme 435, Lipozyme IM and papaya latex lipase as a biocatalyst. The result showed that Novozyme 435 obtained the highest conversion to alkyl esters. The conversion using Novozyme 435 as catalyst was 98% which Lipozyme IM and papaya latex lipase gave about 40 – 50% conversion. The conversion of long chain alcohol was higher than medium chain one. The isopropyl esters of ceambe oil and camelina oil catalyzed by Novozyme 435 and Lipozyme IM had low conversion and these esters could not be formed when the reaction was catalyzed by papaya latex lipase.

Hadzir *et al*. (2001) studied the production of oleyl oleate. The alcoholysis reaction was performed by Lipozyme IM and Novozyme using triolein and oleyl alcohol as a starting material. The effect of parameters such as reaction time, reaction temperature, amount of enzyme, molar ratio of substrate, various organic solvents used and initial water activities were investigated. The optimum conditions were 5 hours of reaction time, 50 ^{O}C for Lipozyme IM and 60 ^{O}C for Novozyme, 0.3 g of enzyme weight and 6:1 of oleyl alcohol-to-triolein molar ratio. The best solvents were heptane and hexane. The wax esters prepared in the optimum condition using Lipozyme as catalyst gave 75.66% yield.

Salis *et al*. (2003) studied the synthesis of wax esters by lipase-enzyme catalyst. Triglyceride containing in sheep milk fat was reacted with cetyl alcohol using n-hexane as a solvent. The products were cetyl myristate, cetyl palmitate, cetyal oleate and cetyl stearate which were determined by HPLC/ELSD. The result indicated that Novozyme 435 was less active than Lipozyme *RM* IM.

Radzi *et al*. (2005) studied esterification reaction to synthesis oleyl oleate from oleic acid and oleyl alcohol by using lipase enzyme as a catalyst. The result showed that the optimum condition produced more than 90% of wax esters at 30 minutes of reaction time, 50 ^{O}C of reaction temperature, 90 g of enzyme amount, 400 rpm of agitation speed, two numbers of impeller tips and 1:2 of molar ratio of oleic acid to oleyl alcohol.

Sin *et al*. (2008) studied the synthesis of palm esters from palm oil fraction with oleyl alclohol through lipase enzyme tranesterification for cosmetic industry. The result showed that the optimum condition gave more than 80% yield when the reaction was run about 5 hours.

Chobset (2010) studied the production of palm wax esters from palm fatty acid distillate with oleyl alcohol by using amberlyst 15 as a catalyst. Chobset investigated the optimum condition with five variables. The experiments were done by changing one parameter whilst another parameter was kept constant. The result showed that the optimum condition was 1 hour of reaction time, 60 ^{O}C of reaction temperature, 30% w/w of amount of catalyst, 1:2 molar ratio of PFAD to alcohol and 250 rpm of agitation speed. This condition gave about 56% of wax esters yield.

The wax esters were further identified by gas chromatograph. The properties of wax esters were determined followed standard method. The summary of these works were shown as following:

Allawzi *et al.* (1998) investigated physicochemical characteristic and thermal stability of Jordanian jojoba oil as natural wax esters. Results showed that jojoba oil had low ash and water content but both flash point and viscosity index were high value. Viscosity and specific gravity were slightly changed with increasing temperature. The thermal stability was studied in the temperature range of 40 – 200 ^{O}C. The result showed that the chemical structure, kinematic viscosity and refractive index remained constant under these temperatures.

Gunawan *et al.* (2005) studied the synthesis of palm-based wax esters by enzymatic reaction and optimization using the RSM method. The wax esters were analyzed by gas chromatograph (GC). The GC was performed using Rtx-65TG capillary column and helium as a carrier gas. The temperature program was maintained at 150 ^{O}C for 2 minute, increased to 300 ^{O}C with the ramping rate of 20 ^{O}C/minute and held constant at 300 ^{O}C for 10 minutes. It showed that the percentage yield at optimum condition was 84.6%.

Sin *et al.* (2005) studied the scale-up synthesis of palm-based wax esters through enzymatic reaction and the use of RSM for optimization. The gas chromatograph (GC) with Rtx-65TG capillary column was used to determine wax esters. The temperature of injector and detector were set at 250 ^{O}C and 300 ^{O}C, respectively. Oven temperature was maintained at 150 ^{O}C for 2 minutes, increased to 300 ^{O}C with the ramping rate of 20 ^{O}C/minute and held constant for 14 minutes. Nitrogen was used as a carrier gas. This condition of GC presented 92.3% of percentage yield.

Sin *et al.* (2008) reported the properties of several palm esters for cosmetic industry. The palm ester was synthesized through enzymatic transesterification between oleyl alcohol and various palms raw material such as RBD palm oil, palm olein, palm sterin, palm kernel oil and palm kernel olein. Simultaneous differential scanning calorimeter-thermal gravity was used to study thermal stability. The physicochemical properties such as reflective index, density, surface tension, slip melting point, saponification value, iodine value and acid value using standard test method were analyzed and also determined dermal irritancy test. The results showed that palm esters were suitable for cosmetic ingredient.

Rahman *et al* (2011) studied the synthesis of Engkabang fat esters for cosmetic industry. This wax esters were synthesized by Engkabang fat and oleyl alcohol using Lipozyme *RM* IM as a catalyst. The gas chromatograph (GC) was performed to analyze wax esters. The column for GC was Rtx-65TG capillary column and helium was used as a carrier gas. Injector and detector temperature were set at 300 ^{O}C and 320 ^{O}C, respectively. Oven temperature was maintained at 180 ^{O}C for 2 minute, increased to 300 ^{O}C with the ramping rate of 20 ^{O}C/minute and held constant for 10 minutes. This condition of GC presented 100% and 91.5% yield in heptane and hexane medium, respectively.

4. Response Surface Methodology (RSM)

Response surface methodology (RSM) is a collection of statistical and mathematical techniques using for developing, improving and optimizing process. The variables of RSM can be divided into two groups. The first group is input variables or sometimes calls independent variables or factors. The second group is responses or sometimes calls dependent variables. Most real-world application of RSM involves more than one response. Normally, the graphic from RSM could be plotted as a three-dimensional including two independent variables and one response on x, y, z axis and it is called response surface plot. But the two-dimensional is also preformed and it is known as contour plot (Myers and Montgomery, (1995)).

The general equation of first, second and third-order model are shown in equations 1, 2 and 3, respectively.

$$y = b_0 + \sum_{i=1}^{n} b_i x_i + \sum^{n-1} \sum_{i<j=1}^{n} b_{ij} x_{ij} \tag{1}$$

$$y = b_0 + \sum_{i=1}^{n} b_i x_i + \sum_{i=1}^{n} b_{ii} x_i^2 + \sum^{n-1} \sum_{i<j=1}^{n} b_{ij} x_{ij} \tag{2}$$

$$y = b_0 + \sum_{i=1}^{n} b_i x_i + \sum_{i=1}^{n} b_{ii} x_i^2 + \sum_{i=1}^{n} b_{iii} x_i^3 + \sum_{i<j}^{n-1} \sum_{j=1}^{n} b_{ij} x_{ij} + \sum_{i<j}^{n-2} \sum_{j<k}^{n-1} \sum_{k=1}^{n} b_{ijk} x_{ijk} \tag{3}$$

Where:

y = Response

x_i, x_{ij}, x_{ijk} = Coded or uncoded independent variables

b_0 = Constant

b_i = Linear term coefficients

b_{ii} = Quadratic term coefficients

b_{iii} = Cubic term coefficients

b_{ij} = Cross-product coefficients for quadratic term

b_{ijk} = Cross-product coefficients for cubic term

4.1 Advantages of second-order model

The second-order model is widely used in response surface methodology for several reasons. These reasons are: (Source: Myers and Montgomery, (1995)):

4.1.1 The second-order model is very flexible. It can be generated a various functional forms of response surface. Thus, it is carried out to approximate the true response surface. The forms of response surface are usually find in the form of mound-shaped, stationary, rising ridge and saddle.

4.1.2 It is easy to estimate the coefficient parameter in the second-order model. The method of least squares can be used for this purpose.

4.1.3 The second-order model can be solved a real response surface problem in many researches.

4.2 Experimental design for second-order model

The experimental designs for second-order model are as following (Janchud, (2009)):

4.2.1 Experimental of 2^k factorial in completely randomized design (CRD) or randomized complete block design (RCD, RCBD, RBD) which cross product is used to generate the second-order model. The factorial can be used to investigate several factors at the same time and also studied interaction of factor. A 2^k factorial means that this factorial has k factor and two levels of each factor. The CRD is random treatment and manages to a unit of experimental. But RCBD is randomized a treatment into a block for regular inside a block. The one confinement is not interactive between block and treatment.

4.2.2 The second-order model is generated by experimental of 3^k factorial in completely randomized design (CRD) or randomized complete block design (RCD, RCBD, RBD). A 3^k factorial means that this factorial has k factor and three levels of each factor.

4.2.3 The second-order model is performed using experimental of fractional factorial in completely randomized design (CRD) or randomized complete block design (RCD, RCBD, RBD). The fractional factorial considers some treatments such as 2^{k-1} fractional factorial will consider a half of all treatments.

4.2.4 Rotatable design is designed that the distance of every treatment from center point of geometry has the same level. Normally, the treatment is built from geometric symmetry such as circle, sphere, square or cube. All of 2^k factorials are rotatable but only some of 3^k factorials are rotatable. The coordination at the angle of geometry is used as a code value. The code level of two factors rotatable design is presented in Figure 21.

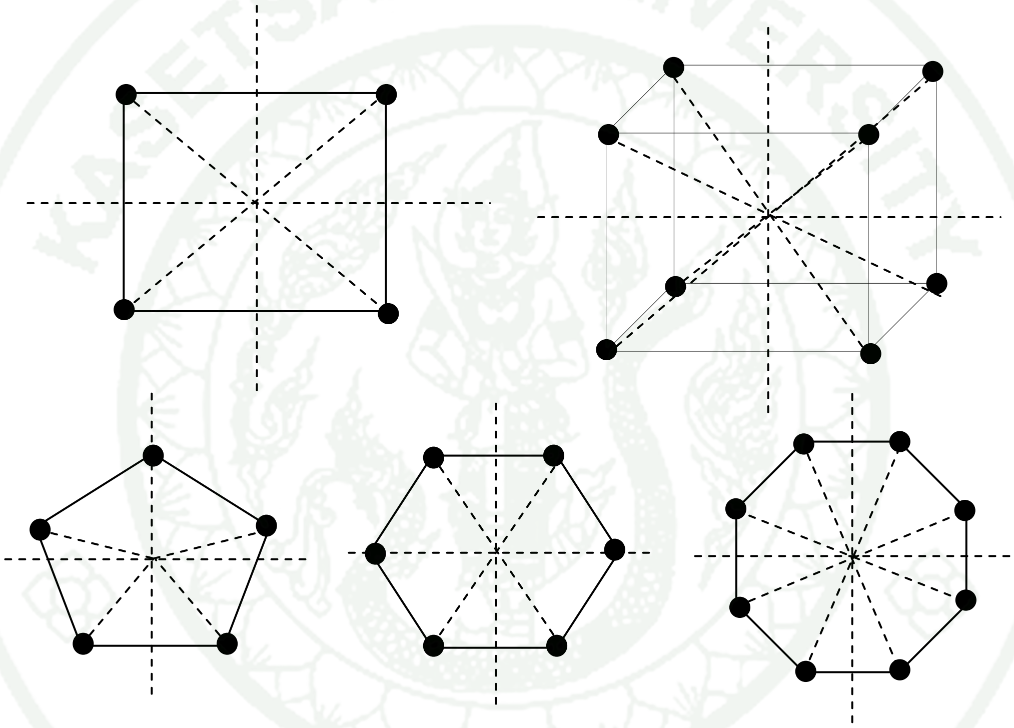


Figure 21 The code level of two factors for rotatable design

Source: Janchud (2009)

4.2.5 Central composite design (CCD) is the experimental which is added treatment between the levels of factor to generate the high order of a model such as the third-order model. Normally, CCD is duplicated at a center point of each factor to estimate the error of the experiment. The performance of CCD begins to build 2^k factorial and then adds the coordination which is a code level as ±α. The treatment is finally randomized into a unit of experiment. The 2^2 factorial of CCD has also been a rotatable design. The code levels of two and three factor CCD are shown in Figure 22.

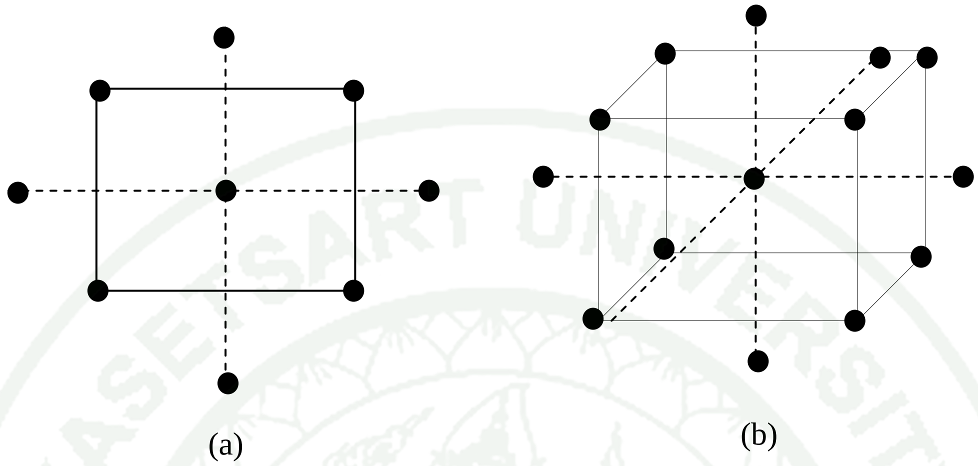


Figure 22 The code level of central composite design; (a) two factors; (b) three factors

Source: Janchud (2009)

In some cases of α = 1, each point of experiment is added on the face center which is known as face-centered central composite design. This design will reduce the number of each factor such as a 5-level of CCD becomes three level of face-centered central composite design. The advantages of face-centered CCD can save the cost and time for performing the experiment. The code level of three factors for face-centered central composite design is demonstrated in Figure 23.

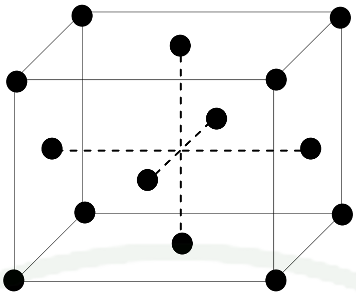


Figure 23 The code level of three factors for face-centered central composite design

Source: Janchud (2009)

4.2.6 Box-Behnken design is one choice of 3^k factorial. These experimental designs are combination of 2^k factorial and balanced incompletely block design (BIB) together. The code level of three factors Box-Behnken design is illustrated in Figure 24.

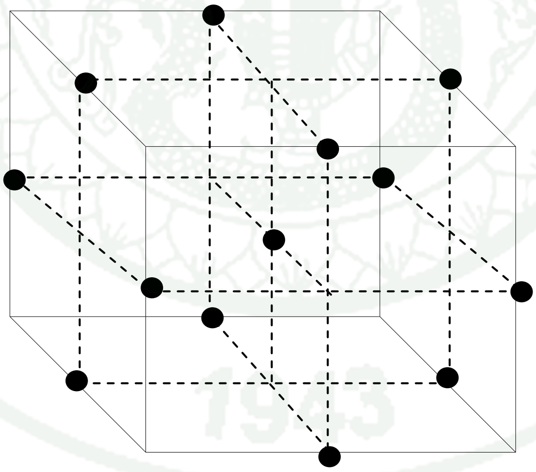


Figure 24 The code level of three factors for Box-Behnken design

Source: Janchud (2009)

4.2.7 Mixture design is simple experiment to study more than two factors. It is suitable for developing process and normally uses a three factor in each performing experiment. The basic of each axis has a value as zero and the top of each factor is value as one. The code level of mixture design is presented in Figure 25.

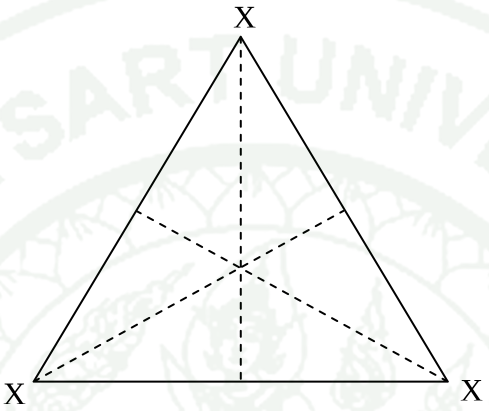


Figure 25 The trilinear coordinate system in mixture design

Source: Janchud (2009)

Response surface methodology is a useful statistical technique which has been applied in research into complex variable process. It employs multiple regression and correlation analyses as tools to assess the effect of two or more independent factors on the dependent variables. Its principle advantage is to reduce number of experimental runs required to generate sufficient information for a statistically acceptable results. RSM has successfully been applied to optimize the reactions by several researchers.

Chu *et al.* (2002) investigated the optimization of PFAD reaction which performed at 65±1 ^{O}C. A 5-level-4-factor central composite design was employed on percentage yield of FFA and vitamin E concentration. These factors were lipase concentration, reaction time and water content of the reactant. The optimum conditions which gave a maximum yield were 2.5% (w/w) of lipase and 45.2 – 47.3% (v/w) of water for 5.5 – 5.7 hours.

Gunawan *et al.* (2005) studied the synthesis of wax esters using response surface methodology (RSM). Wax esters were synthesized by RBD palm oil and oleyl alcohol using lipozyme IM as a catalyst. RSM based on a 5-level-4-factor central composite rotatable design (CCRD) was performed to evaluate the interactive effect on percentage yield of wax esters. The factors for this synthesis were 2.5 – 10 hours of reaction time, 30 – 70 ^{O}C of temperature, 0.1 – 0.2 g of enzyme amount and 1:1 – 1:5 of substrate molar ratio. The optimum conditions derived via RSM were 7.38 hours of reaction time, 53.9 ^{O}C of temperature, 0.149 g of enzyme amount and 1:3.41 of substrate molar ratio. The experimental and predicted yields were 84.6% and 86.4%, respectively.

Sin *et al.* (2005) used RSM to optimized wax esters synthesis from palm oil and oleyl alcohol using lipozyme as a catalyst. RSM based on a 5-level-5-factor central composite rotatable design (CCRD) was used to evaluate the effect of synthesis parameters such as temperature, amount of palm oil, amount of oleyl alcohol and agitation speed on percentage yield of wax esters and amount of productivity. The optimum conditions were 50.4 ^{O}C of temperature, 16% of enzyme amount, 200 mmol of palm oil amount, 600 mmol of oleyl alcohol and 242.1 rpm of agitation speed. The actual value of percentage yield and productivity were 92.3% and 110.8 mmol/hour, respectively. It was compared between 91.5% of predicted percentage yield and 106.4 mmol/h of predicted productivity.

Ghadge *et al*. (2006) studied the production of biodiesel using mahau and methanol. A 5-level-3-factor CCRD was performed on esterification reaction which was pretreatment step. The optimum conditions containing less than 1% of acid level in mahau oil were 0.32 v/v methanol-to-oil ratio, 1.24% v/v H_2SO_4, 1.26 hours of reaction time and 60 ^{O}C of temperature. After that, transesterification was performed to synthesize biodiesel. The optimum conditions were 0.25 v/v methanol-to-oil ratio, 0.7 % w/v KOH as a catalyst. The properties of methyl esters were test following both of American and European standards.

Karnasuta *et al.* (2007) used RSM based on 5-levels-3-factors central composite design (CCD) to study pretreatment and biodiesel production from trap grease with methanol. For pretreatment step, the optimum conditions were 2.5% w/v of H_2SO_4 as a catalyst, 0.43 v/v of methanol-to-oil ratio and 4 hours of reaction time that gave 2.66 mgKOH/g of acid value or 1.3% of FFA. In addition, the optimum conditions for transesterification were 2.5% w/v of KOH as a catalyst, 0.26 v/v of methanol-to-oil ratio and 1 hour of reaction time. It gave 95.49% of biodiesel yield.

Bidin *et al*. (2009) reported the synthesis of palm amino acid surfactant using RSM. The enzymatic synthesis was performed using palm kernel olein and L(+)-lysine catalyzed by Lipozyme *RM* IM. A central composite rotatable design was employed to evaluate the effect of each parameter. The studying parameters were temperature (40 – 70 ^{O}C), impeller speed (100 – 400 rpm), substrates ratio (1 – 4 mmol) and amount of enzyme (5 – 8 g). The optimum conditions derived via RSM were 24 hours of reaction time, 47.50 ^{O}C of temperature, 323.96 rpm of impeller speed, 3.25 mmol of substrate ratio and 7.25 g of catalyst. The experimental yield was 89.03% under the optimum condition comparing with 93.77% of predicted yield.

Chen *et al*. (2010) studied the optimization of octyl caffeate synthesis. It was synthesized by caffeic acid and octanol and catalyzed by Novozyme 435 from *Candida antarctica*. RSM based on a 5-level-4-factor CCRD was performed to evaluate the interactive effect such as reaction temperature, reaction time, substrate molar ratio and enzyme amount on percentage con effect version. The results showed that reaction temperature and time had significant on the response. The optimum conditions were 55 hours of reaction time, 75 ^{O}C of temperature, 1:78 of substrate ratio and 317 PLU of enzyme amount. The molar conversions of the actual and predicted values were 93.79% and 90.34±1.38%, respectively.

Chou *et al.* (2010) studied the effect of temperature, NaOH concentration and time on solubilization of palm oil mill effluent (POME). RSM based on three factor central composite design was applied to identify the optimum COD solubilization condition. The maximum COD solubilization was 82.63% which was performed at 32.5 ^{O}C, 8.83 g/L of NaOH and 41.23 hours.

Omar and Amin (2011) studied heterogeneous transesterification of waste cooking palm oil to synthesize biodiesel using Sr/ZrO_2 as a catalyst. RSM was employed to study the relationship of each parameter such as methanol-to-oil molar ratio, catalyst loading, reaction time and reaction temperature on free fatty acid conversion and methyl esters yield. The 2^4 full factorial designs central composite with two center point was applied in this experiment. The 79.7% of maximum methyl esters were given at 89 minutes of reaction time, 115.5 ^{O}C of temperature, 29:1 of methanol-to-oil ratio and 2.7 wt% of catalyst loading.

Rahman *et al.* (2011) reported alcoholysis reaction to produce Engabang fat esters. These esters were synthesized by Engabang fat with oleyl alcohol and catalyzed by Lipozyme *RM* IM. The reaction was performed in two solvents including heptane and hexane. RSM based on a 4-factor-5-level central composite design was applied to evaluate the interactive effect on percentage yield. The parameters were temperature, substrate molar ratio, enzyme amount and impeller speed. The optimum conditions in heptane and hexane were 53.9 ^{O}C of reaction temperature, 309.5 and 309.0 rpm of impeller speed, 4.82 and 5.65 g of enzyme amount, 2.94 and 3.39:1 of substrate molar ratio, respectively. The actual yield in heptane and hexane medium were 96.2% and 91.4% comparing with predicted value of 100% and 91.5%, respectively.

5. Design RSM process

The procedure of RSM begins with the selection of the experimental design which is to assign a number and each condition of the experiment. Then, factors and its levels are set. The factor’s value can be classified into two types. The first type is the uncode value which is expressed in natural unit of measurement such as mole and hour. The second type is called the code value which is transformed an uncode value to dimensionless. The experimental data builds the suitable equation which is called model fitting and surface plot to describe the overall reaction.

The suitable equation considering from coefficient of determination (R^2) will be more than 0.70. Moreover, the lack of fit test is one variable to determine the equation. The calculated F-value from the experimental data will be compared with tubular F-value. If the equation has high R^2 value and insignificant lack of fit, it indicates the suitable equation. In the case of high R^2 value but significant lack of fit, validation test should be performed. It performs by running the reaction again and compares the response of experimental value with predicted value. The conditions for validation test are different from conditions that use to generate the equation but it must be within the same range. If both values are similar, the equation is suitable. If both values are quite different, it indicates that the model isn’t suitable. In this case, some term in equation which is insignificant to the model might be cut off and the lack of test and validation test should be performed again. The last solution is running a reaction again by using a new condition to generate a new equation. For the optimization process, the suitable model is further used to generate the response surface plot. Normally, the program creates the response surface plot as a function of two independents variable and response at one time. Thus, if we studies three variables such as A, B and C, the three response surface plots such as A and B, A and C and B and C are generated. The three of them are overlapped to create the optimum region of each variable. Then, the reaction is run again by setting a condition from the optimum region. Finally, the optimum condition is chosen from those conditions. The diagram of RSM process is presented in Figure 26.

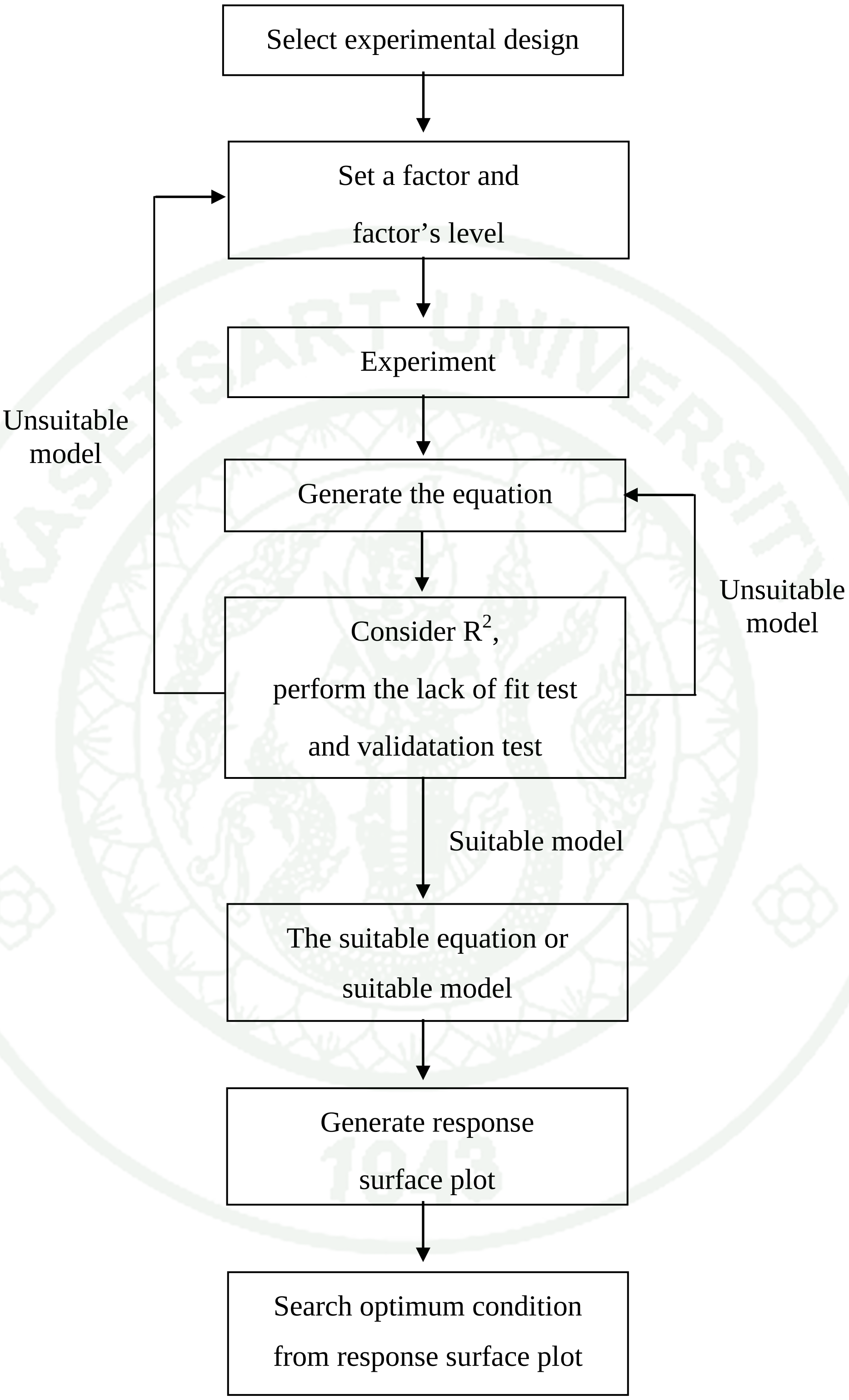


Figure 26 The diagram of RSM process

MATERIALS AND METHODS

Materials

1. Palm fatty acid distillate samples were obtained from Pathum Palm oil Co., Ltd (Thailand)

Figure 27 Palm fatty acid distillate at; room temperature (left); higher than 50 ^{0}C (right)

2. Oleyl alcohol 65% (Commercial grade, Aldrich, USA.)
3. Amberlyst15 (Rohm and Haas Co, Aldrich, USA.)

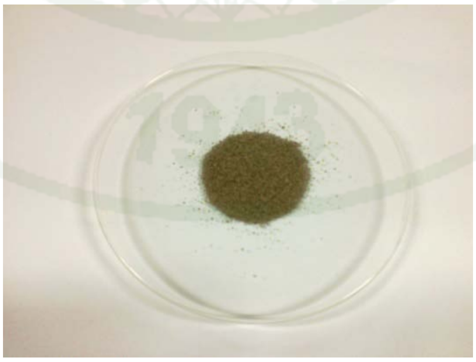


Figure 28 Amberlyst 15 catalyst

4. Reagents
    - 2, 2’-bipyridine 99% (Analytical grade, Sigma-Aldrich, USA)
    - Anhydrous sodium sulphate (Analytical grade, APS, Australia)
    - Boron trifluoride in methanol (BF_3 14%v/v, Supelco, USA)
    - Chloroform (Analytical grade, RCl Labscan, Thailand)
    - Ethanol 98% (Analytical grade, Merck, Germany)
    - Hexane (Analytical grade, QRëC, New Zealand)
    - Iron (III) chloride hexahydrate 97% (Analytical grade, Sigma-Aldrich, USA)
    - Isopropanol (Analytical grade, Carlo erba, Italy)
    - n-Heptane (GC grade, QRëC, New Zealand)
    - Methanol 99% (Analytical grade, Merck, Germany)
    - Phenolphthalein (Analytical grade, Merck, Germany)
    - Potassium hydroxide (Analytical grade, J.T. Baker, USA)
    - Potassium iodine (Analytical grade, QRëC, New Zealand)
    - Sodium chloride (Analytical grade, APS, Australia)
    - Sodium hydroxide (Analytical grade, J.T. Baker, USA)
    - Sodium thiosulfate (Analytical grade, APS, Australia)
    - Starch solution (Analytical grade, Merck, Germany)
    - Toluene (Analytical grade, Carlo erba, Italy)
    - Wijs solution (Analytical grade, Fluka, USA)

5. Standard chemical compounds
    - Fatty acid methyl esters mixture (C_8–C_{24}) (GC grade, Supelco, USA)
    - Methyl laurate 99.5% (GC grade, Aldrich, USA)
    - N-Methyl-N-trimethylsilytrifluoroacetamide (GC grade, Restek, USA)
    - Oleyl linoleate 99% (GC grade, Sigma, USA)
    - Oleyl oleate 99% (GC grade, Sigma, USA)
    - Oleyl palmitate 99% (GC grade, Chemos GmbH, USA)
    - α-Tocopherol (GC grade, Fluka, USA)
    - Tricaprin (GC grade, Restek, USA)

Equipments

1. A 4-digit balance (Percisa, 120A, USA.)
2. Fourier-Transform Infrared Spectrometry (EQUINOX 55, Bruker)
3. Gas chromatography (Agilent Technology, 6890N, USA.)
4. Hot air oven (Binder, Germany)
5. UV-Visible spectroscopy (PG Instrument, China)

Methods

1. **Synthesis of wax esters**

1.1 Synthesis of esters

The esterification reactions were conducted in 100 ml three-necked round bottom flask equipped with a reflux condenser and a thermometer. The flask was charged with 1.50 g of PFAD and preheated with set temperature including stirred at 400 rpm with a magnetic stirrer. After achieving the setting temperature of the reactant, certain quantities of Amberlyst 15 catalyst and oleyl alcohol were added into the flask. The reaction parameters were designed as shown in Table 6. After the reaction finished, the wax esters product and catalyst were separated by filtration. The product was heated at 100 ^{O}C for about 5 minute to remove water. The product was further analyzed for percentage conversion of PFAD by comparing between initial and final values of percentage FFA. In addition, wax esters product was determined in terms of percentage yield with calculation the total weight of wax esters by gas chromatography. The reaction apparatus is set as Figure 29.

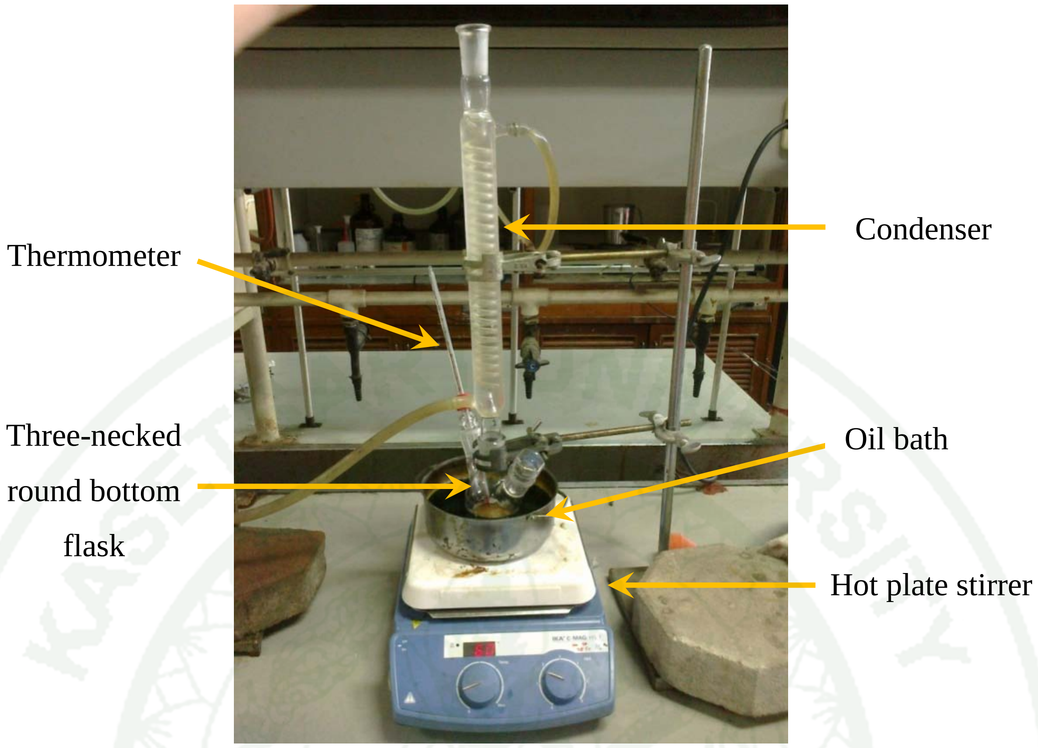


Figure 29 The apparatus for esterification reaction

In case of absence of catalyst, the synthesis of wax esters without Amberlyst 15 catalyst was carried out by comparing the reaction at two temperatures which was 70 ^{O}C and 150 ^{O}C. The percentage conversion of PFAD at 70 ^{O}C and 150 ^{O}C were 1.85% and 18.12%, respectively when the other parameters were 3.5 moles of oleyl alcohol-to-PFAD molar ratio and 2.5 hours of reaction time. The results indicated that the wax esters could occur at high temperature without catalyst. Because increasing of temperature promoted higher kinetic energy of molecule resulting in that the molecule of substrate could be moved faster than the molecule at room temperature and it increased a probable collision of substrate to generate a product. When comparison of percentage conversion at 70 ^{O}C and 150 ^{O}C, it was found that higher temperature promoted a larger of percentage conversion. Thus, Amberlyst 15 as a catalyst must be needed in this reaction.

1.2 Experimental design

A five-level-four-factor central composite design (CCD) was employed in this study, requiring 30 experiments including 16 factorial points, 8 axial points and 6 center points. The variables and their selected levels are presented in Table 6.

Table 6 Independent variables and level used for central composite design (CCD) for esterification reaction

Independent variables	Symbol	Levels				
		-2 (-α)	-1	0	1	2 (+α)
Amberlyst 15 amount (%wt/wt)	A	10.00	17.50	25.00	32.50	40.00
Oleyl alcohol-to-PFAD molar ratio (mol)	B	1.00	1.75	2.50	3.25	4.00
Reaction time (h)	C	0.00	1.00	2.00	3.00	4.00
Reaction temperature (^{O}C)	D	40.00	50.00	60.00	70.00	80.00

The 30 treatments for optimization of wax esters using central composite design are presented in Table 7.

Table 7 The conditions for optimization of wax esters using CCD

Treatment	Coded value				Uncoded value			
	A	B	C	D	A	B	C	D
					(%wt)	(mol)	(h)	(^{O}C)
1	-1	-1	-1	-1	17.50	1.75	1.00	50.00
2	-1	-1	-1	1	17.50	1.75	1.00	70.00
3	-1	-1	1	-1	17.50	1.75	3.00	50.00
4	-1	-1	1	1	17.50	1.75	3.00	70.00
5	-1	1	-1	-1	17.50	1.75	1.00	50.00
6	-1	1	-1	1	17.50	1.75	1.00	70.00
7	-1	1	1	-1	17.50	1.75	3.00	50.00
8	-1	1	1	1	17.50	1.75	3.00	70.00
9	1	-1	-1	-1	32.50	3.25	1.00	50.00
10	1	-1	-1	1	32.50	3.25	1.00	70.00
11	1	-1	1	-1	32.50	3.25	3.00	50.00
12	1	-1	1	1	32.50	3.25	3.00	70.00
13	1	1	-1	-1	32.50	3.25	1.00	50.00
14	1	1	-1	1	32.50	3.25	1.00	70.00
15	1	1	1	-1	32.50	3.25	3.00	50.00
16	1	1	1	1	32.50	3.25	3.00	70.00
17	-2	0	0	0	10.00	2.50	2.00	60.00
18	2	0	0	0	40.00	2.50	2.00	60.00
19	0	-2	0	0	25.00	1.00	2.00	60.00
20	0	2	0	0	25.00	4.00	2.00	60.00
21	0	0	-2	0	25.00	2.50	0.00	60.00
22	0	0	2	0	25.00	2.50	4.00	60.00
23	0	0	0	-2	25.00	2.50	2.00	40.00
24	0	0	0	2	25.00	2.50	2.00	80.00

Table 7 (Continued)

Treatment	Coded value				Uncoded value			
	A	B	C	D	A (%wt)	B (mol)	C (h)	D (^{O}C)
25	0	0	0	0	25.00	2.50	2.00	60.00
26	0	0	0	0	25.00	2.50	2.00	60.00
27	0	0	0	0	25.00	2.50	2.00	60.00
28	0	0	0	0	25.00	2.50	2.00	60.00
29	0	0	0	0	25.00	2.50	2.00	60.00
30	0	0	0	0	25.00	2.50	2.00	60.00

The resulting data was performed by SPSS package version 12.0 to generate the equation which was fitted with this result. The general equation of second-order polynomial and third-order polynomial were presented in equation 4 (Gunawan *et al*., 2005) and equation 5 (Sin *et al*., 2005), respectively.

$$y = b_0 + \sum_{i=1}^{4} b_i x_i + \sum_{i=1}^{4} b_{ii} x_i^2 + \sum^{3} \sum_{i<j=1}^{4} b_{ij} x_{ij} \qquad (4)$$

$$y = b_0 + \sum_{i=1}^{4} b_i x_i + \sum_{i=1}^{4} b_{ii} x_i^2 + \sum_{i=1}^{4} b_{iii} x_i^3 + \sum_{i<j}^{3} \sum_{j=1}^{4} b_{ij} x_{ij} + \sum_{i<j}^{2} \sum_{j<k}^{3} \sum_{k=1}^{4} b_{ijk} x_{ijk} \qquad (5)$$

Where:

y = The response (conversion (%), yield (%))
x_i, x_{ij}, x_{ijk} = Uncoded independent variables
b_0 = Constant
b_i = Linear term coefficients
b_{ii} = Quadratic term coefficients
b_{iii} = Cubic term coefficients

b_{ij} = Cross-product coefficients for quadratic term
b_{ijk} = Cross-product coefficients for cubic term

The model was proved by validation test. A verifying method was performed by running a new reaction. This condition was within a range of each parameter but it was not the same condition that gave the model. The response value from experiment was compared with the predicted value from the model. The conditions of validation method were depicted in Table 3.

Table 8 The conditions of validation test

Treatment	A (%wt)	B (mol)	C (h)	D (^{O}C)
1	20.00	1.50	1.50	55.00
2	30.00	1.80	2.50	65.00
3	35.00	3.50	3.75	75.00
4	28.00	2.70	1.25	65.00
5	15.00	3.80	2.25	55.00

The suitable model was used to generate a response surface plot which gave from STATISTICA software.

1.3 Investigation the efficiency of Amberlyst 15 catalyst

The efficiency of Amberlyst 15 catalyst was determined in terms of recycling number. The catalyst was recovered and used to synthesize wax esters at the same condition including 30% of amount of Amberlyst 15 catalyst, 2.5:1 of oleyl alcohol-to-PFAD molar ratio, 2.5 hours of reaction time and 70 ^{O}C of reaction temperature. The reaction was performed as same as section 1.1. After the reaction was completed, the Amberlyst 15 catalyst was separated by filtration. Then, Amberlyst 15 catalyst was refluxed with excess hexane for 30 minutes and washed with excess methanol to remove some substance such as polar and non-polar materials that adsorbed on catalyst surface. Amberlyst 15 catalyst was dried at 105 ±5 ^{O}C for 4 hours. Finally, the Amberlyst 15 catalyst was reused as a catalyst again. The reaction was run 20 cycles.

2. Analytical methods

2.1 Analysis of fatty acid composition in palm fatty acid distillate

The fatty acid composition of PFAD was performed by AOCS Official Method Ce 2-66.

PFAD was weighed about 250 mg and put into 50 ml round bottom flask with boiling chips. Then, 4 ml of 0.5 N methanolic sodium hydroxide was added to a sample flask which attached with a condenser. A solution mixture was refluxed at 90 ^{O}C until fat globules disappeared which was about 5 to 10 minutes. After that, 5 ml of borontrifluoride solution (BF_3, 14%v/v) was added through a condenser and continued boiling for 2 minutes. Then, 5 ml of n-heptane was added to a flask and still boiled 1 minute longer. After completely reaction, a solution was cooled down and then added 15 ml of saturated sodium chloride solution including shake for 15 seconds. A mixture was allowed to stand for separation into two layers. The upper layer of solution was determined for fatty acid composition by GC.

GC analysis was performed by inject 1 µl of aliquot into DB-wax capillary column (30 m × 0.32 mm i.d.; film thickness 0.1 µm; Agilent Technologies, USA.) in a split mode equipped with a flame-ionization detector. The temperature of injector and detector were set at 270 ^{O}C. Oven temperature was maintained at 80 ^{O}C for 2 minutes, increased to 290 ^{O}C with ramping rate 20 ^{0}C/minute and constantly held at this temperature for 10 minutes. Helium was used as a carried gas with a flow rate of 2 ml/minute. The fatty acid composition was calculated by percentage area of each fatty acid.

2.2 Determination of mono, di and triglyceride in palm fatty acid distillate

Mono, di and triglyceride which contained in palm fatty acid distillate were investigated by ASTM 6584-07 standard method

100 mg of PFAD was weighed into septa vial. Then, 100 µl of tricaprin was added and followed by 100 µl of N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA). After that, the mixture was shaken and allowed to stand for 15 to 20 minutes at room temperature. Finally, 8 ml of n-heptane was added to a vial and analyze mono, di and triglyceride by GC.

GC analysis was done by injecting 1 µl of the mixture into DB-5HT capillary column (30 m × 0.32 mm i.d.; film thickness 0.1 µm; Agilent Technologies, USA.) in a cool on column mode equipped with a flame-ionization detector. The temperature of injector and detector were set at 380 ^{O}C. Oven temperature was maintained at 50 ^{O}C for 2 minutes and increased to 270 ^{O}C with ramping rate 20 ^{O}C/minute, raised to 380 ^{O}C with ramping rate 3 ^{O}C/minute and constantly held at this temperature for 10 minutes. Helium was used as a carried gas with a flow rate of 3 ml/minute. The mono, di and triglyceride were calculated by using peak area of each compound following equations 6, 7 and 8.

$$\text{Monoglyceride (\%)} = \frac{\left(a_m \times \left(\frac{A_m}{A_{is}}\right) + b_m\right) \times m_{is} \times 100}{m_s} \qquad (6)$$

Where:

a_m = Slope of calibration function
A_m = Peak area of monoglyceride
A_{is} = Peak area of internal standard
b_m = Intercept of the calibration function
m_{is} = Weight of internal standard (mg)

m_s = Weight of sample (mg)

$$\text{Diglyceride (\%)} = \frac{\left(a_d \times \left(\frac{A_d}{A_{is}}\right) + b_d\right) \times m_{is} \times 100}{m_s} \tag{7}$$

Where:

a_d = Slope of calibration function
A_d = Peak area of diglyceride
A_{is} = Peak area of internal standard
b_d = Intercept of the calibration function
m_{is} = Weight of internal standard (mg)
m_s = Weight of sample (mg)

$$\text{Triglyceride (\%)} = \frac{\left(a_t \times \left(\frac{A_t}{A_{is}}\right) + b_t\right) \times m_{is} \times 100}{m_s} \tag{8}$$

Where:

a_t = Slope of calibration function
A_t = Peak area of triglyceride
A_{is} = Peak area of internal standard
b_t = Intercept of the calibration function
m_{is} = Weight of internal standard (mg)
m_s = Weight of sample (mg)

2.3 Investigation of percentage conversion of palm fatty acid distillate

The percentage conversion of PFAD was determined in terms of the initial percentage FFA containing in PFAD and the final percentage of FFA within product. The FFA (%) was analyzed using AOCS Official Method Ca 5a-40.

The Erlenmeyer flask was charged with about 1 g of sample. Then, the sample was melted and added 50 ml of ethanol. Lastly, the sample mixture was analyzed by titration with 0.1 N NaOH using 1% phenolphthalein as an indicator. The percentage FFA of the sample which had palmitic acid as a major component was calculated by equation 9.

$$\text{FFA (\%)} \quad = \quad \frac{(\text{A} - \text{B}) \times \text{N} \times 25.6}{\text{W}} \qquad (9)$$

Where:

FFA = Free fatty acid (%)
A = Volume of 0.1 N NaOH required to titrate sample (ml)
B = Volume of 0.1 N NaOH required to titrate blank (ml)
N = Normality of NaOH solution (N)
W = Weight of sample (g)

The percentage conversion of PFAD could be determined following equation 10 (Marchetti *et al*., 2007).

$$\text{Conversion (\%)} \quad = \quad \frac{\text{F}_\text{i} - \text{F}_\text{f}}{\text{F}_\text{i}} \times 100 \qquad (10)$$

Where:

F_i = Initial FFA content of PFAD
F_f = Final FFA content of product

2.4 Analysis of percentage yield of wax esters

Wax esters were weighed about 50 mg into a septa vial and mixed with 0.5 ml of methyl laurate (C_{12}) as an internal standard which had a concentration of 3000 ppm. The vial was shaken to homogenize and the sample was taken to analyze by GC.

GC analysis was conducted by injecting 1 µl of aliquot into DB-5HT capillary column (30 m × 0.32 mm i.d.; film thickness 0.1 µm; Agilent Technologies, USA.) in a split mode equipped with a flame-ionization detector. The temperature of injector and detector were set at 300 and 250 ^{O}C, respectively. The oven temperature was maintained at 150 ^{O}C for 2 minutes, increased to 270 ^{O}C with ramping rate 3 ^{O}C/minute and held for 16 minutes at 290 ^{O}C. Helium was used as a carried gas with a flow rate of 0.8 ml/minute. The concentration of each oleyl ester was quantified by equation 11 (Gunawan *et al*., 2005).

$$C_x = \left(\frac{A_x}{A_{is}}\right) \times \left(\frac{C_{is}}{R_F}\right) \tag{11}$$

Where:

C_s = Concentration of component x
C_{is} = Concentration of internal standard
A_x = Peak area of component x
A_{is} = Peak area of internal standard
R_F = Response factor

The percentage of wax esters was calculated by equation 12 (Gunawan *et al*., 2005).

$$\text{Yield (\%)} \quad = \quad \frac{\text{Weight of all wax esters wihtin sample (g)}}{\text{Weight of PFAD used (g)}} \times 100 \qquad (12)$$

2.5 Fourier-Transform Infrared Spectrometry (FT-IR) analysis

The functional group of PFAD and other product were investigated by FT-IR. A Bruker Equinox 55 FT-IR spectrometer equipped with Attenuated Total Reflectance (ATR) apparatus was used to analyze liquid sample. One or two drops of liquid sample were applied on Zn-Se crystal. The sample background was collected and the sample was measured. The spectra were scanned from 4000 to 400 cm^{-1}. All spectra were collected at 4 cm^{-1} of spectral resolution by using one minute sample and one minute background collection times.

2.6 Determination of physicochemical characteristics

The physicochemical properties of palm fatty acid distillate and wax esters were determined to define its characteristic.

2.6.1 Specific gravity

Specific gravity is define in terms of the ratio between mass per unit volume of sample with mass per unit volume of water at 25 ^{O}C. This value was analyzed according to AOCS Official Method Cc 10b-25.

2.6.2 Acid value

Acid value is a measure of the number of fatty acids within the sample. This value is the amount of potassium hydroxide required to neutralize one gram of sample. Acid value was investigated according to AOCS Official Method Cd 3d-63.

2.6.3 Iodine value

The iodine value is defined in terms of grams of iodine adsorbed per 100 grams of sample. This value is a determination as a function of double bond amount within sample. If a sample has high iodine value indicated that this sample has more unsaturated fatty acid. Iodine value was analyzed following AOCS Official Method Cd 1b-25.

2.6.4 Saponification value

The saponification value is amount of alkali required to saponify a definite quantity of sample. It is demonstrated as the number of milligrams of potassium hydroxide which reacts with one gram of sample. This value was determined according to AOCS Official Method Cd 3-25.

2.6.5 Kinematic viscosity

The kinematic viscosity is defined as a function of resistance of liquid to flow. This value was performed following ASTM 445 method.

2.6.6 Water content

Water content of sample is measures in terms of percentage quantified water containing in sample. This value was investigated by weighing about 1 g of sample into aluminum pan sealed with a cover which was partially close. Then, aluminum pan was taken to oven setted at the temperature of 105 ^{0}C. Finally, the pan was weighed every hour until the weight of sample was constant.

Figure 30 Aluminum pan for water content detection

2.7 Determination of Vitamin E content

Vitamin E content was determined by UV-VIS spectrophotometer. The 20 mg of palm fatty acid distillate or 200 mg of wax esters were weighed into 10 ml volumetric flask. Then, 5 ml of toluene was added by pipette and the solution was shaken. After that, 3.5 ml of 0.07% w/v in 95% aqueous ethanol of 2,2'-bipyridine and 0.5 ml of 0.2% w/v in 95% aqueous ethanol of $FeCl_3.6H_2O$ were added into the flask. After standing for one minute, the adsorption at 520 nm was determined using a reference blank solution which prepared as above but omitting the sample. The solution should be protected from strong light during color development.

The calibration curve was performed by preparing standard containing 0 – 240 ppm of pure α-tocopherol standard in 10 ml of toluene and analyzed as above.

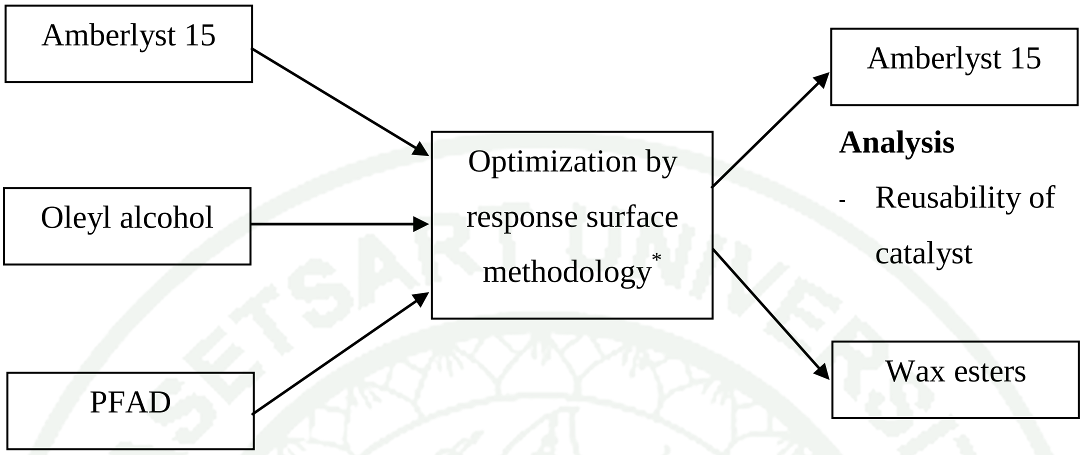
The concentration of vitamin E as α-tocopherol in the sample was calculated by equation 13 (Wong, *et al.*, 1988)

$$\text{Vitamin E content} = \frac{(A - B)}{M \times W} \qquad (13)$$

Where:

A = Absorbance of sample in 10 mm. cell
B = Absorbance of blank in 10 mm. cell
M = Gradient of absorbance and weight from calibration curve
W = Weight of sample (g)

The summary experiment is presented in Figure 31



Analysis

- Fatty acid composition
- Mono, di, triglyceride content
- FFA (%)
- FT-IR
- Physicochemical properties
- Vitamin E content

Analysis

- Reusability of catalyst

Analysis

- Conversion (%)
- Yield (%)
- FT-IR
- Physicochemical properties
- Vitamin E content

* Response surface methodology was performed by using five-level-four-factor CCD which constant variable was 400 rpm of agitation speed. A five-level-four-factor included:

- Amberlyst 15 amount (10.00, 17.50, 25.00, 32.50 and 40.00 wt%)
- Oleyl alcohol-to-PFAD molar ratio (1.00:1, 1.75:1, 2.50:1, 3.25:1 and 4.00:1 mol)
- Reaction time (0, 1, 2, 3 and 4 hours)
- Reaction temperature (40, 50, 60, 70 and 80 ^{0}C)

Figure 31 The summary diagram for experiment

RESULTS AND DISCUSSION

1. Properties of palm fatty acid distillate (PFAD)

The main component of palm fatty acid distillate (PFAD) is fatty acid. The rest of the components are monoglyceride, diglyceride and triglyceride. The components of PFAD are shown in Table 9. The fatty acid and other component of our PFAD was higher than those from other sources (Top, 2010), the total glyceride was lower but other components were higher when comparing with other sources.

Table 9 The components of PFAD

Component	Value (%)	
	Experiment	Typical composition*
FFA (as plamitic acid, %)	88.40	81.7
Total triglyceride (%)	1.35	14.4
Other	10.25	3.9

Source: *Top (2010)

The fatty acid composition of PFAD was investigated by gas chromatograph. The retention time of each peak of PFAD was compared with standard compound peaks. The chromatogram of fatty acid composition is shown in Figure 32.

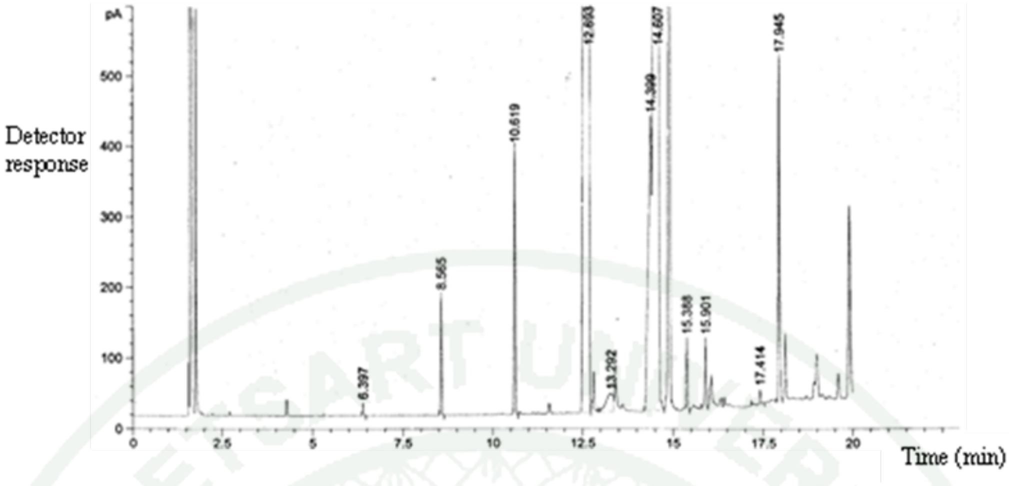


Figure 32 The chromatogram of fatty acid composition in PFAD

From Figure 32, the result showed that the main fatty acids were palmitic acid, oleic acid and linoleic acid which contained 45.60%, 37.86% and 7.98%, respectively. The minor fatty acids were octanoic acid, decanoic acid, lauric acid, myristic acid, stearic acid, linolenic acid, arachidic acid, behenic acid and erucic acid as shown in Table 10.

Table 10 The percentage of each fatty acids content in PFAD

Compound	Symbol	Retention time (min)	Weight (%)
Octanoic acid	C8:0	4.292	0.07
Decanoic acid	C10:0	6.397	0.06
Lauric acid	C12:0	8.565	0.58
Myristic acid	C14:0	10.619	1.36
Palmitic acid	C16:0	12.693	45.60
Stearic acid	C18:0	14.399	3.95
Oleic acid	C18:1	14.607	37.86
Linoleic acid	C18:2	14.918	7.98

Table 10 (Continued)

Compound	Symbol	Retention time (min)	Weight (%)
Linolenic acid	C18:3	15.388	0.36
Arachidic acid	C20:0	16.063	0.34
Behenic acid	C22:0	17.414	0.07
Erucic acid	C22:1	17.945	1.76

In addition, the molecular weight of PFAD was determined as a function of average molecular weight result of contained fatty acids in PFAD. The average molecular weight of PFAD was 269.59 g/mol as calculated by equation 14.

$$\text{Average molecular weight of PFAD} = \frac{\sum(\%\ \text{Area} \times \text{MW})}{100} \tag{14}$$

Where:

$\sum$ = Summation
%Area = Area of each fatty acid that analyzed by GC
MW = Molecular weight of each fatty acid

Besides fatty acid, palm fatty acid distillate contained mono, di, triglyceride which were investigated in the form of total glyceride. The internal standards for this analysis were tricaprin and N-methyl-N-trimethylsilytrifluoroacetamide (MSTFA). The total glyceride was analyzed by gas chromatography as shown in Figure 33 and Table 11.

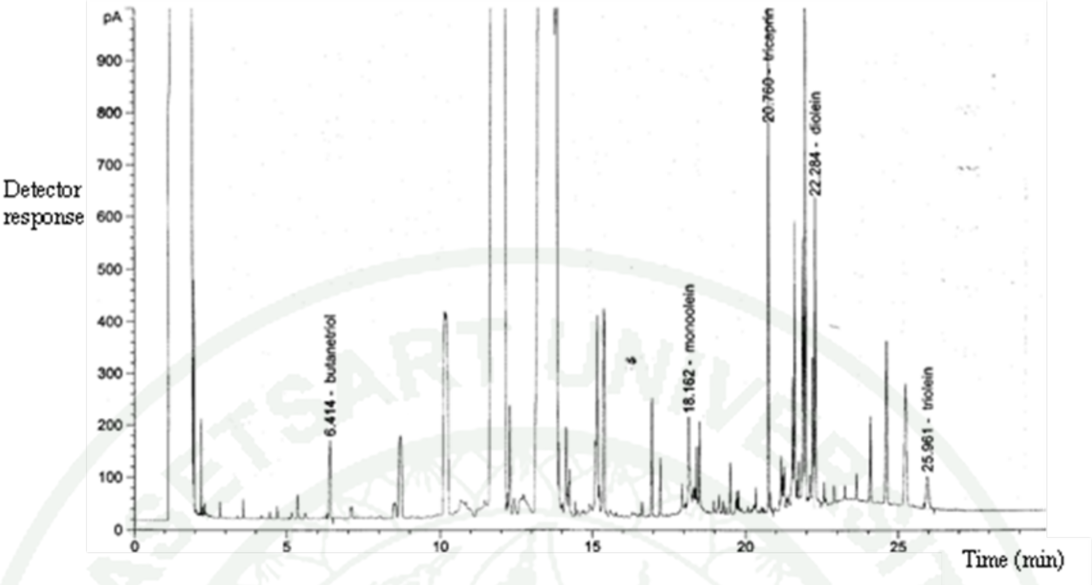


Figure 33 The chromatogram of mono, di and triglyceride in PFAD

Table 11 The percentage of glyceride compound in palm fatty acid distillate

Compound	Retention time (min)	% (wt)
Monoglyceride	18.162	0.3383
Diglyceride	22.284	0.8605
Triglyceride	25.961	0.1475
Total	-	1.3463

The other physicochemical properties of palm fatty acid distillate are presented in Table 12.

Table 12 Physicochemical properties of palm fatty acid distillate

Characteristics	Value	
	Experiment	Survey by Bonnie & Mohtar[a]
Acid value (mg KOH/g)	193.60	-
Iodine value (g I_2/100g sample)	51.54	46.3 – 57.6
Saponification value (mg KOH/g)	200.46	200.3 – 249.4
Specific gravity at 25 ^{O}C	0.8685	-
Kinematics viscosity at 40 ^{O}C (cSt)	n.d.[b]	-
Water content (%wt)	0.05	0.03 – 0.24

Note: [b]n.d. is not detected

Source: [a]Top (2010)

Acid value of PFAD was 192.79 mg KOH/g. The high acid value of PFAD indicated that it was suitable to use as a raw material for esterification reaction. Normally, if they had palmitic acid as a major component, the acid value was usually 2.19 times of percentage free fatty acid.

Iodine value represents the number of unsaturated fatty acids in PFAD. In this case, the iodine value of PFAD was 51.54 g I_2 per 100 g of sample. Higher iodine value indicated that the molecule of sample contained large amount of unsaturated fatty acid.

Saponification value presents the number of ester bonds in molecule. If the molecule contains more ester bond, it usually has high saponification value such as palm oil which contains three ester bonds within molecule, it presented above 600 mg KOH per one gram of sample. Saponification value of PFAD was 200.46 mg KOH per one gram of sample.

Kinematics viscosity implies the resistance of liquid to flow. This value is not detected due to the standard method of kinematics viscosity performed at 40 ^{O}C and palm fatty acid distillate was still as a solid at this temperature. The density of PFAD was 0.8685 g/cm^3.

Water content is one of the important values. The esterificaiton reaction which is reaction to synthesize wax esters is reversible reaction and it gives water as a by-product. From the principle of Le Chatelier, a high of water content can more the equilibrium backward resulting low percentage yield of wax esters.

2. Statistical data

The response of esterification reaction was detected as the form of percentage conversion of PFAD and percentage yield of wax esters. Both of responses were calculated from 30 experimental values to generate a quadratic polynomial model.

2.1 Percentage conversion

The percentage conversion of PFAD was calculated by comparison between the initial FFA(%) of PFAD and the final FFA(%) of product. The experimental data for esterification reaction of PFAD and oleyl alcohol is given in Table 13.

Table 13 Central composite design (CCD) arrangement along with percentage conversion for esterification reaction

Treatment	A	B	C	D	Conversion (%)	
					Experimental	Predicted
1	-1	-1	-1	-1	60.51	58.83
2	-1	-1	-1	1	73.82	78.80
3	-1	-1	1	-1	67.19	64.06

Table 13 (Continued)

Treatment	A	B	C	D	Conversion (%)	
					Experimental	Predicted
4	-1	-1	1	1	92.37	90.11
5	-1	1	-1	-1	75.43	71.60
6	-1	1	-1	1	82.36	85.81
7	-1	1	1	-1	78.41	75.16
8	-1	1	1	1	93.77	95.45
9	1	-1	-1	-1	66.38	65.17
10	1	-1	-1	1	83.29	84.24
11	1	-1	1	-1	72.88	67.22
12	1	-1	1	1	88.13	92.37
13	1	1	-1	-1	77.28	77.33
14	1	1	-1	1	87.14	90.64
15	1	1	1	-1	82.24	77.72
16	1	1	1	1	97.71	97.11
17	-2	0	0	0	73.94	76.72
18	2	0	0	0	84.59	84.71
19	0	-2	0	0	67.44	69.07
20	0	2	0	0	85.21	86.57
22	0	0	2	0	87.31	93.93
23	0	0	0	-2	41.24	51.61
24	0	0	0	2	98.21	90.97
21	0	0	-2	0	85.87	82.23
25	0	0	0	0	80.23	80.49
26	0	0	0	0	80.18	80.49
27	0	0	0	0	80.38	80.49
28	0	0	0	0	80.07	80.49
29	0	0	0	0	80.38	80.49
30	0	0	0	0	80.58	80.49

The predicted value was determined from model fitting technique using SPSS package (version 12.0). The Analysis of variance (ANOVA) for esterification reaction which was percentage conversion as response value is shown in Table 14.

Table 14 Analysis of variance (ANOVA) for the second order polynomial model of esterification reaction along with percentage conversion

Model	Sum of Squares	Degree of freedom	Mean sum of square	F-value	Sig.
Regression	3406.350	14	243.311	9.243	0.000[a]
Residual	394.843	15	26.323	-	-
Total	3801.193	29	-	-	-
Pure error	2.870	5	0.574	-	-
Lack of fit	391.973	10	39.197	68.288[b]	-

Note: [a]Significant at “Sig.” less than 0.05

[b]Tubular F-value was 4.74

The computed model F-value of 9.243 which was higher than tubular F-value, indicated the model was significant at 95% confidence level. The correlation coefficient (R^2) of the model was 0.897 which should be more than 0.75 for using the empirical model to represent the real relationship between variable and response (Omar *et al*., 2011). In this case, 0.897 of R^2 imply that the independent variable could be explained percentage conversion of 89.7% and only 10.3% of the response value was no defined by studied variables.

For lack of fit test, F-value of the model was 68.288 which was higher than tubular F-value ($F_{(0.05,10,5)} = 4.74$), indicated that the model was insignificant. Hence, the suitable model was proven by validation test.

The validation test was done by running the reaction at different conditions. These conditions must be in the same range of 30 experiments but they were not the same condition. The conditions of validation test are present in Table 15.

Table 15 The experimental data for validation test of percentage conversion

Treatment	A	B	C	D	Conversion (%)		Difference
	(%wt)	(mol)	(h)	(^{O}C)	Experimental	Predicted	(%)
1	20.00	1.50	1.50	55.00	65.99	64.35	-2.52
2	30.00	1.80	2.50	65.00	84.44	84.47	0.03
3	35.00	3.50	3.75	75.00	99.06	105.98	6.75
4	28.00	2.70	1.25	65.00	80.11	85.14	6.08
5	15.00	3.80	2.25	55.00	80.06	80.20	0.17

The experimental and predicted values were plotted as a graph to show the comparison as shown in Figure 34. Actually, if the predicted model was excellent, the experimental and predicted values should be the same. So, the data point in the graph was nearby the x = y line.

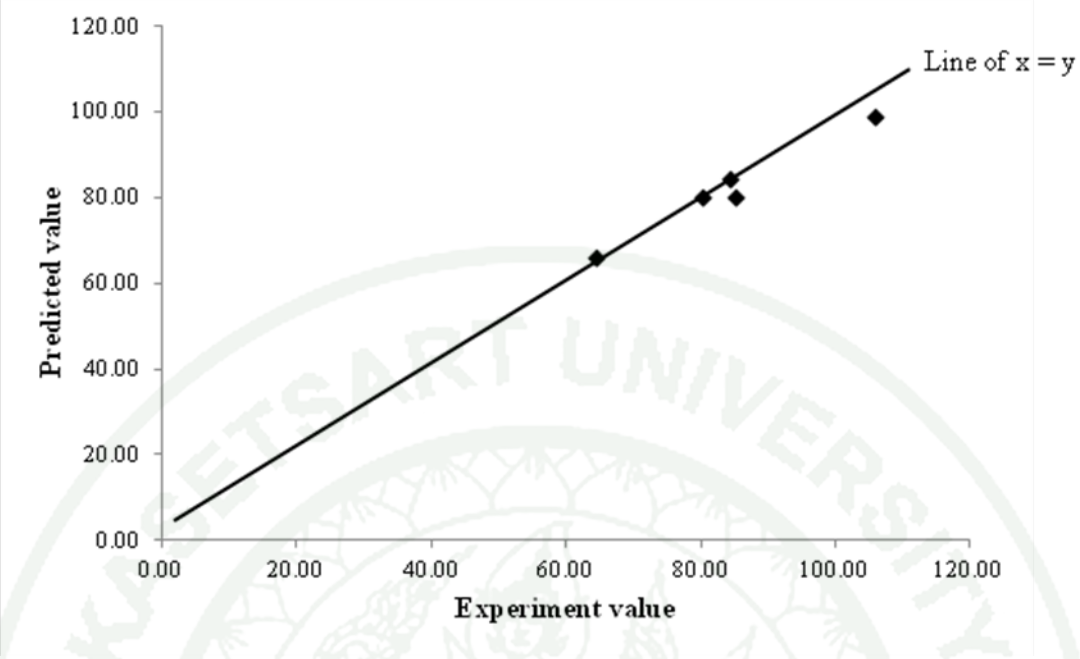


Figure 34 The comparison of experimental value and predicted value for validation test of percentage conversion

In Figure 34, the predicted value that was calculated from the model was not far from experimental value. So, high R^2 and a different least value between experimental and predicted value of validation confirmed that this model was suitable.

The coefficients of predicted model for esterification reaction, which was percentage conversion as a response, was generated by SPSS package. The coefficients are demonstrated in Table 16.

Table 16 Coefficients of the model for percentage conversion

Model	Unstandardized Coefficients		Standardized Coefficients	t-test	Sig
	B	Standard error	Beta		
Intercept	-112.475	60.429	-	-1.861	0.082[b]
A	0.676	1.508	0.403	0.448	0.660[b]
B	25.081	15.075	1.495	1.664	0.117[b]
C	-9.752	10.594	-0.775	-0.921	0.372[b]
D	3.995	1.351	3.175	2.958	0.010[a]
A^2	0.001	0.017	0.036	0.068	0.947[b]
B^2	-1.189	1.742	-0.359	-0.683	0.505[b]
C^2	1.898	0.980	0.625	1.937	0.072[b]
D^2	-0.023	0.010	-2.220	-2.367	0.032[a]
AB	-0.027	0.228	-0.057	-0.116	0.909[b]
AC	-0.106	0.171	-0.252	-0.621	0.544[b]
AD	-0.003	0.017	-0.113	-0.160	0.875[b]
BC	-0.554	1.710	-0.131	-0.324	0.750[b]
BD	-0.192	0.171	-0.791	-1.122	0.279[b]
CD	0.152	0.128	0.768	1.182	0.256[b]

Note: [a] Significant at “Sig.” less than 0.05

[b] Insignificant at “Sig.” more than 0.05

From Table 16, the significant of each variable was defined by t-value and significant (Sig). The linear and quadratic effects of temperature have effect on percentage conversion since Sig. values of both variables were less than 0.05. The standardized coefficient or beta was unit less value which could be used to compare between variables. In this case, linear effect of temperature had more effective than quadratic effect of temperature because beta value of linear effect of temperature was higher than quadratic effect of temperature.

The data was fitted to a quadratic polynomial model. The predicted model for percentage conversion of wax esters (y) in terms of the uncode factor was depicted in equation 15.

$$\begin{aligned} \text{Conversion (\%)} = &- 112.475 + 0.676A + 25.081B - 9.752C + 3.995D + 0.001A^2 \\ &- 1.189B^2 + 1.898C^2 - 0.023D^2 - 0.027AB - 0.106AC \\ &- 0.003AD - 0.554BC - 0.192BD + 0.152CD \end{aligned} \quad (15)$$

Where:

A = Amount of Amberlyst 15 catalyst
B = Oleyl alcohol-to-PFAD molar ratio
C = Reaction time
D = Reaction temperature

2.2 Percentage yield

The percentage yield of wax esters was determined by gas chromatography which used methyl laurate as an internal standard. The interpretation on chromatogram was performed by comparison with chromatogram of standard oleyl esters. The chromatogram of wax esters in every condition was the same pattern but it was different on the peak area of each oleyl ester including oleyl palmitate, oleyl oleate and oleyl linieate. Figure 35 demonstrated the chromatogram of wax esters from the first condition which was 17.5 wt% of Amberlyst 15, 1.75:1 oleyl alcohol-to-PFAD molar ratio, one hour of reaction time and 50 ^{O}C of reaction temperature.

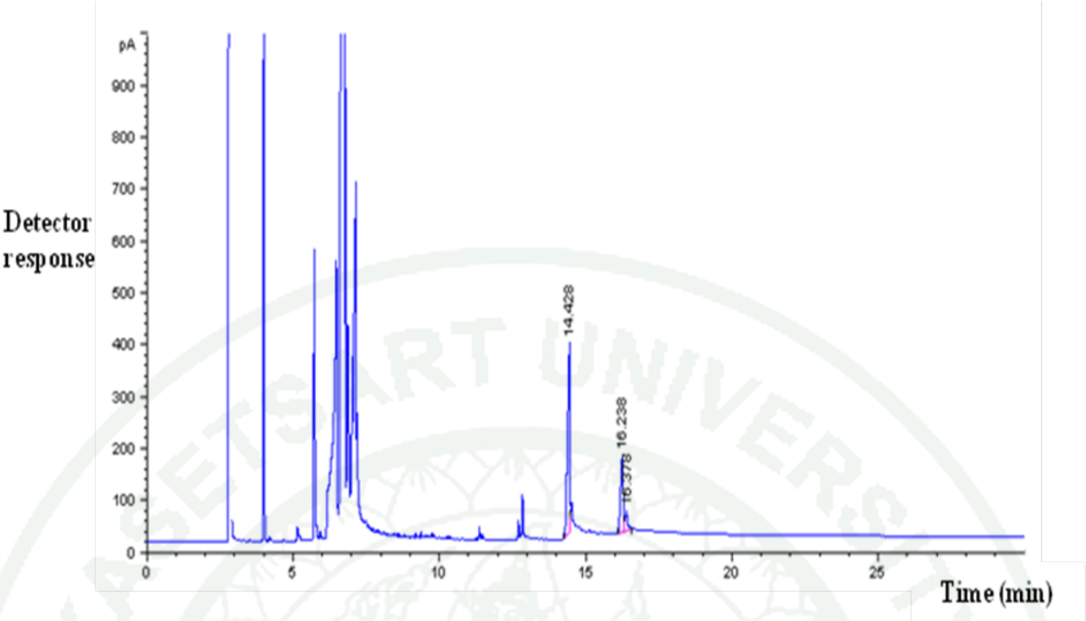


Figure 35 The chromatogram of wax esters generated at 17.5 wt% of Amberlyst 15, 1.75:1 oleyl alcohol-to-PFAD molar ratio, one hour of reaction time and 50 ^{O}C of reaction temperature

Figure 35 showed that the peak of heptane as a solvent at 2.811 min, an internal standard at 3.982 minute, unreacted PFAD at 5.710 minute, a group of excesses of oleyl alcohol at about 6 minute, oleyl palmitate at 14.428 minute, oleyl olealte at 16.238 minute and oleyl linoleate at 16.378 minute. The percentage of esters product was calculated by equations 11 and 12 which was 14.53%. The central composite design conditions and percentage yield of wax esters are given in Table 17.

Table 17 Central composite design (CCD) arrangement along with percentage yield for esterification reaction

Treatment	A	B	C	D	Yield (%)	
					Experimental	Predicted
1	-1	-1	-1	-1	14.53	11.00
2	-1	-1	-1	1	26.56	25.79
3	-1	-1	1	-1	21.32	22.17
4	-1	-1	1	1	30.54	32.55
5	-1	1	-1	-1	45.35	48.10
6	-1	1	-1	1	61.13	56.13
7	-1	1	1	-1	52.82	59.61
8	-1	1	1	1	67.37	63.25
9	1	-1	-1	-1	26.58	26.93
10	1	-1	-1	1	55.29	42.31
11	1	-1	1	-1	48.00	46.91
12	1	-1	1	1	64.55	57.90
13	1	1	-1	-1	66.46	58.38
14	1	1	-1	1	71.76	67.01
15	1	1	1	-1	81.73	78.71
16	1	1	1	1	85.61	82.95
17	-2	0	0	0	37.64	31.59
18	2	0	0	0	57.04	67.22
19	0	-2	0	0	17.85	20.83
20	0	2	0	0	81.78	82.98
21	0	0	-2	0	19.96	28.06
22	0	0	2	0	59.07	55.16
23	0	0	0	-2	46.71	42.49
24	0	0	0	2	53.20	61.51

Table 17 (Continued)

Treatment	A	B	C	D	Yield (%)	
					Experimental	Predicted
25	0	0	0	0	55.13	54.80
26	0	0	0	0	56.12	54.80
27	0	0	0	0	55.93	54.80
28	0	0	0	0	56.58	54.80
29	0	0	0	0	55.31	54.80
30	0	0	0	0	55.48	54.80

Actually, percentage conversion of free fatty acid in Table 17 and percentage yield of wax esters in Table 13 should be equivalent. However, both values in this experiment were quite different because percentage yield was calculated using only the three standards of oleyl esters. This standard included oleyl palmitate, olely olelate and oleyl linoleate which were a major fatty acid in PFAD. Beside three fatty acids, PFAD also contained other fatty acids such as octanoic acid, decanoic acid, lauric acid, myristic acid, stearic acid, linolenic acid, arachidic acid, behenic acid and erucic acid. Hence, percentage yield was lower than a real value. However, both values of percentage conversion and percentage yield were in the same result trend.

The multiple regression coefficients were obtained by employing a least square technique to predict a second-order polynomial model for percentage yield of wax esters. The analysis of variance (ANOVA) for esterification reaction which was percentage yield as a response was presented in Table 18.

Table 18 Analysis of variance (ANOVA) for the second order polynomial model of esterification reaction along with percentage yield

Model	Sum of Squares	Degree of freedom	Mean sum of square	F-value	Sig.
Regression	9976.556	14	712.611	14.714	0.000
Residual	726.466	15	48.431	-	-
Total	10703.022	29	-	-	-
Pure error	1.488	5	0.298	-	-
Lack of fit	724.978	10	7.250	243.28	-

Note: [a]Significant at “Sig.” less than 0.05
[b]Tubular F-value was 4.74

The model characteristics and the coefficients indicated the predictability of the model at 95% confidence level. Table 18 shows result of the analysis of variance ANOVA indicating that the model was highly significant as the model. F-value of 14.714 with Sig. was 0.000 was which less than 0.05 for the model. It implied that the model was significant. The high correlation coefficient (R^2 = 0.932) indicated that the model was suitable to represent the real relationship among the variables studied. In this case, the value of the correlation coefficient (R^2 = 0.932) indicated that the sample variation of 93.20% for the esterification reaction was attributed to the independent variables and only 6.80% of the total variations could not explained by this model.

Moreover, the significant lack-of-fit test ($F_{(0.05,10,5)}$ = 243.28) indicated that the model was not suitable to represent the experimental data using the design experimental data. So, the verify method was chosen to prove the model. The conditions of validation test are presented in Table 19.

Table 19 The experimental data for validation test of percentage yield

Treatment	A	B	C	D	Yield (%)		Difference
	(%wt)	(mol)	(h)	(^{O}C)	Experimental	Predicted	(%)
1	20.00	1.50	1.50	55.00	19.33	17.69	-8.88
2	30.00	1.80	2.50	65.00	51.89	51.91	0.05
3	35.00	3.50	3.75	75.00	80.97	87.89	8.20
4	28.00	2.70	1.25	65.00	59.87	56.90	-5.09
5	15.00	3.80	2.25	55.00	68.34	68.48	0.20

The experimental values were plotted against the predicted values as presented in Figure 36. Actually, the excellent model should have a data point in the graph nearby the x = y line.

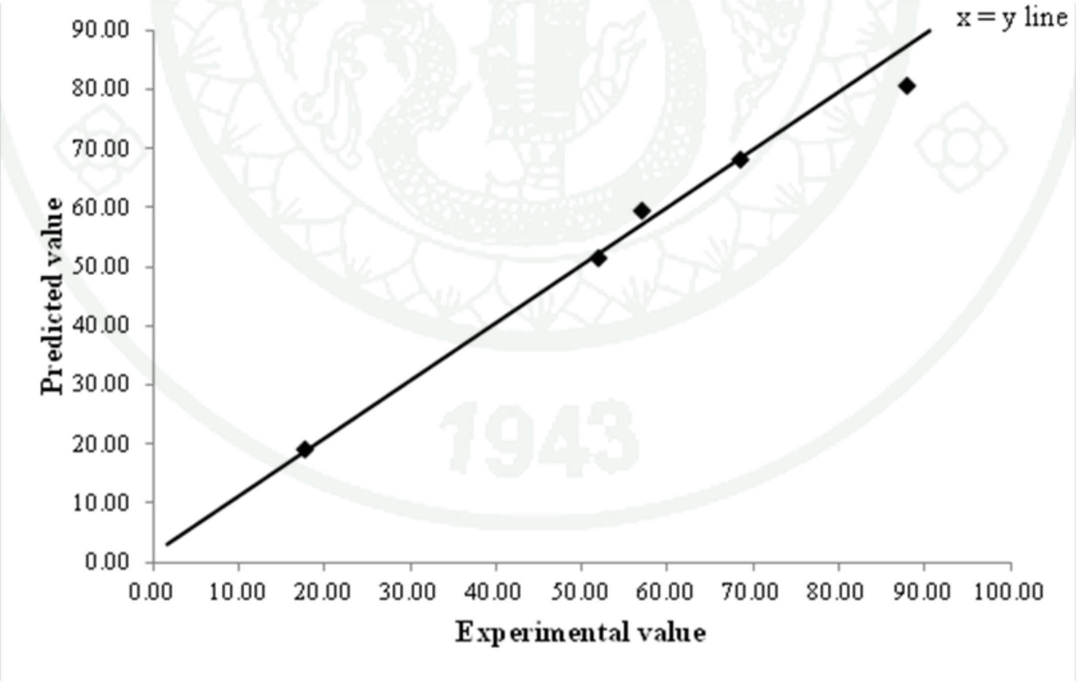


Figure 36 The comparison of experimental and predicted values for validation test of percentage yield

In Figure 36, the predicted values that calculated from the model was close to experimental values. The results of validation test and high R^2 confirmed that the model was suitable.

The coefficients of predicted model for esterification reaction which was percentage yield as a response, were generated by SPSS package. The coefficients were depicted in Table 20.

Table 20 Coefficients of the model for percentage yield

Model	Unstandardized Coefficients		Standardized Coefficients	t-test	Sig
	B	Standard error	Beta		
Intercept	-174.560	81.967	-	-2.130	0.050[b]
A	2.307	2.045	0.819	1.128	0.277[b]
B	46.703	20.449	1.659	2.284	0.037[a]
C	18.931	14.370	0.896	1.317	0.207[b]
D	2.048	1.832	0.970	1.118	0.281[b]
A^2	-0.024	0.024	-0.430	-1.011	0.328[b]
B^2	-1.289	2.362	-0.232	-0.546	0.593[b]
C^2	-3.299	1.329	-0.648	-2.483	0.025[a]
D^2	-0.007	0.013	-0.394	-0.519	0.611[b]
AB	-0.251	0.309	-0.320	-0.811	0.430[b]
AC	0.294	0.232	0.415	1.269	0.224[b]
AD	0.002	0.023	0.059	0.103	0.919[b]
BC	0.116	2.320	0.016	0.050	0.961[b]
BD	-0.225	0.232	-0.553	-0.971	0.347[b]
CD	-0.110	0.174	-0.333	-0.633	0.536[b]

Note: [a] Significant at “Sig.” less than 0.05

[b] Insignificant at “Sig.” more than 0.05

Table 20 shows the significance of two variables which were the linear effect of oleyl alcohol-to-PFAD molar ratio and quadratic effects of temperature. It meaned that both variables had effect on percentage yield. The standardized coefficient indicated that the quadratic effects of temperature was less effective than linear effect of oleyl alcohol-to-PFAD molar ratio due to that the standardized coefficient of linear effect of oleyl alcohol-to-PFAD molar ratio was higher than quadratic effects of temperature.

The experimental data was fitted to a second-order polynomial model. The predicted model for percentage yield of wax esters (y) in terms of the coded factors is shown in equation 18.

$$\begin{aligned}\text{Yield (\%)} = {} & -174.560 + 2.307A + 46.703B + 18.931C + 2.048D - 0.024A^2 \\ & - 1.289B^2 - 3.299C^2 - 0.007D^2 - 0.251AB + 0.294AC \\ & + 0.002AD + 0.116BC - 0.225BD - 0.110CD \qquad (18)\end{aligned}$$

Where:

A = Amount of Amberlyst 15 catalyst
B = Oleyl alcohol-to-PFAD molar ratio
C = Reaction time
D = Reaction temperature

3. Response surface plot

The optimized levels of variables were determined by constructing three-dimensional surface plots according to equations 12 and 13. Two variables were plotted at any one time on the x_1, and x_2 axes, respectively, with the other remaining variables set at their centre point values (coded level : 0). The result of effect of Amberlyst 15 and oleyl alcohol-to-PFAD molar ratio on percentage conversion and percentage yield are presented in Figure 37.

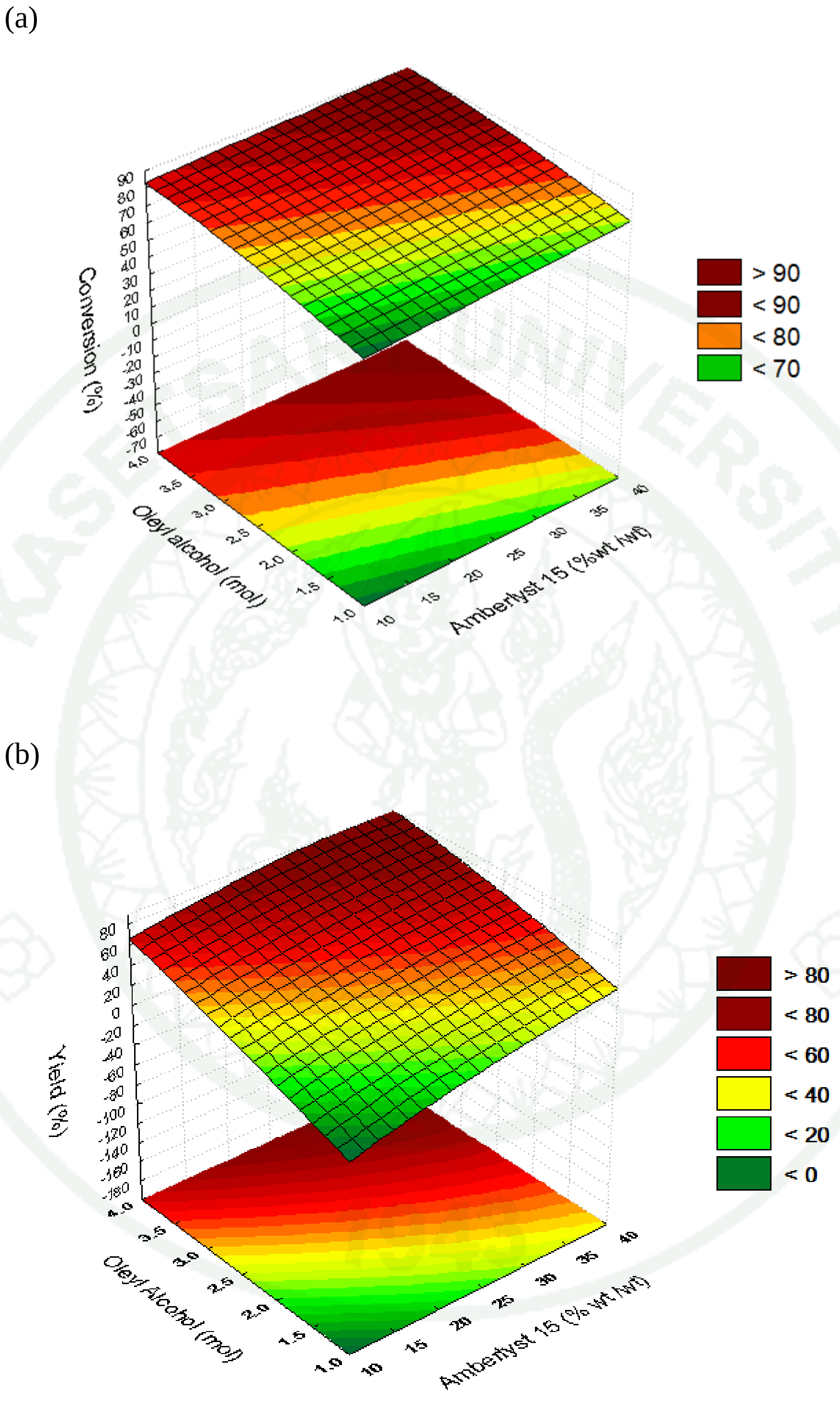


Figure 37 Response surface plots showing the effect of amount of Amberlyst 15, oleyl alcohol-to-PFAD molar ratio and their mutual effect on the synthesis of wax esters: (a) conversion (%); (b) yield (%). Other variables are constant: 2 hour of reaction time and 60 ^{O}C of reaction temperature.

Figure 37 showed the effect of varying amount of Amberlyst 15 and oleyl alcohol-to-PFAD molar ratio on percentage conversion of PFAD (37a) and percentage yield of wax esters (37b) at 2 hours and 60 ^{O}C. The increasing of Amberlyst 15 amount and oleyl alcohol-to-PFAD molar ratio increased percentage conversion(%) and percentage yield(%). The highest values of both responses were above 25%wt of Amberlyst 15 and 3:1 molar ratio of oleyl alcohol-to-PFAD.

The result of effect of Amberlyst 15 and reaction time on percentage conversion and percentage yield was shown in Figure 38.

(a)

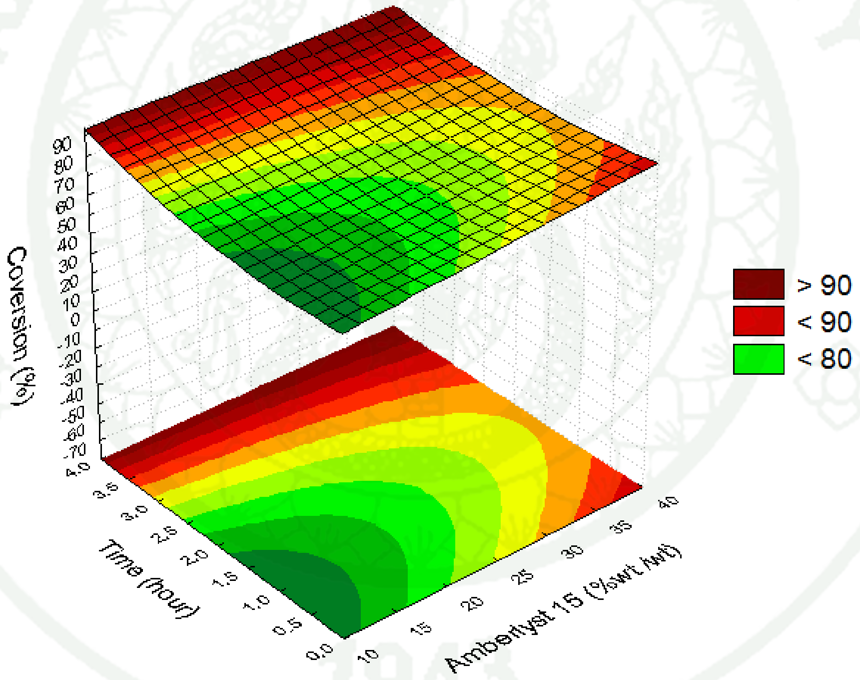


Figure 38 Response surface plots showing the effect of amount of Amberlyst 15, reaction time and their mutual effect on the synthesis of wax esters: (a) conversion (%); (b) yield (%). Other variable are constant: 2.5:1 of oleyl alcohol-to-PFAD molar ratio and 60 ^{O}C of reaction temperature.

(b)

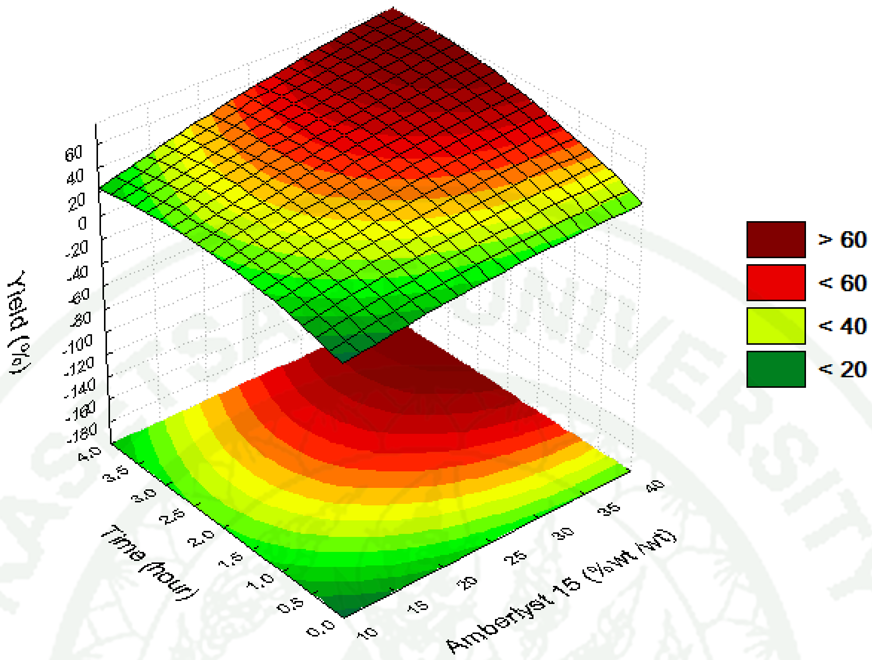


Figure 38 (Continued)

Figure 38a represented response surface plot as a function of catalyst amount, reaction time and interaction on percentage conversion of PFAD at oleyl alcohol-to-PFAD molar ratio,1:2.5 and temperature, 60 ^{O}C. The reaction time from initial time to 3.25 hours showed that percentage conversion of PFAD increases with increasing amount of Amberlyst 15, too. After this condition, the amount of Amberlyst 15 was not effective to percentage conversion. The maximum percentage conversion(%) was more than 3.5 hours at every catalyst amount condition.

The effect of Amberlyst 15 amount and reaction time on percentage yield of wax esters at 1:2.5 of oleyl alcohol-to-PFAD molar ratio and 60 ^{O}C was shown in Figure 38b. At low value of both variables, the percentage yield(%) wasn't clearly change. A large variant of percentage yield(%) started at 25%wt of catalyst amount and 1.5 hours of time. From Figure 10b, it indicated that the highest percentage yield was 30%wt of Amberlyst 15 and 2 hours of reaction time.

The result of effect of Amberlyst 15 and reaction temperature on percentage conversion and percentage yield were demonstrated in Figure 39.

(a)

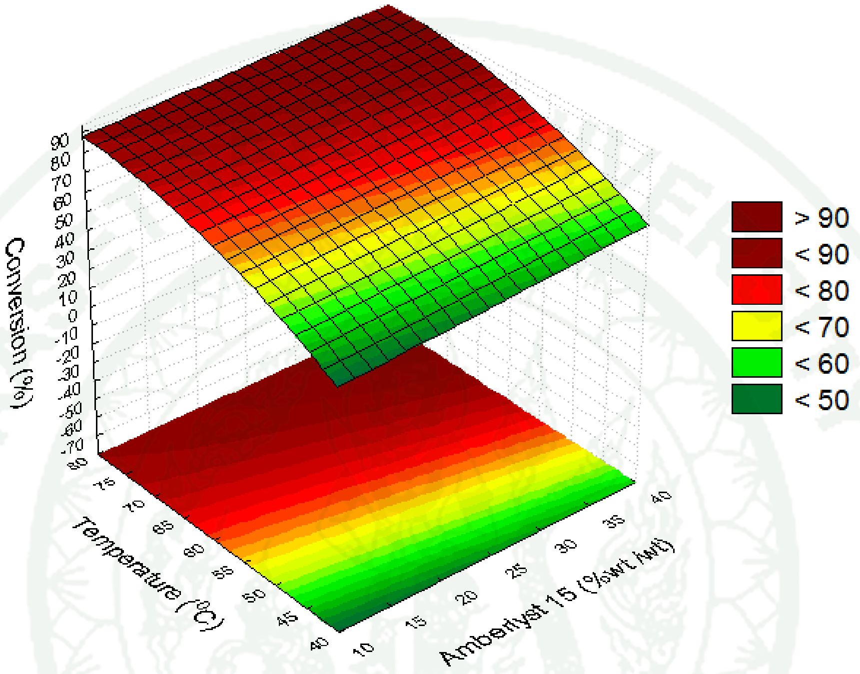


Figure 39 Response surface plots showing the effect of amount of Amberlyst 15, reaction temperature and their mutual effect on the synthesis of wax esters: (a) conversion (%); (b) yield (%). Other variable are constant: 2.5:1 of oleyl alcohol-to-PFAD molar ratio and 2 hour of reaction time.

(b)

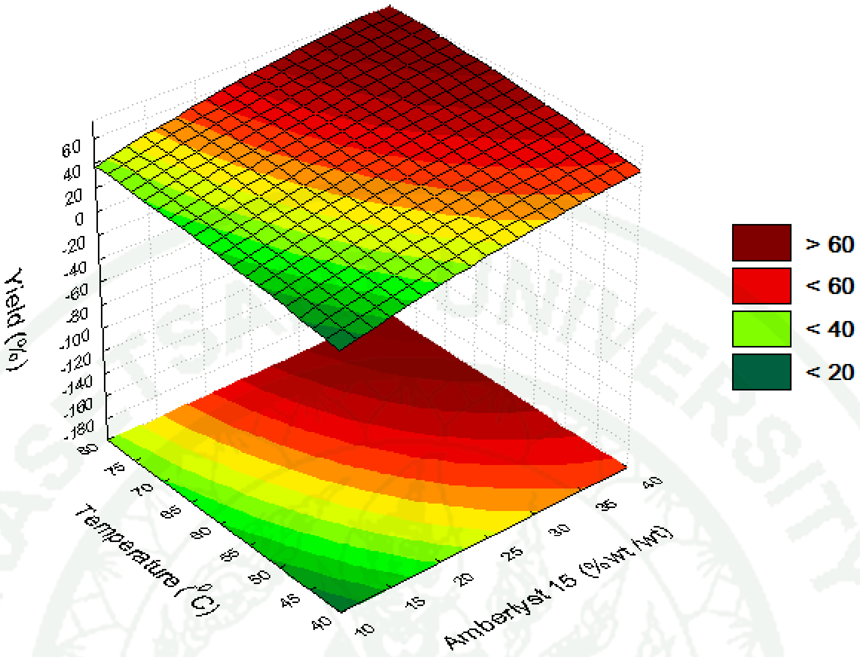


Figure 39 (Continued)

The response surface plotted on percentage conversion of PFAD at 2.5:1 mole of oleyl alcohol-to-PFAD and 2 hours of time was presented in Figure 39a. The amount of Amberlyst 15 did not affect percentage conversion(%) at all reaction temperature. The temperature that is higher than 70 ^{O}C gave maximum conversion (%).

Figure 39b depicted response surface plots as a function of catalyst amount, temperature and interaction of both parameters on percentage yield of wax esters at 2.5 mole of oleyl alcohol to one mole of PFAD and 2 hours of reaction time. The effect of reaction temperature was less effective than the amount of catalyst. The value of Amberlyst 15 amount and temperature which gave the highest percentage yield(%) was 30 %wt and 65 ^{O}C, respectively.

The effect of oleyl alcohol-to-PFAD molar ratio and reaction time on percentage conversion and percentage yield were depicted in Figure 40.

The effect and interaction of oleyl alcohol-to-PFAD molar ratio and time on response at 25 %wt of Amberlyst 15, 60 ^{O}C of reaction temperature were shown in Figure 40. In Figure 40a, the effect of molar ratio of oleyl alcohol-to-PFAD on percentage conversion(%) was the same as the effect of time. The maximum percentage conversion(%) was 3 hours of reaction time and 2.5 moles of oleyl alcohol to one mole of PFAD.

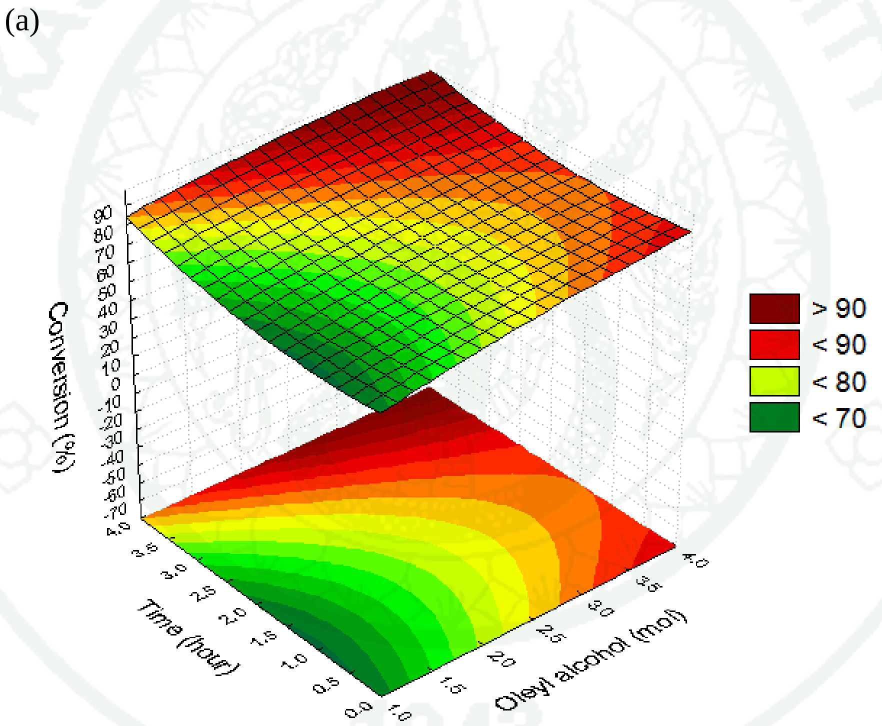


Figure 40 Response surface plots showing the effect of oleyl alcohol-to-PFAD molar ratio, reaction time and their mutual effect on the synthesis of wax esters: (a)conversion (%); (b) yield (%). Other variable are constant: 25 %wt of Amberlyst 15 amount and 60 ^{O}C of reaction temperature.

(b)

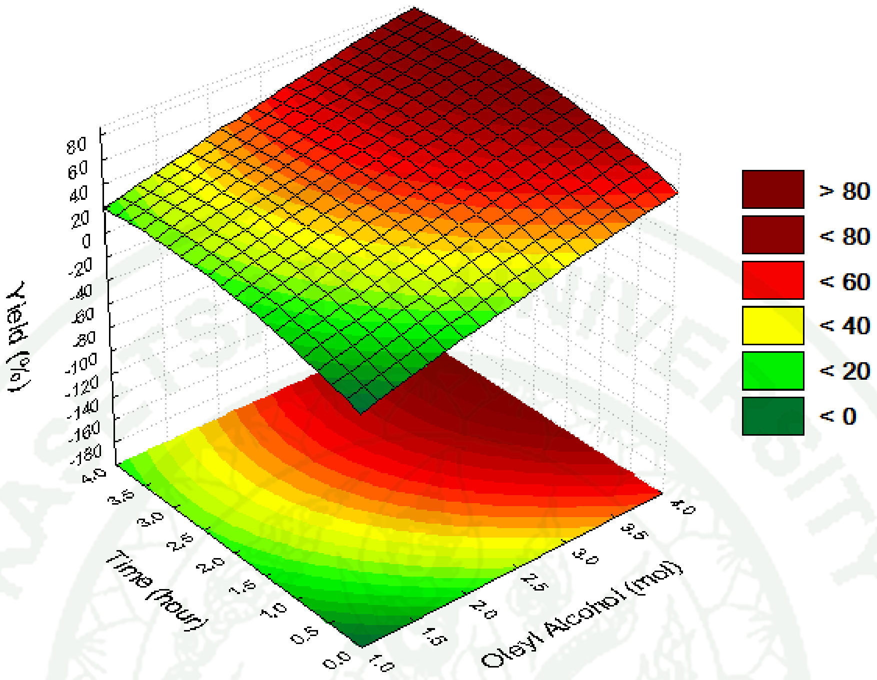


Figure 40 (Continued)

From Figure 40b for percentage yield(%), oleyl alcohol-to-PFAD molar ratio was more effective than reaction time. Increasing of oleyl alcohol-to-PFAD molar ratio made a large change of percentage yield(%) but percentage yield(%) had not change when the reaction time was increased at oleyl alcohol-to-PFAD constancy. The maximum conversion of percentage yield(%) was 3.5:1 molar ratio of alcohol-to-PFAD and more than 1.5 hours of reaction time.

The effect oleyl alcohol-to-PFAD molar ratio and temperature on percentage conversion and percentage yield were shown in Figure 41.

Figure 41 represented the effect and interactive effect of oleyl alcohol-to-PFAD molar ratio and temperature at 25 %wt of catalyst and 2 hours of reaction time. In Figure 41a, percentage conversion(%) increased with increasing both variables. The result showed that 2:1 of oleyl alcohol-to-PFAD molar ratio and 65 ^{O}C of temperature was maximum conversion condition.

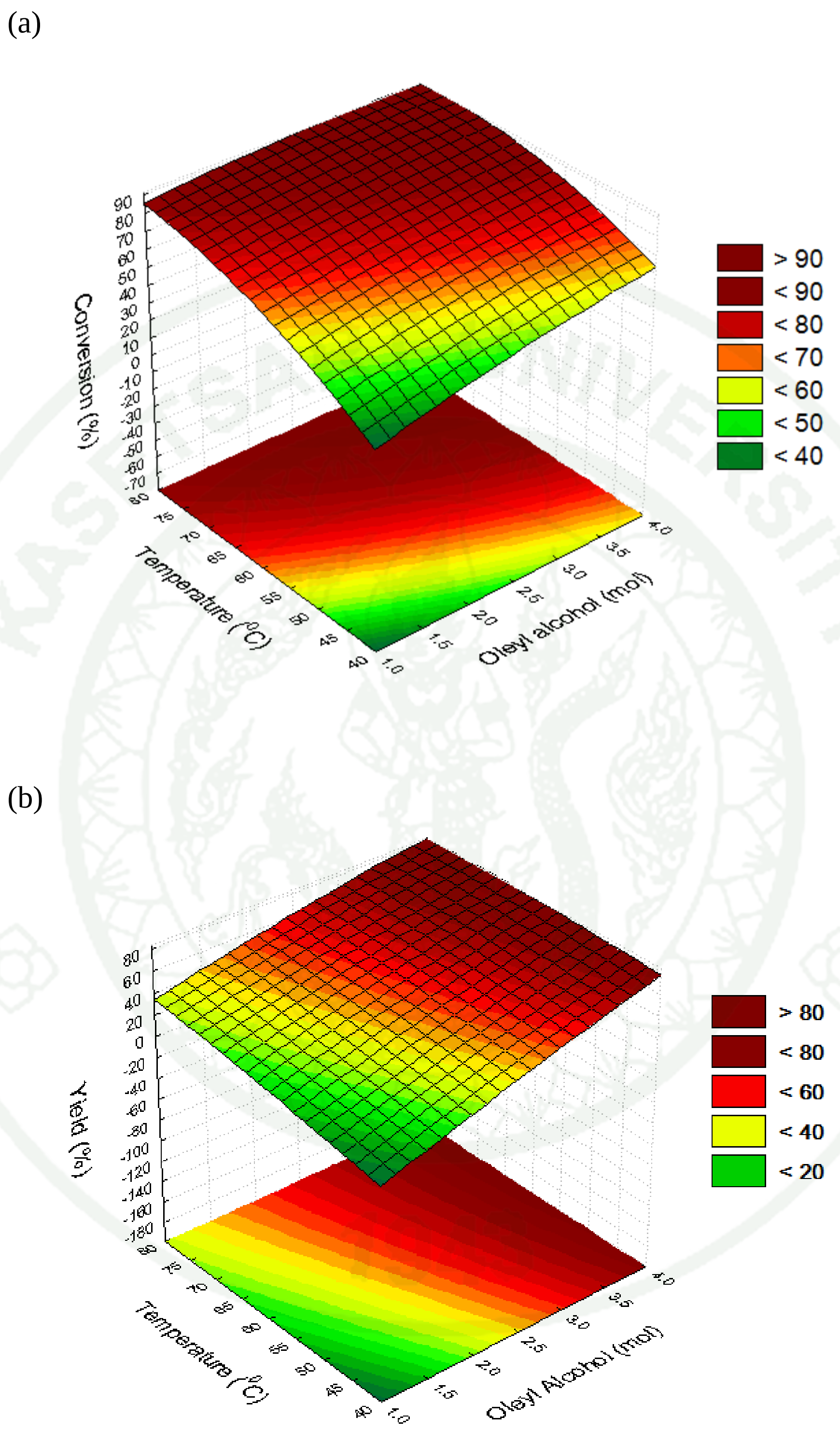


Figure 41 Response surface plots showing the effect of oleyl alcohol-to-PFAD molar ratio, reaction temperature and their mutual effect on the synthesis of wax esters:(a)conversion (%); (b) yield (%). Other variable are constant: 25 %wt of Amberlyst 15 amount and 2 hour of reaction time.

From Figure 41b, it showed that temperature was less effective on percentage yield(%) than oleyl alcohol-to-PFAD molar ratio. And also showed at 3.75 moles of oleyl alcohol, percentage yield(%) wasn't responded from temperature and this point gave the highest value of percentage yield (%).

The effect of reaction time and reaction temperature on percentage conversion and percentage yield were demonstrated in Figure 42.

The response surface plot as a function of time, temperature and interaction at 25%wt of catalyst and 2.5 moles of alcohol are depicted in Figure 42. The percentage conversion (%) was low at low temperature for all of reaction time. The percentage conversion(%) increased with increasing temperature and it gave the maximum conversion at 2.5 hours of reaction time and 65 ^{O}C of reaction temperature.

(a)

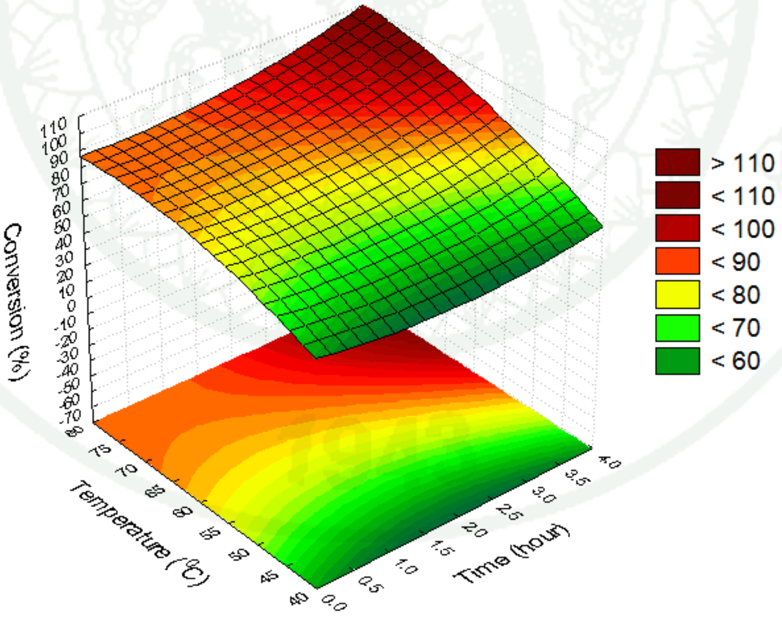


Figure 42 Response surface plots showing the effect of reaction time, reaction temperature and their mutual effect on the synthesis of wax esters:(a)conversion (%); (b) yield (%). Other variable are constant: 25 %wt of Amberlyst 15 amount and 2.5:1 of oleyl alcohol-to-PFAD molar ratio.

(b)

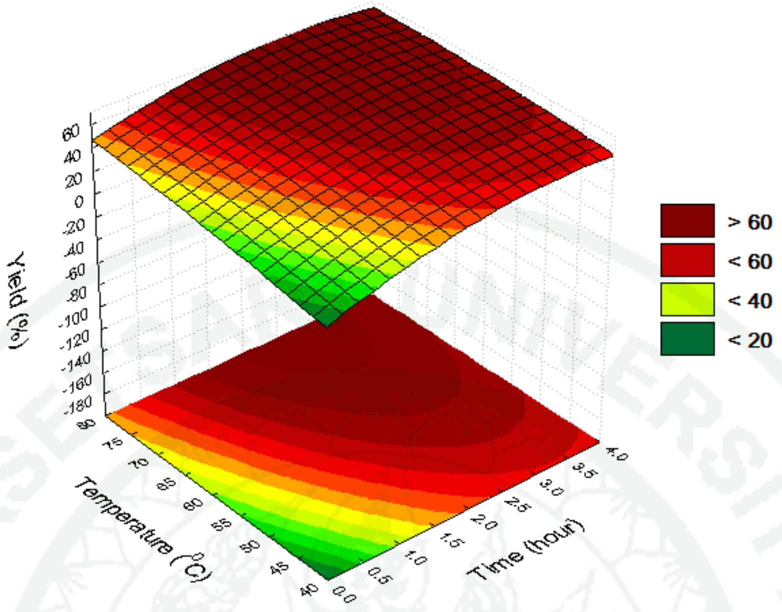


Figure 42 (Continued)

In Figure 42b, the effect of temperature on percentage yield(%) was lower than the effect of time. The percentage yield(%) raised with increasing both variables. The highest percent yield was shown at above 60 ^{O}C of reaction temperature and above 1.5 hours of reaction time.

4. Optimum condition

The optimum condition of wax esters was performed by including the best area of each response surface plot together. The results from Figure 37 to 42 indicated the optimum value of Amberlyst 15 catalyst amount, oleyl alcohol-to-PFAD molar ratio, reaction time and temperature in the range: 30 – 40 % (wt/wt), 3:1 – 4:1 mol, 2.5 – 3.5 h and 65 -75 ^{O}C, respectively. The range of the best area was chosen to run reaction again for the investigation of the optimum condition.

The optimum conditions for esterification reaction from PFAD were predicted using the optimization function of the SPSS software. These are presented in Table 21 along with their predicted and experimental values.

Table 21 Solution of optimum conditions

Experiments No.	Optimized condition				Percentage (%)	
	A (%wt/wt)	B (mol)	C (h)	D (^{O}C)	Conversion	Yield
1	40.00	3.00	2.50	65.00	92.76	81.23
2	30.00	3.50	2.50	70.00	93.89	83.93
3	30.00	3.50	3.00	65.00	94.26	83.05
4	32.00	3.20	3.00	75.00	99.01	81.96
5	35.00	3.00	3.50	65.00	96.20	81.16

Among the various optimum conditions, the highest percentage yield (83.93%) from experiment No. 2 was chosen as the optimum condition although it gave lowest percentage conversion. Because this condition used less amount of catalyst and short reaction time in PFAD conversion than other experiments. This optimum condition was 2.5 hours of reaction time, 30% of amount of Amberlyst 15 catalyst, 3.5:1 of oleyl alcohol-to-PFAD molar ratio and 70 ^{O}C of reaction temperature. The chromatogram of wax esters at optimum condition was shown in Figure 43.

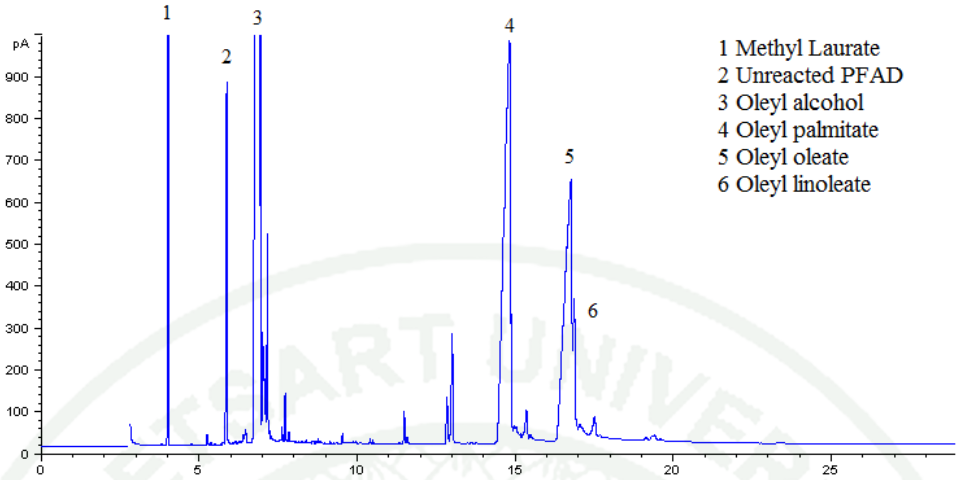


Figure 43 The chromatogram of wax esters at optimum condition

The analysis of chromatogram in Figure 43 was carried out by comparing the retention time of each peak with the authentic oleyl esters standard. Methyl laurate with retention time (RT) 3.903 minute was used as an internal standard for quantitative of three oleyl esters in wax esters product. The retention times of oleyl palmitate, oleyl oleate and oleyl linoleate were 14.496, 16.387 and 16.502 minute, respectively. The result of percentage yield showed that oleyl palmitate had the highest percentage yield (43.29%) followed by oleyl oleate (34.63%) and oleyl linoleate (6.01%). The total percentage yield of three oleyl esters in wax esters product wax 83.93%

This optimum condition was compared with Chobset (2010) who synthesized wax esters from the same starting material and using conventional method. The optimum condition by conventional method was 30% wt/wt of amount of catalyst, 1:2 moles of oleyl alcohol-to-PFAD molar ratio, one of reaction time and 60 ^{O}C of reaction temperature which gave wax esters about 56%. The result showed that the optimum condition from RSM method was presented at a higher condition than conventional method resulting to the percentage yield of wax esters from RSM method had more than the conventional method.

5. Fourier Transform Infrared Spectrophotometer analysis

Fourier Transform Infrared Spectrophotometer (FT-IR) was used to confirm wax esters. The FT-IR spectrums of PFAD and wax esters were presented in Figure 44.

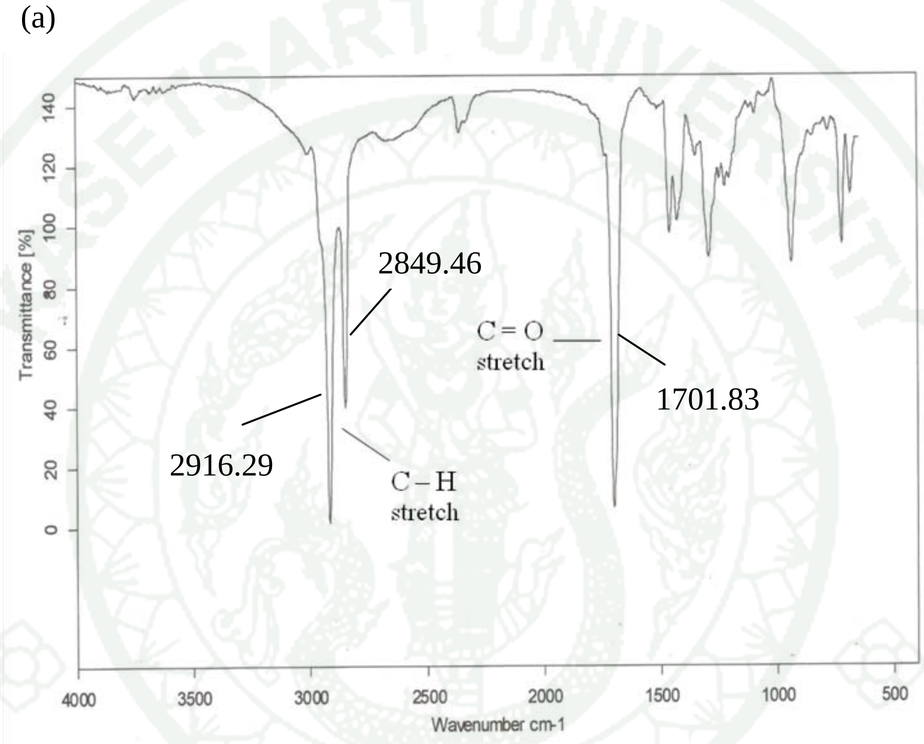


Figure 44 The infrared spectrum of: (a) PFAD; (b) wax esters

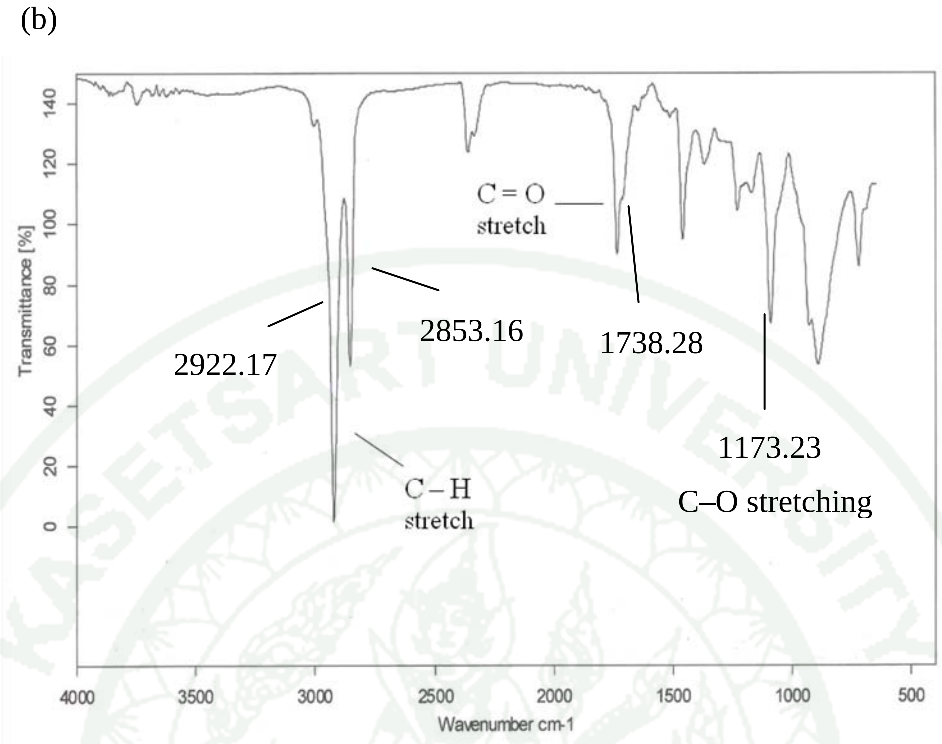


Figure 44 (Continued)

The result from Figure 44 presented that the region of IR spectrum of PFAD and wax esters product about 1400 – 600 cm^{-1} was similar and it was commonly called fingerprinting region. The confirmed spectrum of product was two different peaks. Firstly, the absorption band at 1701.83 cm^{-1} which was C=O stretching of acid that presented in PFAD raw material. In wax esters product, this band shifted to 1738.28 cm^{-1} which assigned to C=O stretching of esters. Lastly, the absorption band at 1173.28 cm^{-1} of product represented C–O stretching of esters which was absent in PFAD spectrum. The absorption bands confirmed that the product was ester.

6. Properties of wax esters at optimum condition

In section 4, the optimum condition gave 5.40% of percentage FFA. These conditions were run again in a large scale to use as a stock for analyzed properties. In this batch, 20 g of PFAD was performed to synthesize wax esters. The reaction produced a larger amount of water than previous reaction. Thus, time to remove this water was longer than usual. It was about 15 – 20 minutes. After removing water, the sample was analyzed percentage FFA again. It found that the percentage FFA reduced from 5.40% to 0.50%. The reason for this change will discuss in section 9.

The physicochemical characteristics of wax esters indicated a qualitative product. The properties were investigated by standard method. These values were presented in Table 22.

Table 22 The physicochemical properties of wax esters

Characteristics	PFAD	PFAD esters	Palm oil esters[a]	Palm olein esters[a]	Jojoba oil[b]
Specific gravity at 25 ^{O}C	0.8685	0.8427	0.8390	0.838	0.861
Acid value (mg KOH/g)	192.79	1.10	0.60	0.80	-
Iodine value (g I_2/100g)	51.54	83.44	69.70	71.60	81.0
Saponification value (mg KOH/g)	200.46	82.02	93.10	84.00	88.0
Kinematic viscosity at 40 ^{O}C (cSt)	n.d.[c]	18.72	-	-	24.75
Water content (%wt)	0.05	0.05	-	-	0.64

Note: [c] n.d. is not detected

Source: [a] Keng *et al*., 2009

[b] Allawzi *et al*., 1998

The physicochemical properties of esters from palm fatty acid distillate were compared with various types of palm's esters (Keng *et al*., 2009) and jojoba oil (Allawzi *et al*., 1998). The density of product was lower than PFAD due to that density was mass per unit volume and raw material as solid substance was less mass when compare with PFAD's wax esters at same volume.

Acid value indicates the free fatty acid (FFA) in ester's molecule. Large amount of FFA was disadvantageous because it was oxidized with oxygen and produced rancidity (Frega *et al*., 1999). The acidity value of PFAD's ester was 11.84 mg KOH/g sample which was highest score of all palm base esters due to that the condition for esters synthesis was not the same.

The iodine value is parameter indicating the number of double bond within molecule. Most of esters must contain more valued than that of raw material result from oleyl alcohol which had double bond in molecule reacts with starting material such as FPAD to generate wax esters. The iodine value of esters from PFAD was 83.44 g I_2/100 g sample which was higher than PFAD (51.54 I_2/100 g sample). The advantage of higher iodine value was increasing permeation rate of compound into stratum corneum when applied on the skin (Sin *et al*., 2009).

Saponification value of product was 82.02 mg KOH/g sample which was lower than palm fatty acid distillate. A mole of KOH is required to completely satoponify one mole of esters and raw material. The molecular weight of PFAD was lower than esters and one gram of PFAD was larger moles than esters. Hence, the number of KOH that needed to react with PFAD was higher than esters at the same sample's mass.

Kinematic viscosity of sample depends on molecular weight and temperature. This value increased with increasing molecular weight but decreased with increasing temperature (Noureddini *et al*., 1992). Viscosity at 40 ^{O}C of esters was 18.72 cSt.

Finally, the water content of esters was 0.05 wt%. A higher value of moisture content could be detected as the result of hydrolysis reaction.

Overall physicochemical properties of wax esters from palm fatty acid distillate were similar to those of jojoba oil (Allawzi *et al*., 1998) which was natural wax esters. Hence, these wax esters could be used to replace the natural wax. Moreover, the properties of PFAD's esters were as same as the properties of ester from Sin *et al*. (2008) who studied palm esters for cosmetic industry. Thus, these wax esters could be used as cosmetic's ingredient.

7. UV-Visible Spectrophotometer analysis

The natural vitamin E is contains of two main groups which are tocopherols and tocotrienols. The tocopherols have different forms such as α-, β-, γ- and δ-tocopherol. However, α-tocopherol was used to represent the total tocopherols in this study.

UV-VIS spectrophotometer was used to analyze vitamin E containing in PFAD and synthetic wax esters. The method calibrated using α-tocopherol and the calibration curve is demonstrated in Figure 45.

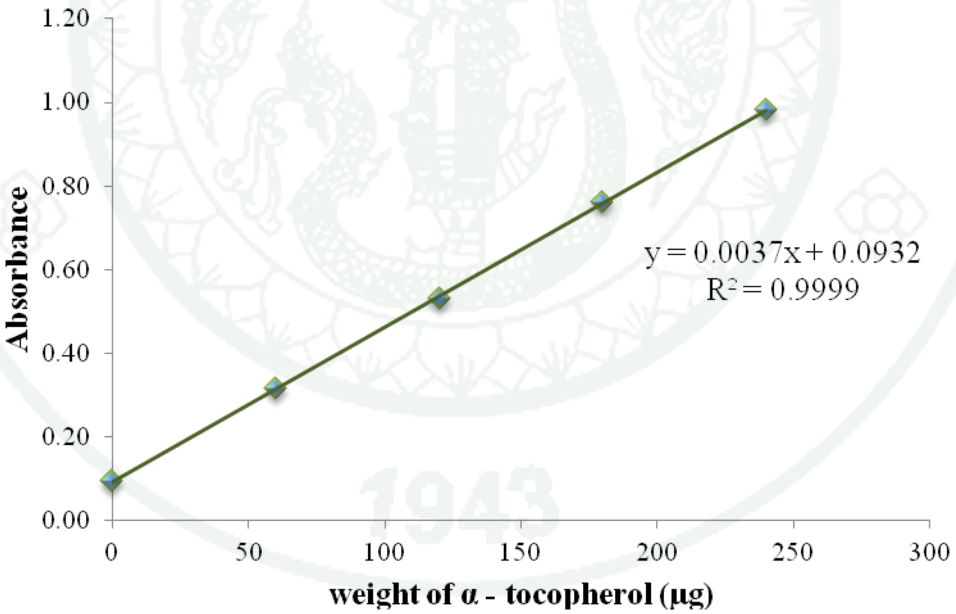


Figure 45 The calibration curve of α-tocopherol

The gradient of calibration curve between weight of α-tocopherol and absorbance was 0.0037. The total tocopherol of palm fatty acid distillate and synthetic wax esters were 5030.80 ppm and 144.31 ppm, respectively. The value of PFAD which was performed by Wong *et al*. (1988) was 7040 ppm. The difference of both values might be result of varietal palm's species, geography of palm's plantation and production of palm fatty acid distillate.

In addition, the result showed that the total tocopherol of synthetic wax esters were lower than palm fatty acid distillate. Because the wax esters were synthesized at high temperature and this point promoted the oxidation of vitamin E (Chu *et al*., 2002).

8. Efficiency of reuse Amberlyst 15 catalyst

The way to save production cost depends on number of catalyst reuse. In this case, the reusability of Amberlyst 15 catalyst was performed by washing the catalyst with hexane and methanol. Then, Amberlyst 15 catalyst was dried at 105 ^{O}C for 4 hours and reused again. The condition of recovering catalyst was run at 30 wt% of Amberlyst 15 catalyst, 2.5:1 of oleyl alcohol-to-PFAD mol ratio, 2.5 hours of reaction time, 70 ^{O}C of reaction temperature and 400 rpm of agitation speed. The efficiency of Amberlyst 15 catalyst was investigated in terms of percentage conversion and shown in Figure 46.

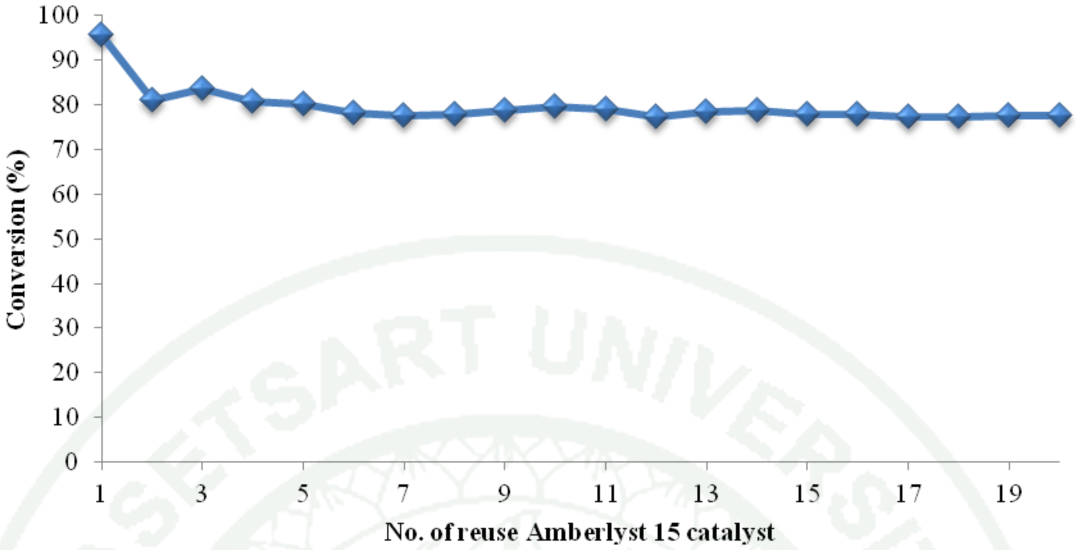


Figure 46 Number of Amberlyst 15 reuse and conversion (%) which performed at 30 wt% of Amberlyst 15 amount, 2.5:1 of oleyl alcohol-to-PFAD molar ratio, 2.5 hours of reaction time, 70 ^{0}C of reaction temperature and 400 rpm of agitator speed

Figure 46 demonstrates the potential of catalyst decreased at the second time and remained constant until the last run. Due to the Amberlyst 15 catalyst had some outer surface pores and many pores of inner surface (Park *et al.*, 2010), the contaminant such as PFAD or esters was coated onto the pore of catalyst. The recovering process of catalyst might be washed the inner surface pore lower than outer surface pore. Thus, the area of catalyst which made the reaction reduced from the first time that made the conversion (%) in second time was 81% which decreased from 96% of first time and remained constant.

CONCLUSIONS AND RECOMMENDATIONS

Conclusions

Palm fatty acid distillate which is a by-product from palm oil refinery industry could be used as a cheap raw material for wax esters synthesis. It was synthesized by esterification reaction with oleyl alcohol using Amberlyst 15 catalyst. These wax esters had similar properties of jojoba oil which is a natural waxes and it could be used in widely application such as cosmetic's ingredient.

The Palm fatty acid distillate consists of fatty acid (88.03%) as a major component, glyceride (1.35%) and other compounds (10.62%). The three main compositions contained of fatty acid in PFAD were palmitic acid, oleic acid and linoleic acid. It also had octanoic acid, decanoic acid, lauric acid, myristic acid, stearic acid, linolenic acid, arachidic acid, behenic acid and erucic acid as minor compositions. The physicochemical properties of palm fatty acid distillate were acid value of 192.79 mg KOH/g sample, iodine value of 51.54 g I_2/100g sample, saponification value of 200.46 mg KOH/g sample, specific gravity of 0.8685 at 25 ^{O}C and water content of 0.05 wt%.

Response surface methodology (RSM) based on a five-level-four-factor central composite design (CCD) was performed to evaluate the interactive effect of this reaction and revealed the optimum condition. The responses of this synthesis were percentage conversion and percentage yield. The percentage conversion was calculated by comparing the initial and the final of free fatty acid content. For the percentage yield, the weight of wax esters was determined by GC using methyl layrate as an internal standard. The optimum condition derived via RSM was 30 wt% of Amberlyst 15 catalyst amount, 2.5:1 of oleyl alcohol-to-PFAD molar ratio, 2.5 hours of reaction time and 70 ^{O}C of reaction temperature. At this condition, the experimental conversion (%) and yield (%) were 93.89% and 83.93%, respectively.

The FT-IR analysis was used to support wax esters product. The absorption band at 1701.83 cm^{-1} was assigned to C=O stretching of acid in PFAD and it slightly shifted to 1738.28 cm^{-1} of product. The absorption band at 1173.28 cm^{-1} of product represented C–O stretching of esters which absented in PFAD spectrum. Both absorption bands confirmed that the reaction was occurred.

The physicochemical characteristics of wax esters were presented the specific gravity of 0.8685 at 25 ^{O}C, acid value of 11.84 mg KOH/g sample, iodine value of 83.44 g I_2/100 g sample, saponification value of 82.02 mg KOH/g sample, kinematic viscosity of 18.72 cSt at 40 ^{O}C and the water content of 0.05%.

In this study, the vitamin E content was investigated in form of total tocopherol and used α-tocopherol as a standard. The total tocopherol of palm fatty acid distillate and synthetic wax esters were 5030.80 ppm and 144.31ppm, respectively.

The number of reused Amberlyst 15 catalyst was investigated by running 20 cycles of reaction. The response of this test was determined on percentage conversion. The result indicated that the percentage conversion at second run drop from the first run and it remained constant until a last run.

Recommendations

The esterification reaction in this research was studied at the temperature of 40 to 80 ^{O}C, so, it should be studied the reaction at the temperature from 90 to 120 ^{O}C because the reaction at higher temperature will reduce the reaction time.

This work propose the synthesis of wax esters using heterogeneous acid catalyst when compared with Gunawan *et al.* (2005) who synthesized wax esters using lipase enzyme as a catalyst and RSM to evaluate the optimum condition. The result showed that the optimum condition of enzyme as a catalyst used lower amount of enzyme loading and temperature than Amberlyst 15 but the time of enzymatic method had longer than Amberlyst 15 about 3 times to produce wax esters as a same scale. For applied in real industry, it was important to realize the cost of production process which depended on several thing such as consumptive energy and catalyst's price for choosing the appropriated catalyst.

In addition, the wax esters in this research were synthesized to replace the natural waxes which were used in various applications. The main propose of synthesized was using as a cosmetic's ingredient. Hence, this waxes should be performed the irritancy test and moisturization test. On the other hand, wax can be used to coat on fruit's surface to keep a fresh fruit. Thus, it should be tested the protected period after the fruit was coated with wax. And it also tested that this wax had the effect on fruit.

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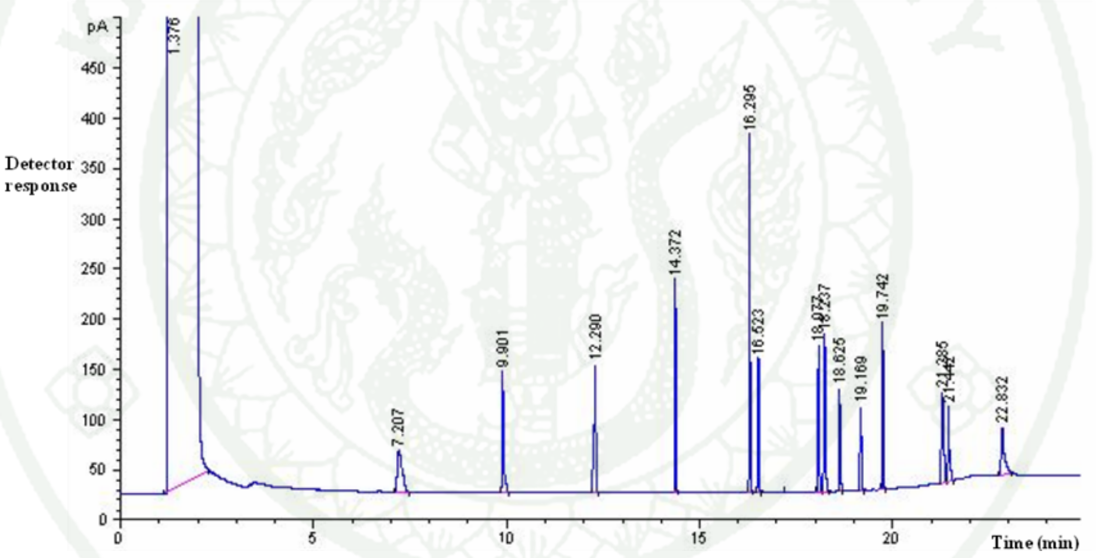
APPENDICES

Appendix A

Standard fatty acid methyl esters

Preparation of standard fatty acid methyl esters

Five concentration of standard fatty acid methyl esters (FAME) mixture (C_8 – C_{24}) was dissolved in heptanes. This concentration included 1000, 2000, 3000, 4000 and 5000 ppm and each mixture was added with 10 mg/ml of methyl heptadecanoate (C_{17}) as an internal standard. The relation between peak area of mixed standard with peak area of internal standard and fatty acid concentrations of mixed standard with concentration of internal standard were plotted on y-axis and x-axis, respectively. The presented of peak with each retention time was specify with each fatty acid methyl esters.



Appendix Figure A1 Chromatogram of standard fatty acid methyl esters mixture (C_8 - C_{24})

Appendix Table A1 The retention time of each fatty acid methyl esters compound

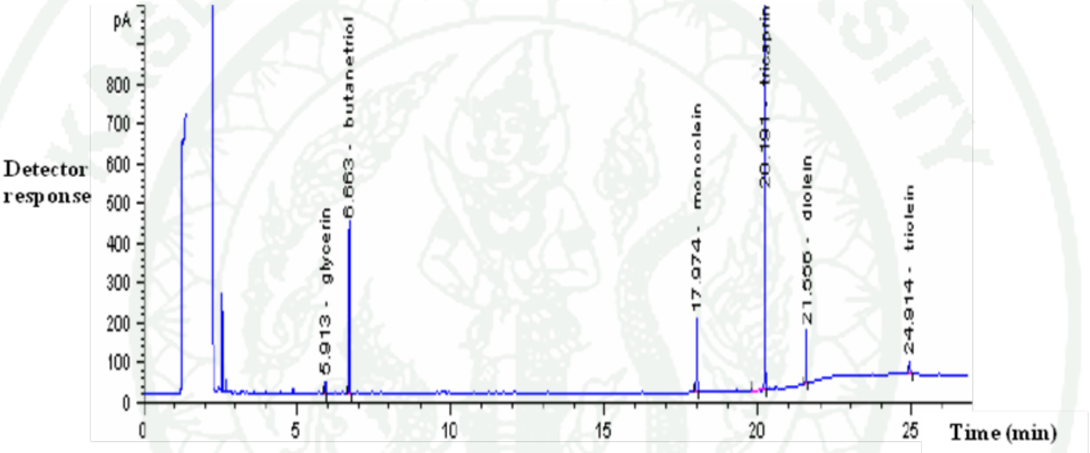
Fatty acid compound	Retention time (min)
Methyl octanoate	7.207
Methyl decanoate	9.901
Methyl laurate	12.290
Methyl myristate	14.372
Methyl palmitate	16.295
Methyl palmitoleate	16.523
Methyl sterate	18.077
Methyl oleate	18.237
Methyl linoleate	18.625
Methyl linolenate	19.169
Methyl arachidate	19.724
Methyl behenate	21.285
Methyl erucate	21.442
Methyl linocerate	22.832

Appendix B

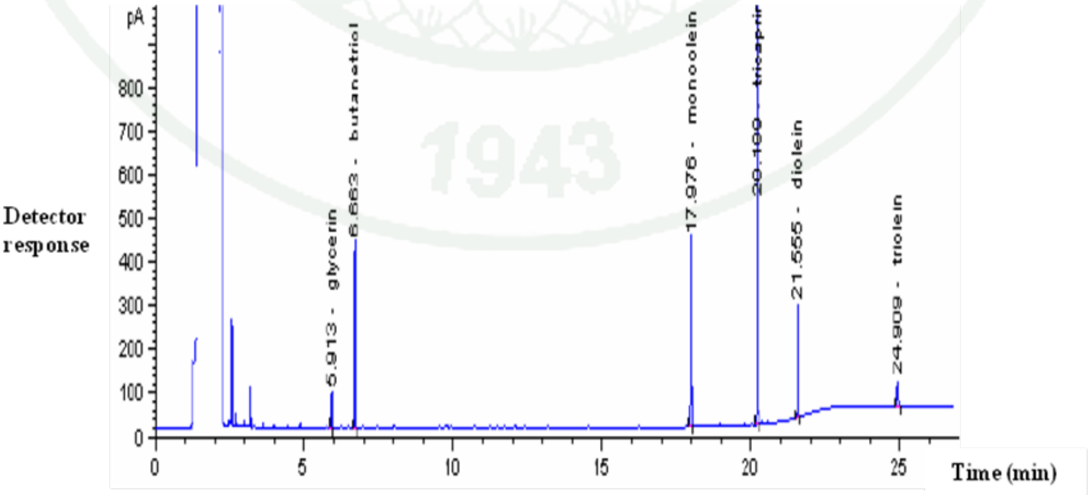
Standard mono, di and triolein

Preparation of standard mono, di and triglyceride

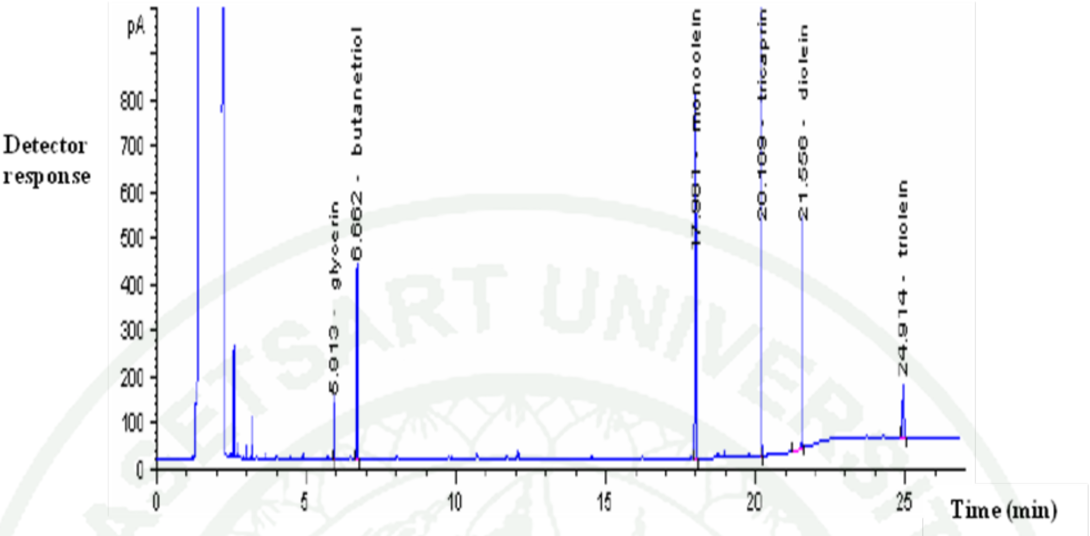
The standard glyceride included monoolein, diolein and triolein. Five concentration of glycerid was dissolved in heptanes. This concentration included 1000, 2000, 3000, 4000 and 5000 ppm and mixture was added with 8 mg/ml of tricaprin as an internal standard. The relation between peak area of each standard with the internal standard and concentration of standard mono, di and triglyceride with concentration of internal standard were plotted on y-axis and x-axis, respectively.



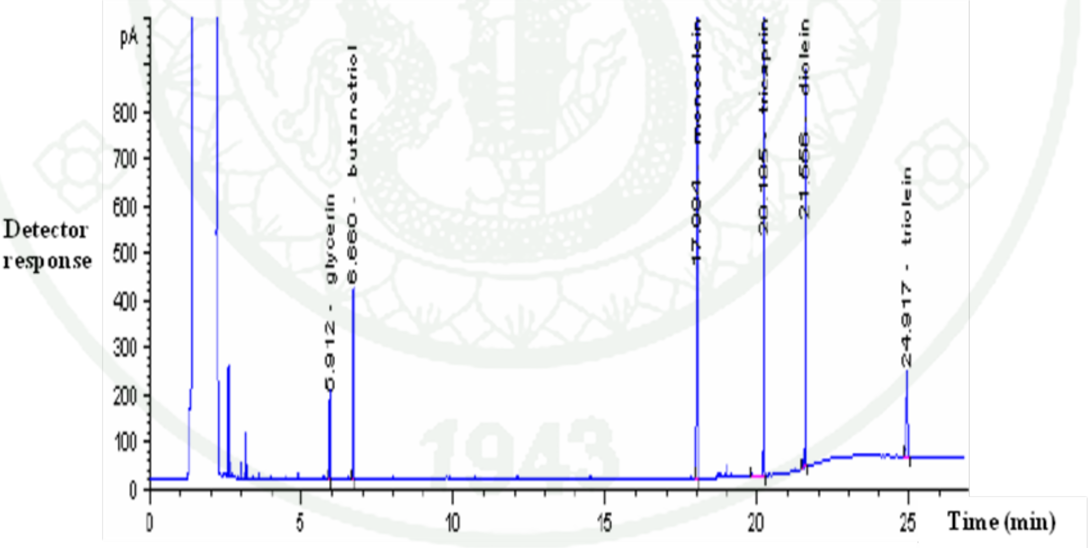
Appendix Figure B1 Chromatogram of standard triglyceride1



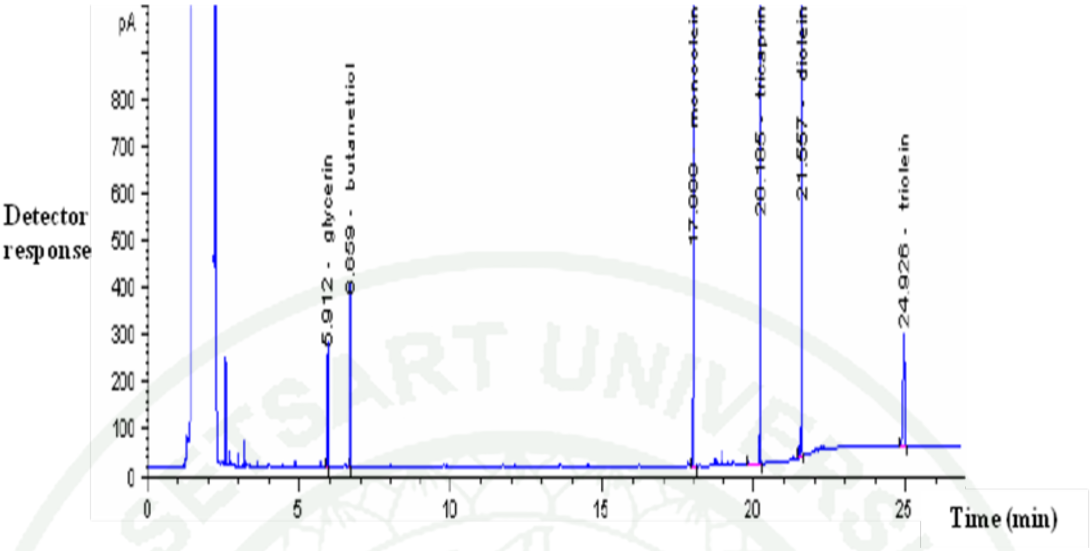
Appendix Figure B2 Chromatogram of standard triglyceride 2



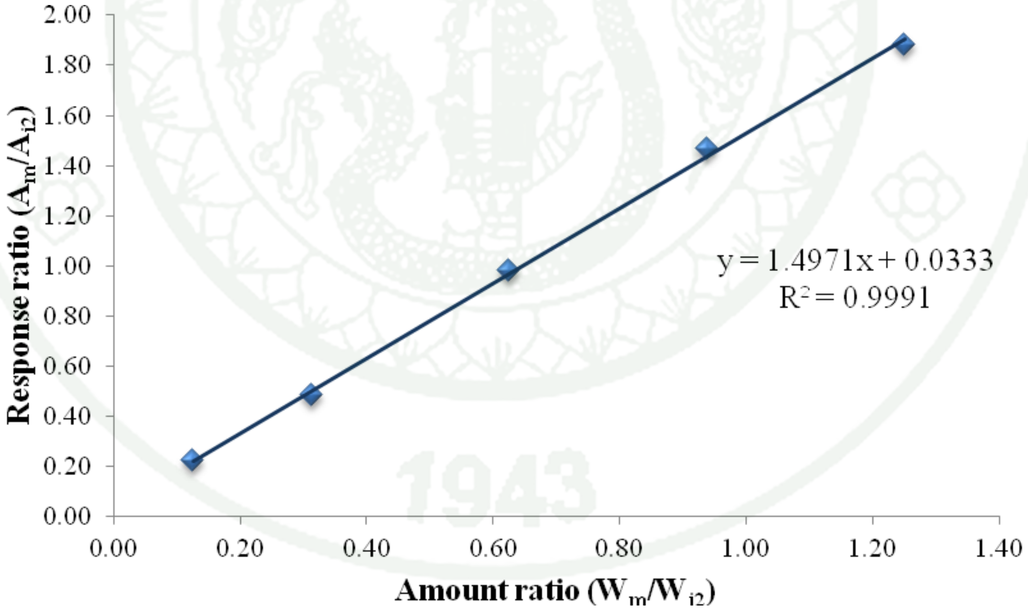
Appendix Figure B3 Chromatogram of standard triglyceride 3



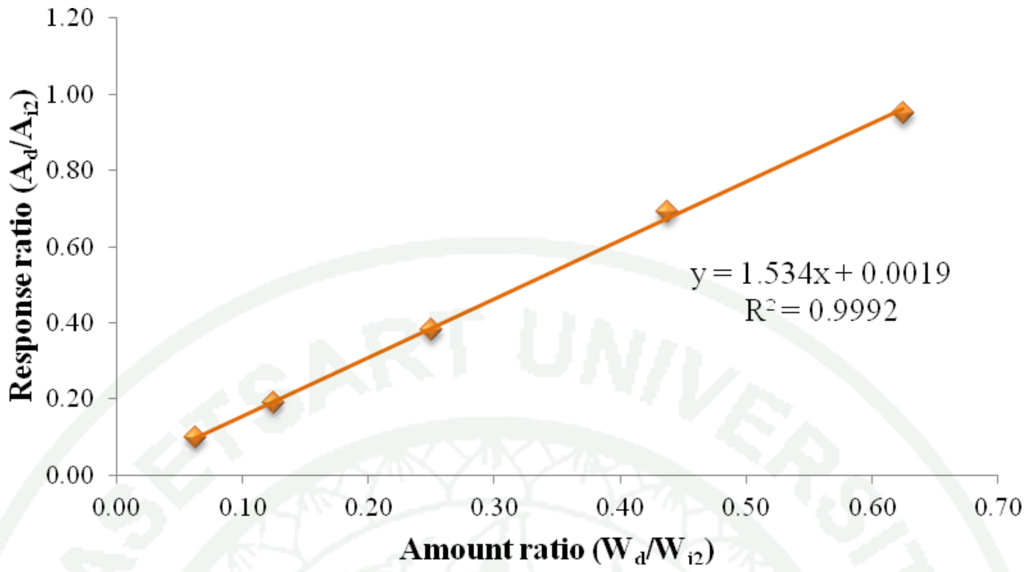
Appendix Figure B4 Chromatogram of standard triglyceride 4



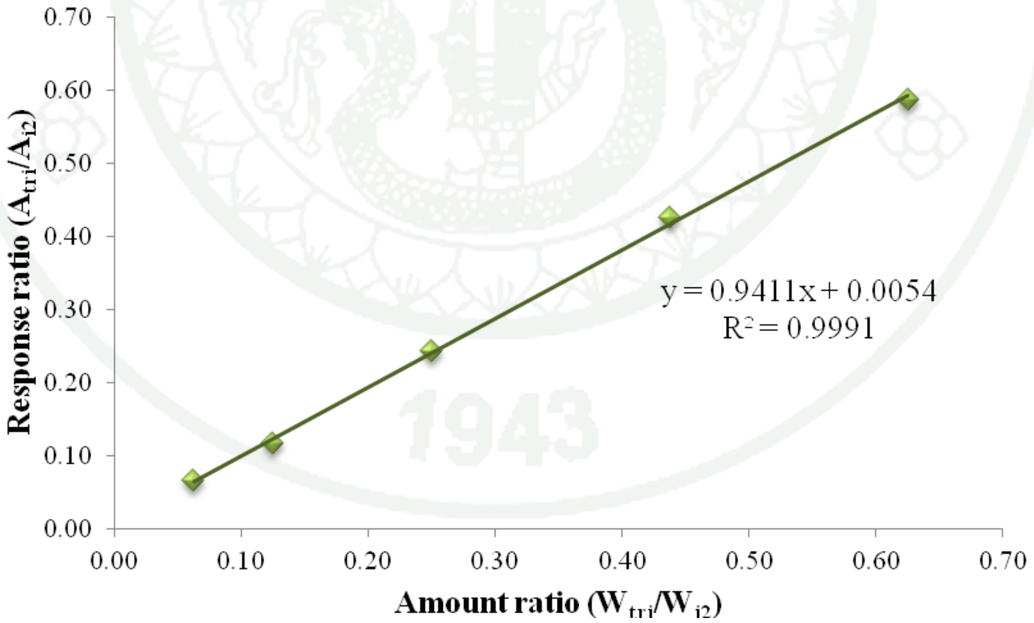
Appendix Figure B5 Chromatogram of standard triglyceride 5



Appendix Figure B6 Calibration curve of monoolein



Appendix Figure B7 Calibration curve of diolein



Appendix Figure B8 Calibration curve of triolein

Appendix C

Standard oleyl fatty acid esters

Preparation of standard oleyl esters

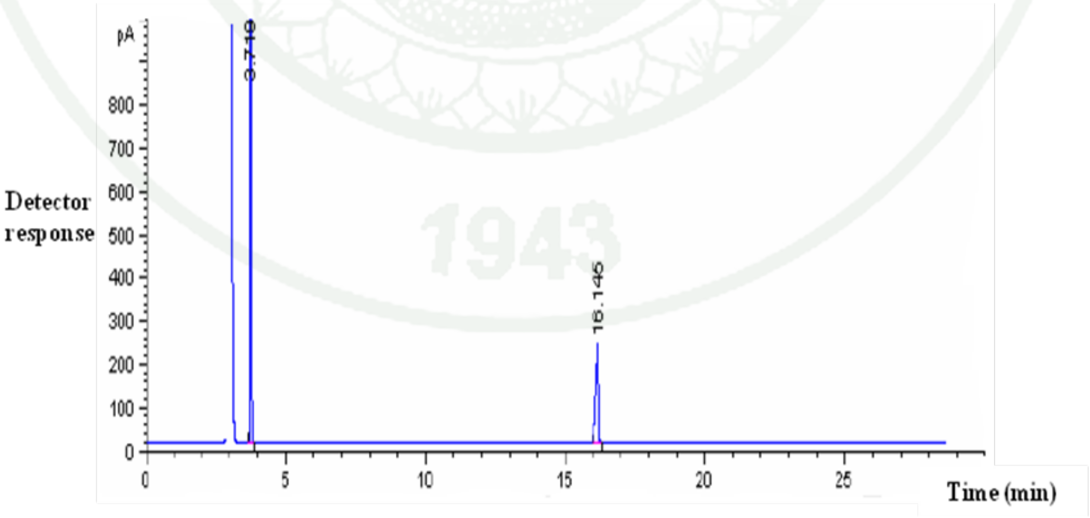
1. Three stock solutions were prepared from oleyl palmitate, oleyl oleate, and oleyl linoleate standards at the original concentrations of 10000, 10,250, and 52,860 mg/L, respectively.

2. A stock solution of an internal standard (methyl laurate) was prepared for the concentration of 3,000 mg/L.

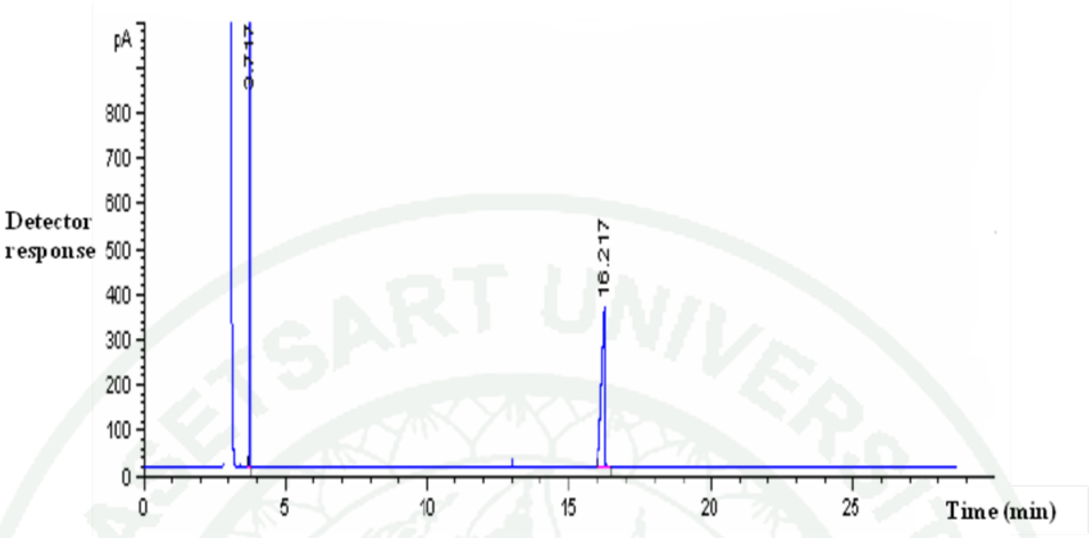
3. Six levels of standard concentration were prepared by dilution of the stock solution. The concentrations of oleyl palmitate were 512.5, 1025, 2050, 3075, 4100, and 5125 mg/L. The concentrations of oleyl oleate were 500, 1,000, 2,000, 3,000, 4,000, and 5,000 mg/L. The concentrations of oleyl linoleate were 528.6, 1057.2, 1585.8, 2114.4, 3171.6, and 4228.6 mg/L.

4. Each standard concentration was put into the vial and added 0.5 ml of internal standard was added into the vial.

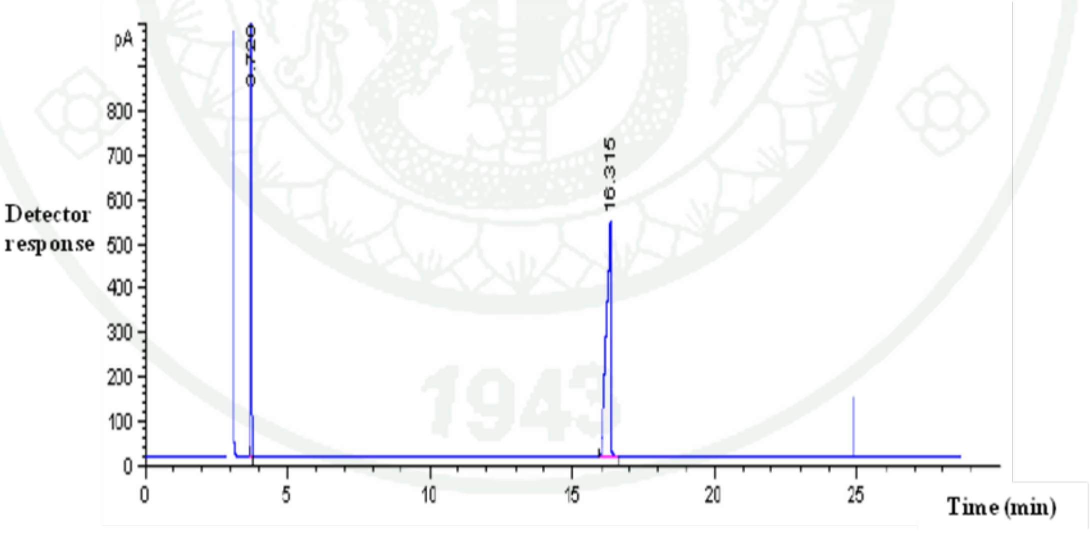
5. Adjusted the final volume of solution was adjusted for 1 ml with n-heptane



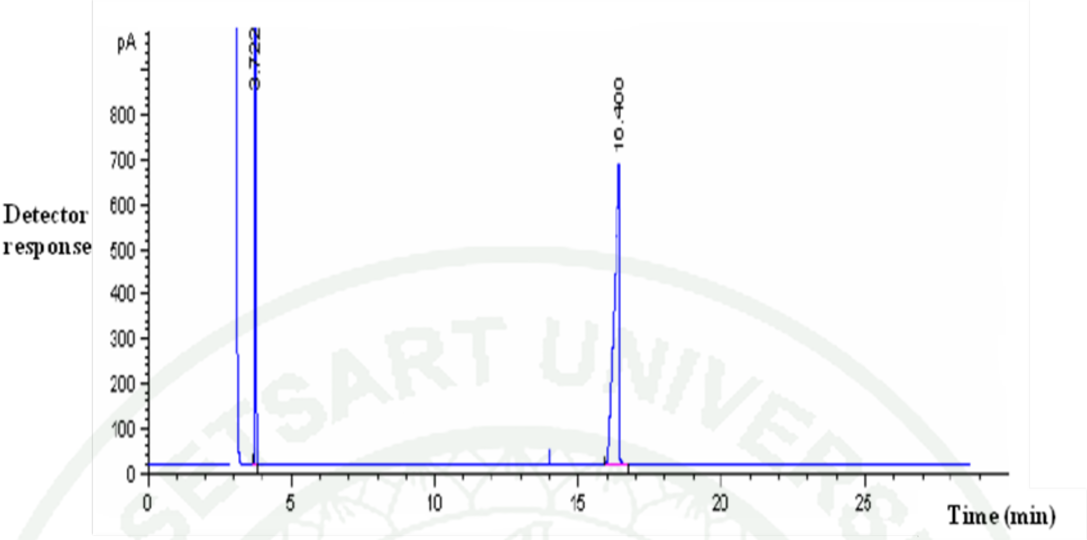
Appendix Figure C1 Chromatogram of standard oleyl palmitate at 500 mg/L of concentration



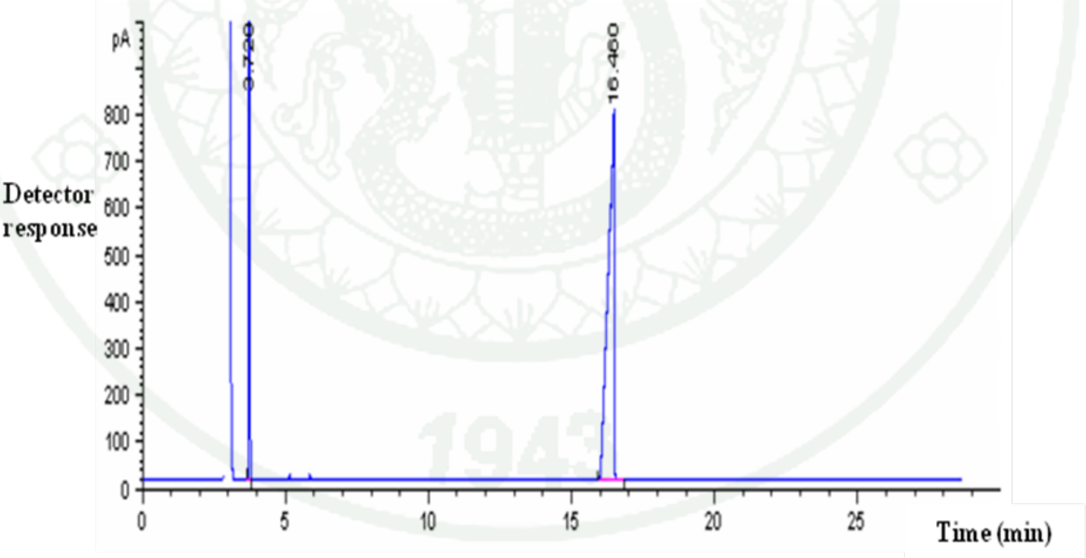
Appendix Figure C2 Chromatogram of standard oleyl palmitate at 1000 mg/L of concentration



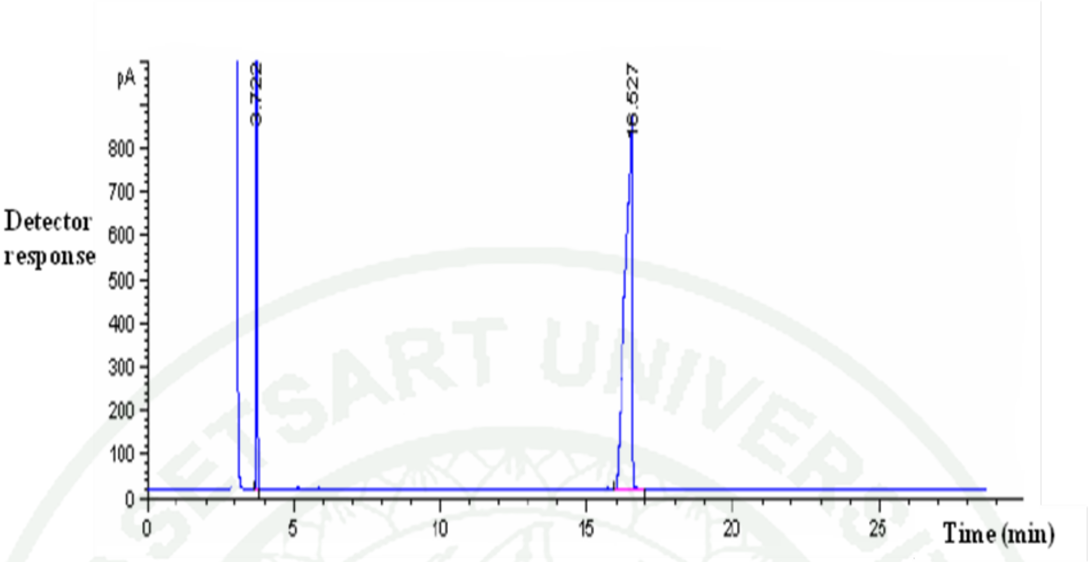
Appendix Figure C3 Chromatogram of standard oleyl palmitate at 2000 mg/L of concentration



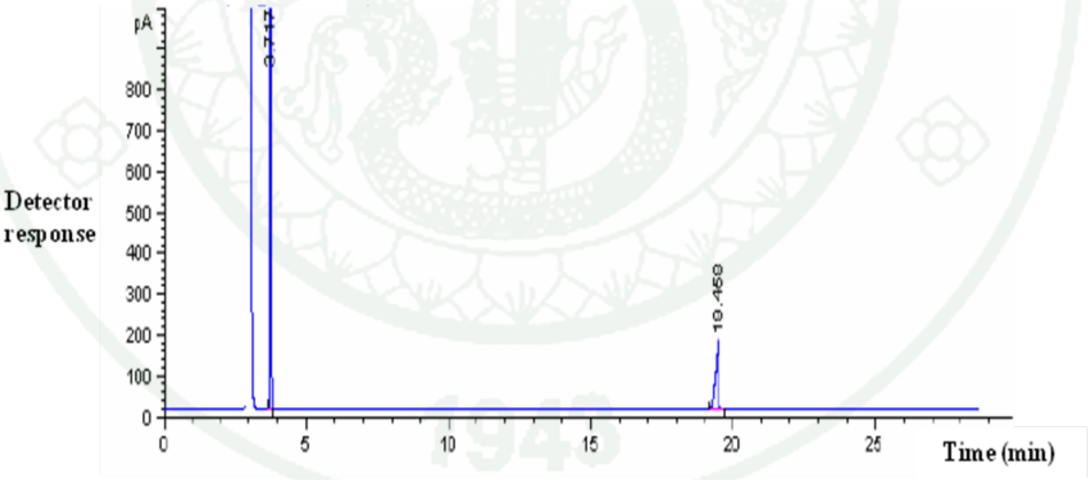
Appendix Figure C4 Chromatogram of standard oleyl palmitate at 3000 mg/L of concentration



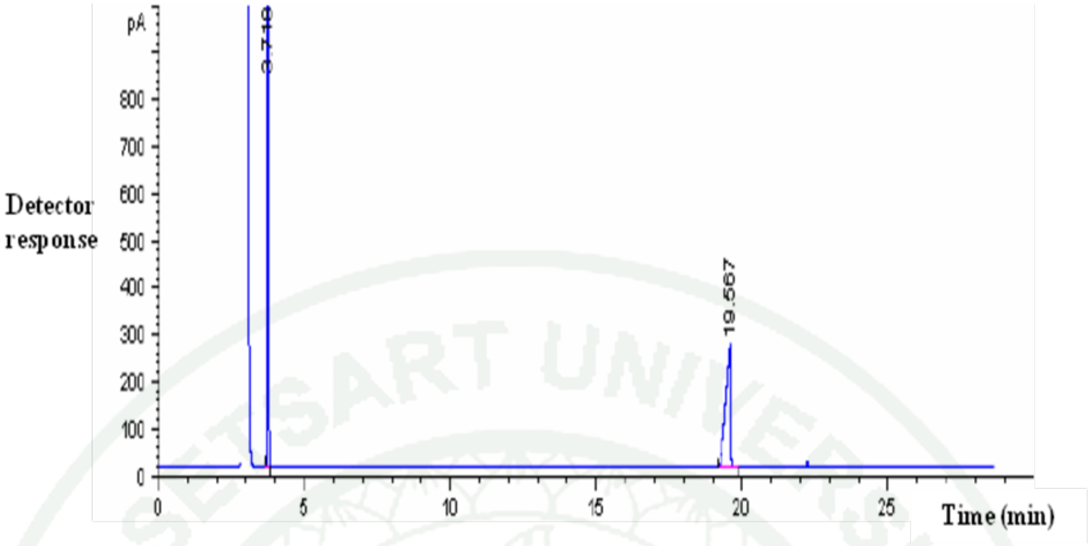
Appendix Figure C5 Chromatogram of standard oleyl palmitate at 4000 mg/L of concentration



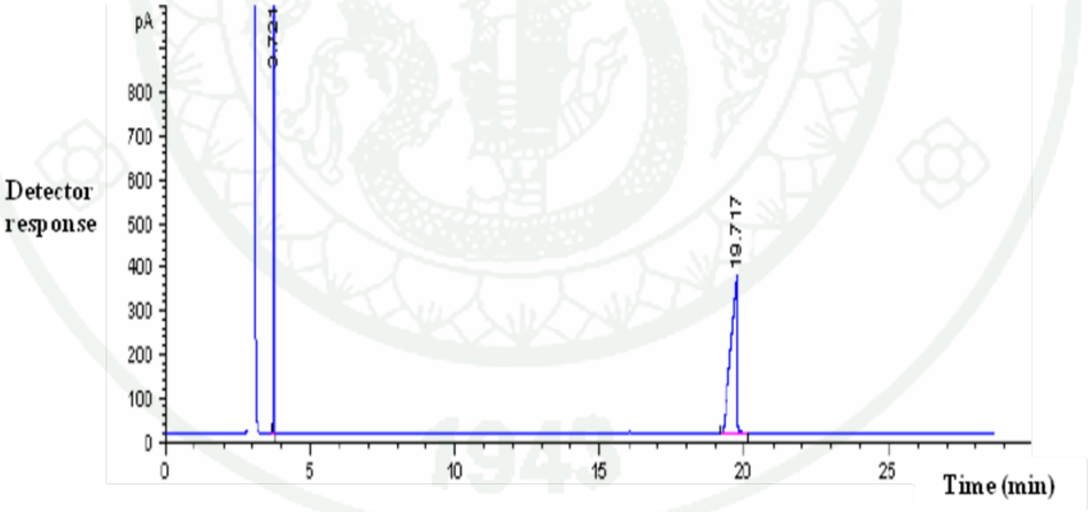
Appendix Figure C6 Chromatogram of standard oleyl palmitate at 5000 mg/L of concentration



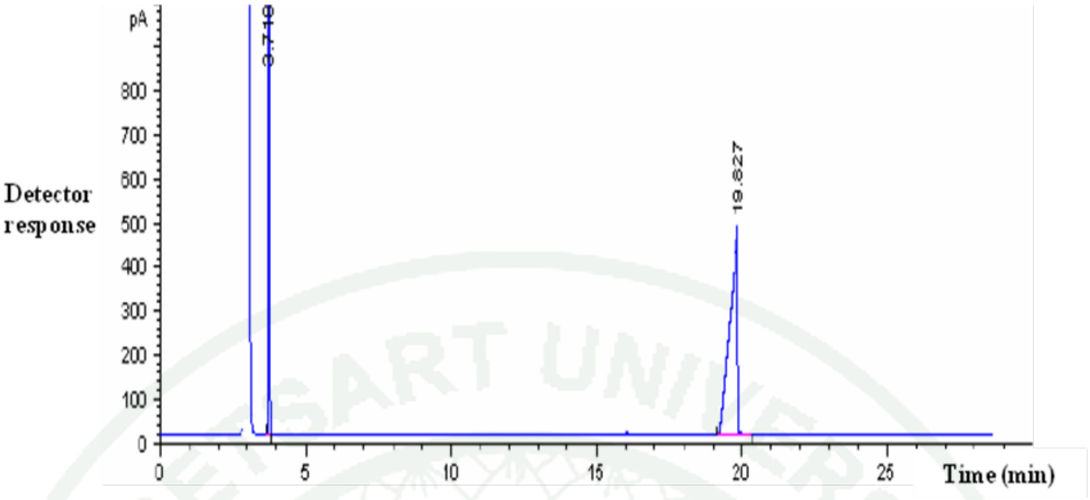
Appendix Figure C7 Chromatogram of standard oleyl oleate at 512.5 mg/L of concentration



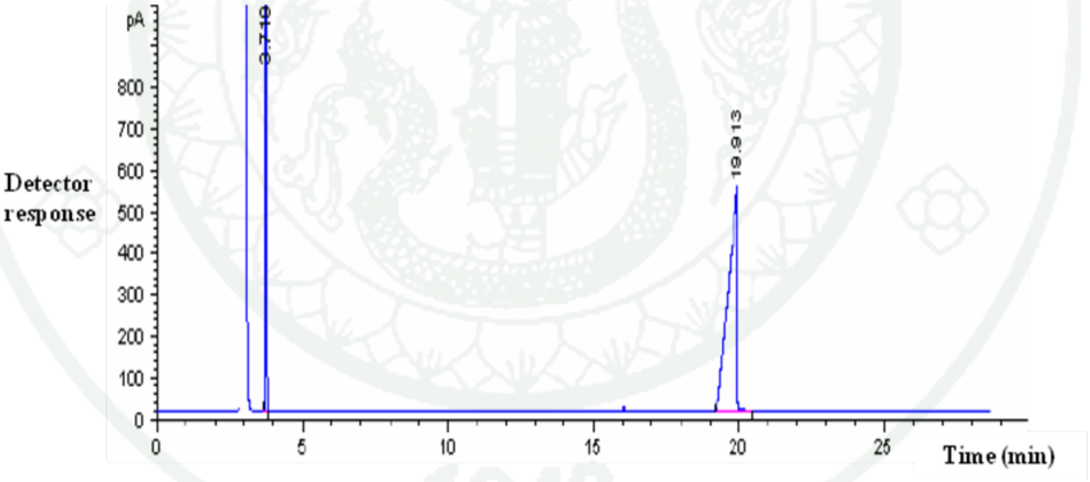
Appendix Figure C8 Chromatogram of standard oleyl oleate at 1025 mg/L of concentration



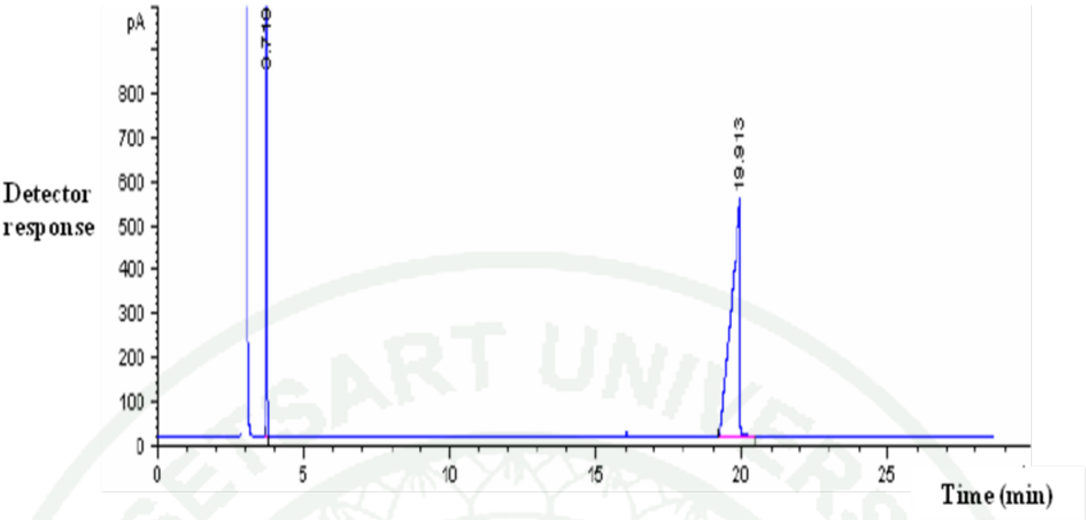
Appendix Figure C9 Chromatogram of standard oleyl oleate at 2050 mg/L of concentration



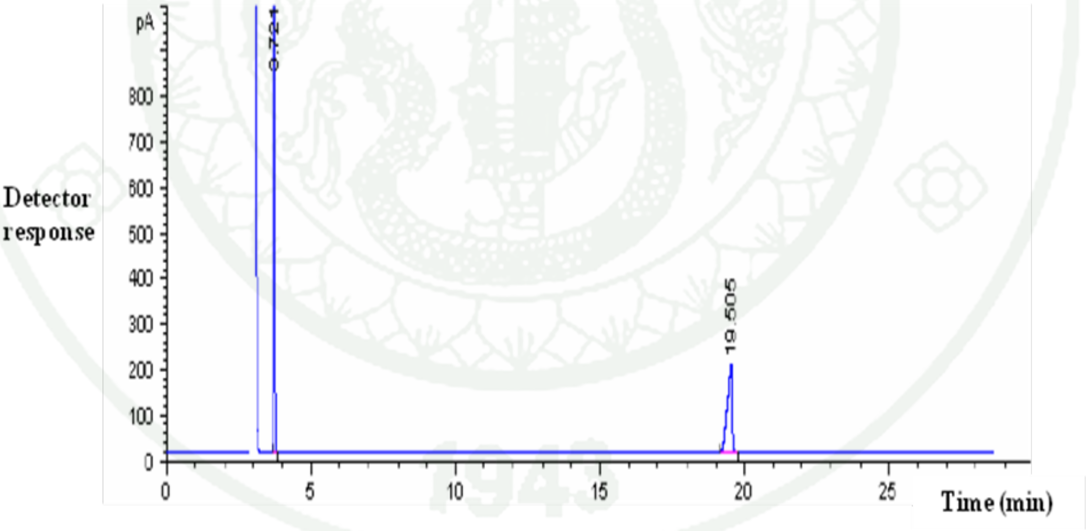
Appendix Figure C10 Chromatogram of standard oleyl oleate at 3075 mg/L of concentration



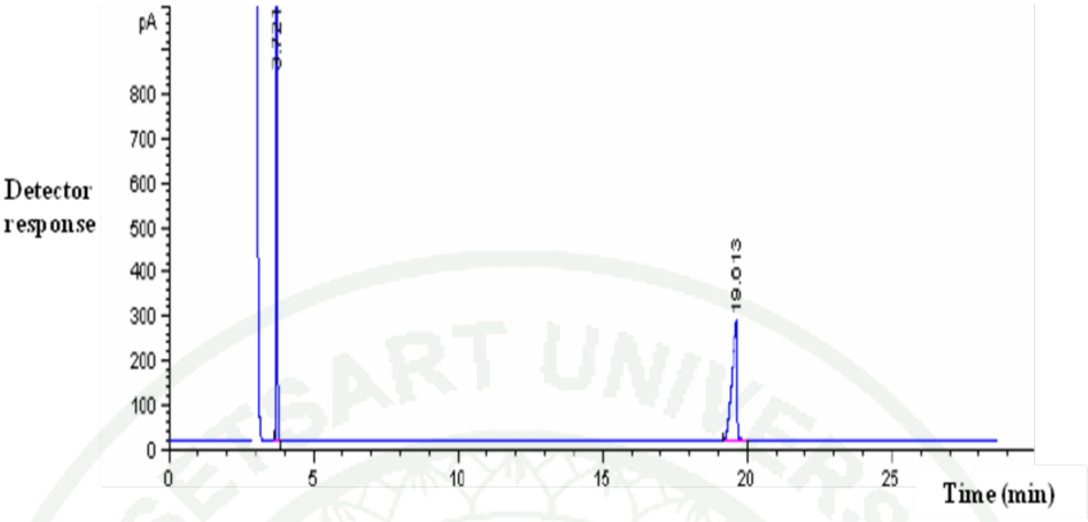
Appendix Figure C11 Chromatogram of standard oleyl oleate at 4100 mg/L of concentration



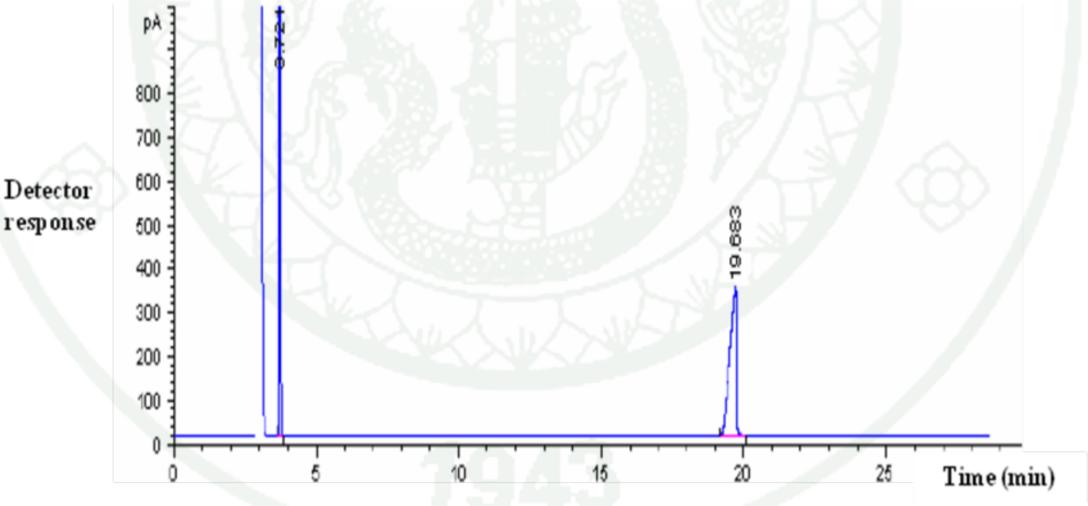
Appendix Figure C12 Chromatogram of standard oleyl oleate at 5125 mg/L of concentration



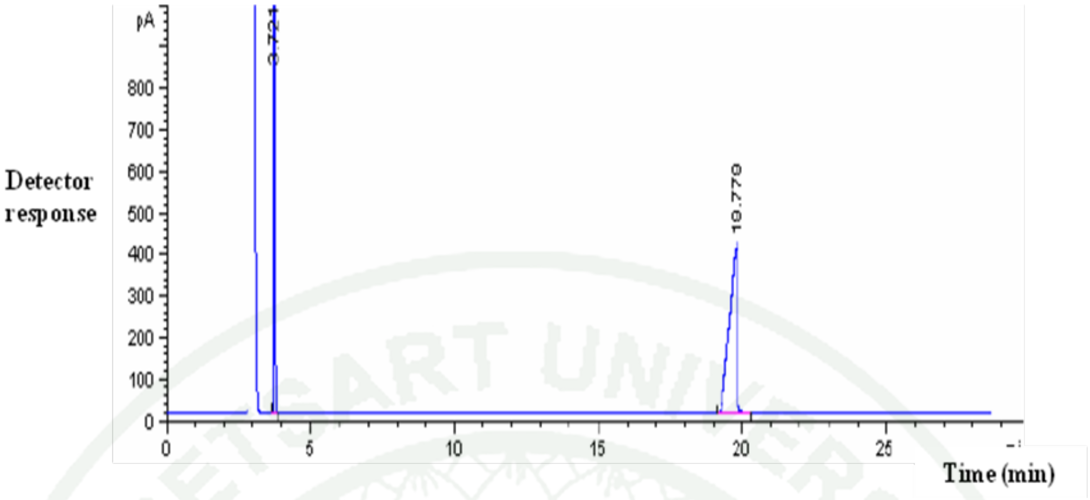
Appendix Figure C13 Chromatogram of standard oleyl linoleate at 528.6 mg/L of concentration



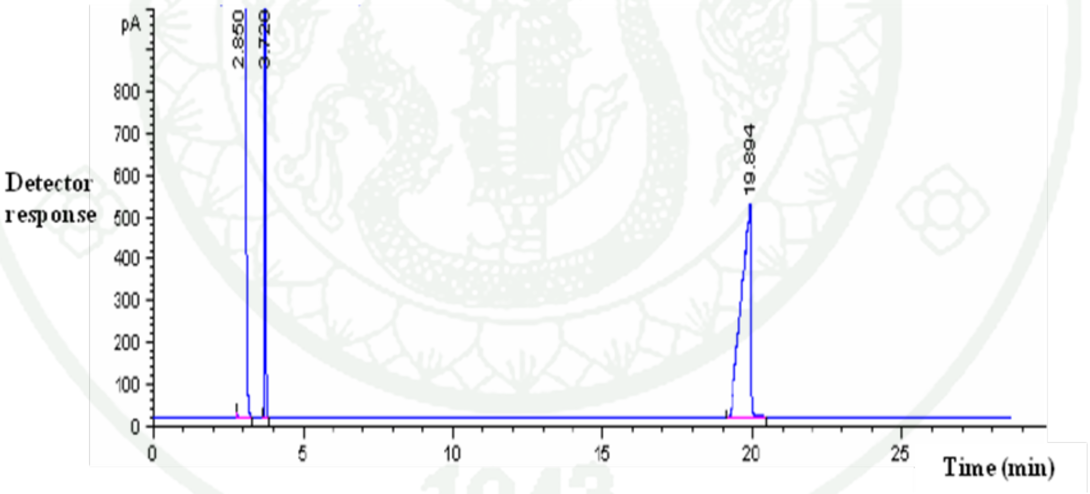
Appendix Figure C14 Chromatogram of standard oleyl linoleate at 1057.2 mg/L of concentration



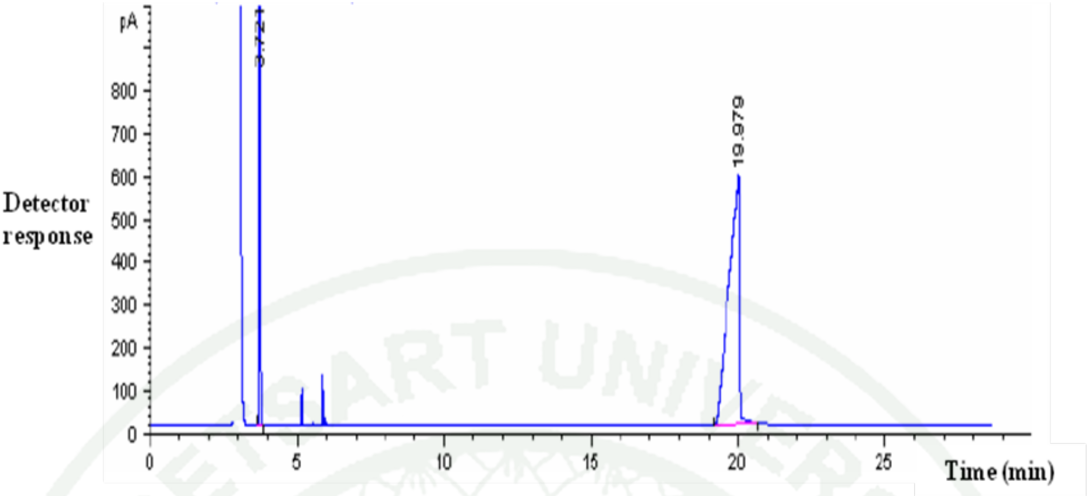
Appendix Figure C15 Chromatogram of standard oleyl linoleate at 1585.8 mg/L of concentration



Appendix Figure C16 Chromatogram of standard oleyl linoleate at 2114.4 mg/L of concentration



Appendix Figure C17 Chromatogram of standard oleyl linoleate at 3176.6 mg/L of concentration



Appendix Figure C18 Chromatogram of standard oleyl linoleate at 4228.6 mg/L of concentration

Appendix Table C1 Detector response factor of oleyl palmitate

Conc. of oleyl palmitate (mg/L) (C_x)	Peak area of Oleyl palmitate (A_x)	Peak area of methyl laurate (A_{is})	Conc. Of methyl laurate (mg/L) (C_{is})	Response factor (RF)
500	1350.08	4034.85	1606	1.0747
1000	2755.70	4249.33	1606	1.0415
2000	5653.88	4326.86	1606	1.0493
3000	8642.70	4258.49	1606	1.0865
4000	11767.00	4339.47	1606	1.0887
5000	14436.50	4236.74	1606	1.0945
Mean	-	-	-	1.0725
sd	-	-	-	0.0202
%cv	-	-	-	1.8809

Appendix Table C2 Detector response factor of oleyl oleate

Conc. of oleyl oleate (mg/L) (C_x)	Peak area of Oleyl oleate (A_x)	Peak area of methyl laurate (A_{is})	Conc. Of methyl laurate (mg/L) (C_{is})	Response factor (RF)
512.5	1397.46	4298.12	1606	1.0189
1025	2947.80	4348.86	1606	1.0620
2050	5369.64	4367.16	1606	0.9632
3075	8883.46	4330.50	1606	1.0714
4100	11576.10	4297.79	1606	1.0551
5125	14742.90	4304.21	1606	1.0734
Mean	-	-	-	1.0407
sd	-	-	-	0.0390
%cv	-	-	-	3.7475

Appendix Table C3 Detector response factor of oleyl linoleate

Conc. of oleyl oleate (mg/L) (C_x)	Peak area of Oleyl oleate (A_x)	Peak area of methyl laurate (A_{is})	Conc. Of methyl laurate (mg/L) (C_{is})	Response factor (RF)
528.6	2098.98	4429.97	1606	1.4395
1057.2	3620.53	4410.26	1606	1.2471
1585.8	5302.11	4411.00	1606	1.2173
2114.4	7661.86	4526.66	1606	1.2856
3171.6	11225.90	4674.47	1606	1.2161
4228.6	14819.20	4639.51	1606	1.2161
Mean	-	-	-	1.2698
sd	-	-	-	0.0800
%cv	-	-	-	6.3002

Appendix D

Experimental data

Appendix Table D1 The percentage conversion of free fatty acid by titration with 0.1 N NaOH along with 30 experimental runs of CCD

Treatment	A (%wt)	B (mol)	C (h)	D (^{O}C)	Initial FFA (%)	Final FFA (%)	Conversion of free fatty acid (%) (as palmitic acid)
1	17.50	1.75	1.00	50.00	88.40	34.91	60.51
2	17.50	1.75	1.00	70.00	88.40	23.14	73.82
3	17.50	1.75	3.00	50.00	88.40	29.00	67.19
4	17.50	1.75	3.00	70.00	88.40	6.74	92.37
5	17.50	1.75	1.00	50.00	88.40	21.72	75.43
6	17.50	1.75	1.00	70.00	88.40	15.59	82.36
7	17.50	1.75	3.00	50.00	88.40	19.09	78.41
8	17.50	1.75	3.00	70.00	88.40	5.51	93.77
9	32.50	3.25	1.00	50.00	88.40	29.72	66.38
10	32.50	3.25	1.00	70.00	88.40	14.77	83.29
11	32.50	3.25	3.00	50.00	88.40	23.97	72.88
12	32.50	3.25	3.00	70.00	88.40	10.49	88.13
13	32.50	3.25	1.00	50.00	88.40	20.08	77.28

Appendix Table D1 (Continued)

Treatment	A (%wt)	B (mol)	C (h)	D (^{O}C)	Initial FFA (%)	Final FFA (%)	Conversion of free fatty acid (%) (as palmitic acid)
14	32.50	3.25	1.00	70.00	88.40	11.37	87.14
15	32.50	3.25	3.00	50.00	88.40	15.70	82.24
16	32.50	3.25	3.00	70.00	88.40	2.02	97.71
17	10.00	2.50	2.00	60.00	88.40	23.04	73.94
18	40.00	2.50	2.00	60.00	88.40	13.62	84.59
19	25.00	1.00	2.00	60.00	88.40	28.78	67.44
20	25.00	4.00	2.00	60.00	88.40	13.07	85.21
21	25.00	2.50	0.00	60.00	88.40	12.49	85.87
22	25.00	2.50	4.00	60.00	88.40	11.22	87.31
23	25.00	2.50	2.00	40.00	88.40	51.94	41.24
24	25.00	2.50	2.00	80.00	88.40	1.58	98.21
25	25.00	2.50	2.00	60.00	88.40	17.48	80.23
26	25.00	2.50	2.00	60.00	88.40	17.52	80.18

Appendix Table D1 (Continued)

Treatment	A (%wt)	B (mol)	C (h)	D (^{O}C)	Initial FFA (%)	Final FFA (%)	Conversion of free fatty acid (%) (as palmitic acid)
27	25.00	2.50	2.00	60.00	88.40	17.34	80.38
28	25.00	2.50	2.00	60.00	88.40	17.62	80.07
29	25.00	2.50	2.00	60.00	88.40	17.34	80.38
30	25.00	2.50	2.00	60.00	88.40	17.17	80.58

Appendix Table D2 The percentage yield of wax esters by GC along with 30 experimental runs of CCD

Treatment	PFAD (g)	Ester	Retention time (min)	Area	Amount (g)	Yield (%)	
						Each esters	Total
1	1.54	Methyl laurate	3.982	2170.2	-	-	-
		Oleyl palmitate	14.428	2327.4	0.1454	9.4429	14.53
		Oleyl oleate	16.238	1035.1	0.0647	4.1997	
		Oleyl linoleate	16.378	217.6	0.0136	0.8829	
2	1.54	Methyl laurate	3.982	2144.7	-	-	-
		Oleyl palmitate	14.515	4849.8	0.2633	17.4391	26.56
		Oleyl oleate	16.381	1870.9	0.1016	6.7274	
		Oleyl linoleate	16.501	666.4	0.0362	2.3963	
3	1.49	Methyl laurate	3.982	2150.0	-	-	-
		Oleyl palmitate	14.484	4525.4	0.1858	12.4701	21.32
		Oleyl oleate	16.334	2729.4	0.1121	7.5211	
		Oleyl linoleate	16.453	481.2	0.0198	1.3260	

Appendix Table D2 (Continued)

Treatment	PFAD (g)	Ester	Retention time (min)	Area	Amount (g)	Yield (%)	
						Each esters	Total
4	1.52	Methyl laurate	3.980	2189.9	-	-	-
		Oleyl palmitate	14.501	6185.3	0.2688	17.6830	30.54
		Oleyl oleate	16.376	3782.3	0.1644	10.8131	
		Oleyl linoleate	16.489	714.8	0.0311	2.0435	
5	1.48	Methyl laurate	3.981	2177.1	-	-	-
		Oleyl palmitate	14.506	4496.7	0.3793	25.6263	45.35
		Oleyl oleate	16.396	2730.1	0.2303	15.5586	
		Oleyl linoleate	16.516	731.7	0.0617	4.1699	
6	1.53	Methyl laurate	3.982	2163.2	-	-	-
		Oleyl palmitate	14.476	7037.3	0.5742	37.5308	61.13
		Oleyl oleate	16.364	3787.8	0.3091	20.2008	
		Oleyl linoleate	16.484	637.1	0.0520	3.3977	

Appendix Table D2 (Continued)

Treatment	PFAD (g)	Ester	Retention time (min)	Area	Amount (g)	Yield (%)	
						Each esters	Total
7	1.54	Methyl laurate	3.981	2202.6	-	-	-
		Oleyl palmitate	14.474	4083.7	0.3992	25.9228	52.82
		Oleyl oleate	16.363	3578.9	0.3499	22.7184	
		Oleyl linoleate	16.484	658.7	0.0644	4.1813	
8	1.52	Methyl laurate	3.982	2144.3	-	-	-
		Oleyl palmitate	14.511	6528.9	0.5543	36.4678	67.37
		Oleyl oleate	16.409	4779.3	0.4058	26.6953	
		Oleyl linoleate	16.522	752.8	0.0639	4.2048	
9	1.50	Methyl laurate	3.981	2250.9	-	-	-
		Oleyl palmitate	14.485	4651.8	0.2561	17.0724	26.57
		Oleyl oleate	16.300	2163.2	0.1191	7.9391	
		Oleyl linoleate	16.423	426.0	0.0235	1.5635	

Appendix Table D2 (Continued)

Treatment	PFAD (g)	Ester	Retention time (min)	Area	Amount (g)	Yield (%)	
						Each esters	Total
10	1.50	Methyl laurate	3.978	2205.2	-	-	-
		Oleyl palmitate	14.553	8746.7	0.4820	32.1312	55.29
		Oleyl oleate	16.430	5391.7	0.2971	19.8066	
		Oleyl linoleate	16.540	911.7	0.0502	3.3492	
11	1.52	Methyl laurate	3.979	2227.7	-	-	-
		Oleyl palmitate	14.525	7115.0	0.4370	28.7494	48.00
		Oleyl oleate	16.381	4034.5	0.2478	16.3021	
		Oleyl linoleate	16.493	729.5	0.0448	2.9477	
12	1.51	Methyl laurate	3.980	2214.0	-	-	-
		Oleyl palmitate	14.574	10185.2	0.5509	36.4813	64.55
		Oleyl oleate	16.471	6748.4	0.3650	24.1714	
		Oleyl linoleate	16.579	1088.9	0.0589	3.9002	

Appendix Table D2 (Continued)

Treatment	PFAD (g)	Ester	Retention time (min)	Area	Amount (g)	Yield (%)	
						Each esters	Total
13	1.53	Methyl laurate	3.980	2198.2	-	-	-
		Oleyl palmitate	14.532	7408.3	0.6178	40.3782	66.46
		Oleyl oleate	16.449	3819.2	0.3185	20.8161	
		Oleyl linoleate	16.560	965.3	0.0805	5.2613	
14	1.55	Methyl laurate	3.978	2219.6	-	-	-
		Oleyl palmitate	14.503	6370.4	0.5774	37.2501	71.76
		Oleyl oleate	16.399	5077.9	0.4602	29.6924	
		Oleyl linoleate	16.519	823.1	0.0746	4.8130	
15	1.49	Methyl laurate	3.978	2241.0	-	-	-
		Oleyl palmitate	14.496	7911.5	0.7040	47.2512	81.73
		Oleyl oleate	16.387	5012.7	0.4461	29.9382	
		Oleyl linoleate	16.502	759.6	0.0676	4.5367	

Appendix Table D2 (Continued)

Treatment	PFAD (g)	Ester	Retention time (min)	Area	Amount (g)	Yield (%)	
						Each esters	Total
16	1.49	Methyl laurate	3.981	2210.4	-	-	-
		Oleyl palmitate	14.538	7761.0	0.6465	43.3905	85.61
		Oleyl oleate	16.463	6569.3	0.5472	36.7279	
		Oleyl linoleate	16.571	982.4	0.0818	5.4924	
17	1.52	Methyl laurate	3.980	2186.8	-	-	-
		Oleyl palmitate	14.462	3779.4	0.2871	18.8878	37.64
		Oleyl oleate	16.342	3182.6	0.2418	15.9052	
		Oleyl linoleate	16.462	570.4	0.0433	2.8506	
18	1.50	Methyl laurate	3.979	2231.2	-	-	-
		Oleyl palmitate	14.521	6321.9	0.4418	29.4533	57.04
		Oleyl oleate	16.413	5105.4	0.3568	23.7857	
		Oleyl linoleate	16.525	815.4	0.0570	3.7989	

Appendix Table D2 (Continued)

Treatment	PFAD (g)	Ester	Retention time (min)	Area	Amount (g)	Yield (%)	
						Each esters	Total
19	1.53	Methyl laurate	3.980	2221.9	-	-	-
		Oleyl palmitate	14.461	3617.4	0.1783	11.6514	17.85
		Oleyl oleate	16.274	1599.1	0.0788	5.1506	
		Oleyl linoleate	16.398	324.8	0.0160	1.0462	
20	1.50	Methyl laurate	3.982	2333.9	-	-	-
		Oleyl palmitate	14.511	5684.3	0.6222	41.4782	81.78
		Oleyl oleate	16.406	4756.3	0.5206	34.7066	
		Oleyl linoleate	16.524	766.4	0.0839	5.5924	
21	1.55	Methyl laurate	3.983	2211.7	-	-	-
		Oleyl palmitate	14.329	2194.2	0.2023	13.0524	19.96
		Oleyl oleate	16.170	1115.1	0.1028	6.6333	
		Oleyl linoleate	16.345	45.5	0.0042	0.2707	

Appendix Table D2 (Continued)

Treatment	PFAD (g)	Ester	Retention time (min)	Area	Amount (g)	Yield (%)	
						Each esters	Total
22	1.54	Methyl laurate	3.981	2243.5	-	-	-
		Oleyl palmitate	14.502	6327.4	0.4861	31.5654	59.07
		Oleyl oleate	16.397	4752.7	0.3651	23.7097	
		Oleyl linoleate	16.508	761.4	0.0585	3.7984	
23	1.55	Methyl laurate	3.980	2170.6	-	-	-
		Oleyl palmitate	14.488	4858.4	0.3885	25.0632	46.71
		Oleyl oleate	16.360	3600.1	0.2879	18.5720	
		Oleyl linoleate	16.475	595.9	0.0476	3.0741	
24	1.52	Methyl laurate	3.980	2250.2	-	-	-
		Oleyl palmitate	14.519	6694.1	0.4222	27.7771	53.20
		Oleyl oleate	16.425	5235.2	0.3302	21.7234	
		Oleyl linoleate	16.530	890.9	0.0562	3.6968	

Appendix Table D2 (Continued)

Treatment	PFAD (g)	Ester	Retention time (min)	Area	Amount (g)	Yield (%)	
						Each esters	Total
25	1.50	Methyl laurate	3.996	2371.2	-	-	-
		Oleyl palmitate	14.563	6955.6	0.4435	29.5669	55.13
		Oleyl oleate	16.495	4273.6	0.3140	20.9315	
		Oleyl linoleate	16.603	945.1	0.0694	4.6290	
26	1.52	Methyl laurate	3.980	2162.0	-	-	-
		Oleyl palmitate	14.488	6943.7	0.4834	31.8046	56.12
		Oleyl oleate	16.359	4686.9	0.3263	21.4676	
		Oleyl linoleate	16.483	622.8	0.0434	2.8526	
27	1.52	Methyl laurate	3.980	2163.1	-	-	-
		Oleyl palmitate	14.503	7435.7	0.4867	32.0180	55.93
		Oleyl oleate	16.389	4830.7	0.3162	20.8009	
		Oleyl linoleate	16.501	721.7	0.0472	3.1076	

Appendix Table D2 (Continued)

Treatment	PFAD (g)	Ester	Retention time (min)	Area	Amount (g)	Yield (%)	
						Each esters	Total
28	1.53	Methyl laurate	3.980	2167.9	-	-	-
		Oleyl palmitate	14.507	7254.7	0.4925	32.1876	56.58
		Oleyl oleate	16.402	4746.9	0.3222	21.0610	
		Oleyl linoleate	16.513	750.4	0.0509	3.3294	
29	1.52	Methyl laurate	3.980	2228.1	-	-	-
		Oleyl palmitate	14.510	6278.7	0.4608	30.3135	55.31
		Oleyl oleate	16.412	4463.5	0.3276	21.5497	
		Oleyl linoleate	16.523	713.8	0.0524	3.4462	
30	1.51	Methyl laurate	3.981	2202.9	-	-	-
		Oleyl palmitate	14.501	5862.2	0.4483	29.6913	55.48
		Oleyl oleate	16.398	4370.0	0.3342	22.1335	
		Oleyl linoleate	16.516	721.5	0.0552	3.6543	

Appendix Table D3 The percentage conversion of free fatty acid by titration with 0.1 N NaOH for validation test

Treatment	A (%wt)	B (mol)	C (h)	D (^{O}C)	Initial FFA (%)	Final FFA (%)	Conversion of free fatty acid (%) (as palmitic acid)
1	20.00	1.50	1.50	55.00	88.40	30.06	65.99
2	30.00	1.80	2.50	65.00	88.40	13.76	84.44
3	35.00	3.50	3.75	75.00	88.40	0.83	99.06
4	28.00	2.70	1.25	65.00	88.40	17.58	80.11
5	15.00	3.80	2.25	55.00	88.40	17.63	80.06

Appendix Table D4 The percentage yield of wax esters by GC for validation test

Treatment	PFAD (g)	Ester	Retention time (min)	Area	Amount (g)	Yield (%)	
						Each esters	Total
1	1.51	Methyl laurate	3.992	3842.9	-	-	-
		Oleyl palmitate	14.776	5968.0	0.1863	12.3405	19.33
		Oleyl oleate	16.640	3067.1	0.0958	6.3421	
		Oleyl linoleate	16.748	311.8	0.0097	0.6447	
2	1.58	Methyl laurate	3.992	3912.5	-	-	-
		Oleyl palmitate	14.761	12980.4	0.4286	27.1295	51.89
		Oleyl oleate	16.634	10864.1	0.3588	22.7064	
		Oleyl linoleate	16.729	982.4	0.0324	2.0533	
3	1.61	Methyl laurate	3.992	3888.9	-	-	-
		Oleyl palmitate	14.690	13697.0	0.7901	49.0768	88.97
		Oleyl oleate	16.565	10419.1	0.6010	37.3320	
		Oleyl linoleate	16.673	713.8	0.0412	2.5576	

Appendix Table D4 (Continued)

Treatment	PFAD (g)	Ester	Retention time (min)	Area	Amount (g)	Yield (%)	
						Each esters	Total
4	1.52	Methyl laurate	3.993	3974.5	-	-	-
		Oleyl palmitate	14.688	13715.3	0.5760	37.8923	59.87
		Oleyl oleate	16.539	7094.0	0.2979	19.5991	
		Oleyl linoleate	16.648	862.6	0.0362	2.3832	
5	1.48	Methyl laurate	3.993	3895.2	-	-	-
		Oleyl palmitate	14.635	9795.8	0.6235	42.1307	68.34
		Oleyl oleate	16.486	5281.9	0.3362	22.7169	
		Oleyl linoleate	16.600	812.0	0.0517	3.4923	

Appendix Table D5 The percentage conversion of free fatty acid by titration with 0.1 N NaOH for optimum condition

Treatment	A (%wt)	B (mol)	C (h)	D (^{O}C)	Initial FFA (%)	Final FFA (%)	Conversion of free fatty acid (%) (as palmitic acid)
1	40	3	2.5	65	88.4	6.40	92.76
2	30	3.5	2.5	70	88.4	5.40	93.89
3	30	3.5	3	65	88.4	5.07	94.26
4	32	3.2	3	75	88.4	0.88	99.01
5	35	3	3.5	65	88.4	3.36	96.20

Appendix Table D6 The percentage yield of wax esters by GC for optimum condition

Treatment	PFAD (g)	Ester	Retention time (min)	Area	Amount (g)	Yield (%)	
						Each esters	Total
1	1.45	Methyl laurate	3.999	2938.2	-	-	-
		Oleyl palmitate	14.687	9432.7	0.6395	44.1065	81.23
		Oleyl oleate	16.531	7013.8	0.4755	32.7959	
		Oleyl linoleate	16.633	925.9	0.0628	4.3294	
2	1.47	Methyl laurate	3.903	3700.4	-	-	-
		Oleyl palmitate	14.496	9533.8	0.6364	43.2914	83.93
		Oleyl oleate	16.387	7625.6	0.5090	34.6265	
		Oleyl linoleate	16.502	1323.4	0.0883	6.0093	
3	1.46	Methyl laurate	3.997	3892.5	-	-	-
		Oleyl palmitate	14.655	12256.6	0.6600	45.2058	83.05
		Oleyl oleate	16.527	9021.2	0.4858	33.2728	
		Oleyl linoleate	16.627	1239.9	0.0668	4.5731	

Appendix Table D6 (Continued)

Treatment	PFAD (g)	Ester	Retention time (min)	Area	Amount (g)	Yield (%)	
						Each esters	Total
4	1.50	Methyl laurate	3.997	3786.3	-	-	-
		Oleyl palmitate	14.678	13196.6	0.6989	46.5930	81.96
		Oleyl oleate	16.536	8743.5	0.4631	30.8705	
		Oleyl linoleate	16.641	1272.3	0.0674	4.4921	
5	1.48	Methyl laurate	3.995	3674.8	-	-	-
		Oleyl palmitate	14.680	14735.6	0.6312	42.6494	81.16
		Oleyl oleate	16.550	11909.0	0.5101	34.4683	
		Oleyl linoleate	16.649	1396.7	0.0598	4.0425	

Appendix Table D7 The absorbance for calibration curve of α-tocopherol by UV-VIS spectrophotometer

Conc. Of α-tocopherol (ppm)	Absorbance
0	0.095
60	0.316
120	0.530
180	0.761
240	0.981

Appendix Table D8 The absorbance of PFAD and wax esters for vitamin E test by UV-VIS spectrophotometer

Sample	Test no.	Weight (mg)	Absorbance	Total tocopherol
Blank	1	-	0.095	-
	2	-	0.095	-
	Average	-	-	-
PFAD	1	30.9	0.670	5029.30
	2	29.7	0.648	5032.31
	Average	-	-	5030.80
Wax esters	1	202.9	0.203	143.86
	2	203.5	0.204	144.76
	Average	-	-	144.31

Appendix Table D9 The relationship of reusable catalyst and percentage conversion of free fatty acid by titration with 0.1 N NaOH

Reuse no.	Initial FFA (%)	Final FFA (%)	Conversion of free fatty acid (%) (as palmitic acid)
1	88.40	3.72	95.79
2	88.40	16.79	81.01
3	88.40	14.59	83.49
4	88.40	16.96	80.81
5	88.40	17.41	80.30
6	88.40	19.38	78.08
7	88.40	19.89	77.50
8	88.40	19.52	77.92
9	88.40	18.67	78.88
10	88.40	18.14	79.48
11	88.40	18.48	79.09
12	88.40	20.06	77.30
13	88.40	19.15	78.33
14	88.40	18.79	78.74
15	88.40	19.66	77.76
16	88.40	19.67	77.74
17	88.40	19.97	77.41
18	88.40	19.94	77.44
19	88.40	19.71	77.70
20	88.40	19.83	77.57

Appendix E

Lack of fit test

Procedure of lack of fit test (LOF) of RSM in SPSS

1. Run regression to find residual sum square (SS) (df1)

2. Run proc glm and choose option LOF to find pure error SS (df2)

3. Calculated LOF sum square (SS) by

$$\text{LOF SS (df3)} = \text{Residual SS} - \text{Pure error}$$

And

$$\text{df3} = \text{df1} - \text{df2}$$

4. Calculated mean square (MS) LOF by

$$\text{LOF MS} = \frac{\text{LOF SS}}{\text{df3}}$$

5. Significant of LOF is tested by

$$\text{F} = \frac{\text{LOF MS}}{\text{Pure error MS}}$$

6. Compared the calculated F-value and tubular F-value at df3 and df2. If calculated F-value has less than tubular F-value, the model is not lack of fit. It indicated that the model is suitable to describe the experiment.

LOF test of percentage conversion

Appendix Table E1 Data for LOF test of percentage conversion

Model	Sum of Squares	Degree of freedom	Mean sum of square
Residual	394.843	15	26.323
Pure error	2.870	5	0.574

Note: this was done at 95% confidence interval

LOF SS (df3) = Residual SS – Pure error

= 393.973

And df3 = df1 – df2

= 10

LOF MS = 39.3973

F = 68.64

F Table $_{(0.05,\ 10,\ 5)}$ = 4.74

From LOF test, the calculated F-value had more than tubular-value resulting to the model of percentage conversion was significant lack of fit.

LOF test of percentage yield

Appendix Table E2 Data for LOF test of percentage yield

Model	Sum of Squares	Degree of freedom	Mean sum of square
Residual	726.466	15	48.431
Pure error	1.488	5	0.298

Note: this was done at 95% confidence interval

LOF SS (df3) = Residual SS – Pure error

= 724.978

And df3 = df1 – df2

= 10

LOF MS = 72.4978

F = 243.28

F Table $_{(0.05, 10, 5)}$ = 4.74

From LOF test, the calculated F-value had more than tubular-value resulting to the model of percentage yield was significant lack of fit.

Appendix F

Standard method for wax esters analysis

Appendix F1 Free fatty acid by AOCS Official Method Ca 5a-40

Procedure

1. Sample must be well mixed and entirely liquid before weighing; however, do not heat the sample more than 10 ^{0}C over the melting point.

2. Use Table below to determine the sample weight for various ranges of fatty acids. Weigh the designated sample size into an oil sample bottle or Erlenmeyer flask.

Appendix Table F1 Free fatty acid, alcohol volume and strength of alkali

FFA range (%)	Sample (g)	Alcohol (ml)	Strength of alkali
0.00 – 0.20	56.40 ± 0.20	50	0.1 N
0.20 – 1.00	28.20 ± 0.20	50	0.1 N
1.00 – 30.00	7.05 ± 0.05	75	0.25 N
30.00 – 50.00	7.05 ± 0.05	100	0.25 or 1.0 N
50.00 – 100.0	3.525 ± 0.001	100	1.0 N

3. Add the specified amount of hot neutralized alcohol and 2 ml of indicator.

4. Titrate with standard sodium hydroxide, shaking vigorously until the appearance of the first permanent pink color of the same intensity as that of the neutralized alcohol before the addition of the sample. The color must persist for 30 seconds.

Calculations

1. The percentage of free fatty acids in most types of fats and oils is calculated as oleic acid, although in coconut and palm kernel oils it is frequently expressed as lauric acid and in palm oil in terms of palmitic acid.

(a) Free fatty acids as lauric (%) $= \dfrac{(A - B) \times N \times 20}{W}$

(b) Free fatty acids as palmitic (%) $= \dfrac{(A - B) \times N \times 25.6}{W}$

(c) Free fatty acids as oleic (%) $= \dfrac{(A - B) \times N \times 28.2}{W}$

Where:

A = ml of alkali required to titrate sample
B = ml of alkali required to titrate blank
N = Normality of alkali solution (N)
W = Weight of sample (g)

2. The free fatty acids are frequently expressed in terms of acid value instead of percentage free fatty acids. The acid value is defined as the number of milligrams of KOH necessary to neutralize 1 g of sample. To convert percentage free fatty acids (as oleic) to acid value, multiply the percentage free fatty acids by 1.99.

Appendix F2 Acid value by AOCS Official Method Cd 3d-63

Procedure

1. Add indicator solution to the required amount of solvent in ratio of 2 ml to 125 ml and neutralize with alkali to a faint but permanent pink color.

2. Determine the sample size from the following table below.

Appendix Table F2 The sample size for acid value

Acid value	Mass of sample (g) (±10%)	Weighing accuracy
0 – 1	20	0.05
1 – 4	10	0.02
4 – 15	2.5	0.01
15 – 75	0.5	0.001
Above 75	0.1	0.002

3. Weigh the specified amount of a well-mixed liquid sample into an Erlenmeyer flask.

4. Add 125 ml of the neutralized solvent mixture. Be sure that the sample is completely dissolved before titrating. Warming may be necessary in some cases.

5. Shake the sample vigorously while titrating with standard alkali to the first permanent pink color of the same intensity as that of the neutralized solvent before the latter was added to the sample. The color must persist for 30 seconds.

6. Perform a blank titration using 125 ml of the neutralized solvent mixture.

Calculations

$$\text{Acid value (mg KOH/g of sample)} = \frac{(A - B) \times N \times 56.1}{W}$$

Where:

A = ml of alkali required to titrate sample
B = ml of alkali required to titrate blank
N = Normality of alkali solution (N)
W = Weight of sample (g)

To express in terms of free fatty acids as percent lauric, oleic, or palmitic, divide the acid value by 1.99, 2.81, or 2.56, respectively.

Appendix F3 Saponification value by AOCS Official Method Cd 3-25

Procedure

1. Melt the sample if it is not already liquid and filter through dry filter paper to remove any impurities and moisture. The sample must be completely dry.

2. Weigh a sample of such size that the back titration is 45 – 55% of the blank. This usually requires a sample of 4 – 5 g. Add 50 ml of alcoholic KOH with a pipet and allows the pipet drain for a definite period of time.

3. Prepare and conduct blank determination simultaneously with sample and similar in all respects, except omitting the fat and oil.

4. Connect the air condenser and boil gently, but steadily, until the sample is completely saponified. This usually requires about 1 hour for normal sample. Make certain that the vapor ring in the condenser does not rise to the top of the condenser, or loss may occur.

5. After the flask and condenser have cooled somewhat, but not sufficiently to form a gel, wash down the inside of the condenser with a small quantity of distilled water. Disconnect the condenser, add about 1 ml of phenolphthalein indicator and titrate with 0.5N HCl until the pink color just disappears. Record the volume of 0.5N HCl required for the titration.

Calculations

$$\text{Saponification value (mg KOH/g of sample)} = \frac{(B - S) \times N \times 56.1}{W}$$

Where:

B = ml of 0.5N HCl required to titrate blank
S = ml of 0.5N HCl required to titrate sample
N = Normality of HCl solution (N)
W = Weight of sample (g)

Appendix F4 Iodine value (Wijs method) by AOCS Official Method Cd 1-25

Procedure

1. Melt the sample, if it is not already liquid (the temperature during melting should not exceed the melting point of the sample by more than 10 ^{O}C), and filter through two pieces of filter paper to remove any solid impurities and the last traces of moisture. The filtration may be performed in an air oven at 100 ^{O}C, but should be completed within 5 min ± 30 seconds. The sample must be absolutely dry. The glass ware must be absolutely clean and completely dry.

2. After filtration, allow the filtered sample to achieve a temperature of 68 – 71 ± 1 ^{O}C before weighing the sample.

3. Once the sample has achieved a temperature of 68 – 71 ± 1 ^{O}C, immediately weigh the sample into a 500 ml iodine flask, using the weights and weighing accuracy in table below

Appendix Table F4 The sample weights

Iodine value expected	Weight (g), ± 0.001
< 5	3.000
5 – 20	1.000
21 – 50	0.400
51 – 100	0.200
101 – 150	0.130
151 - 200	0.100

4. Add 15 ml of carbon tetrachloride on top of the sample and swirl to ensure that the sample is complete dissolved.

5. Dispense 25 ml of Wijs solution using the pipet into flask containing the sample, stopper the flask and swirl to ensure an intimate mixture. Immediately set the timer for 1.0 or 2.0 hours, depending on the iodine value of sample: IV < 150, 1.0 hour; IV ≥ 150, 2.0 hours.

6. Immediately store the flasks in the dark for the required reaction time at a temperature of 25 ± 5 ^{O}C

7. Remove the flasks from storage and add 20 ml of 10% KI solution, followed by 150 ml of distilled water

8. Titrate with 0.1N $Na_2S_2O_3$ solution, adding it gradually and with constant and vigorous shaking. Continue the titration until the yellow color has almost disappeared. Add 1 – 2 ml of 1% starch indicator solution and continue the titration until the blue color just disappears.

9. Prepare and conduct at least one blank determination with each group of sample simultaneously and similar in all respects to the samples.

Calculations

$$\text{Iodine value (g } I_2\text{/100 g of sample)} = \frac{(B - S) \times N \times 12.69}{W}$$

Where:

B = ml of 0.1N $Na_2S_2O_3$ required to titrate blank
S = ml of 0.1N $Na_2S_2O_3$ required to titrate sample
N = Normality of $Na_2S_2O_3$ solution (N)
W = Weight of sample (g)

Appendix F5 Specific gravity by AOCS Official Method Cc 10b-25

Procedure

1. Prepare 7 solutions (50 to 100 ml each) of alcohol and water with densities ranging from 0.940 to 1.000 at 25 ^{O}C, varying by 0.01 specific gravity (sp.gr.) units. Use the Westphal balance to determine the sp. gr. Store in glass stopper bottles, appropriately numbered, from 1 to 7, in order of increasing sp. gr.

2. Melt 5 g of the sample, making certain the melted sample is clear, free of moisture and air bubbles. If the sample is turbid and/or contains moisture, filter through dry filter paper with the aid of a hot water funnel. Allow the sample to cool slowly to room temperature.

3. Cut a small pellet (3 to 5 mm in diameter) from the solidified sample and brush over with a camel hair brush (wet with distilled water) to avoid the development of air bubbles on the surface.

4. Pour alcohol solution 1 into a beaker, adjust to 25 ^{O}C ± 0.1 ^{O}C and immerse the pellet with the aid of forceps. If the pellet sinks, repeat with solution 2, and so on, until a solution is found in which the pellet just floats.

5. When the solution is found in which the pellet floats, slowly add alcohol at 25 ^{O}C from a buret, stirring gently with a glass stirring rod to prevent the formation of air bubbles around the sample pellet. Continue adding the alcohol until the pellet just sinks. If the correct amount of alcohol is exceeded, distilled water may be added drop wise and the endpoint again determined.

6. Determine the sp. gr. of the solution generated in step with the Westphal balance, making certain the solution is at 25 ^{O}C ± 0.1 ^{O}C. The sp. gr. of this solution is identical with the sp. gr. of the sample.

7. The result is confirmed by adding 1 or more pellets to the same solution. If the sample floats, it should slowly sink upon the addition of a drop of alcohol. If the sample sinks, slowly add water drop wise until it just rises and then determine the sp. gr. of the solution.

Appendix F6 Kinematic viscosity by ASTM 445

Procedure

1. Clean the viscometer using suitable solvents, and by passing clean, dry, filtered air through the instrument to remove the final traces of solvents. Periodically, traces of organic deposits should be removed with chromic acid or non-chromium cleaning solution.

2. If there is a possibility of lint, dust, or other solid material in the liquid sample, filters the sample through a sintered glass filter or fine mesh screen.

3. To charge the sample into the viscometer, invert the instrument and apply suction to tube L, immersing tube N in the liquid sample, and draw liquid to mark F. Wipe clean arm N, and turn the instruments to its normal vertical position.

4. Place the viscometer into the holder, and insert it into the constant temperature bath. A viscometer holder which fits the Cannon-Fenske Opaque viscometer and the Cannon-Manning Semi-Micro viscometer will also fit the Cannon-Fenske Routine viscometer. Align the viscometer vertically in the bath by means of a small plumb bob in tube, if a self-aligning holder is not used.

5. Allow approximately 10 minutes for the sample to come to the bath temperature at 40 ^{O}C and 15 minutes at 100 ^{O}C.

6. Apply suction to tube and draw the liquid slightly above mark.

7. To measure the efflux time, allow the liquid sample to flow freely down past mark F, measuring the time for the meniscus to pass from mark E to mark F.

8. A check run may be made by repeating steps 6 and 7.

9. Calculate the kinematic viscosity in mm^2/second (cSt) of the sample by multiplying the efflux time in seconds by the viscometer constants.

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