Optimization of Monoacylglycerol (MAG) Production from Crude Palm Oil (CPO) and Recovery of Carotenoids from MAG-riched CPO

Norholis Majid

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biotechnology

Prince of Songkla University

2009

Copyright of Prince of Songkla University

Thesis Title	Optimization of Monoacylglycerol (MAG) Production from Crude Palm Oil (CPO) and Recovery of Carotenoids from MAG-riched CPO
Author	Mr. Norholis Majid
Major Program	Biotechnology

Major Advisor

…...……….……………………………….

(Asst. Prof. Dr. Benjamas Cheirsilp)

Examining Committee:

…………...……...…....Chairperson

(Assoc. Prof. Dr. Aran H-Kittikun)

… …...……..…………...............

(Asst. Prof. Dr. Benjamas Cheirsilp)

……………..……..……...............

(Asst. Prof. Dr. Somchai Krairak)

The Graduate School, Prince of Songkla University, has approved this thesis as partial fulfillment of the requirements for the Master of Science Degree in Biotechnology.

…………………………………………

(Assoc. Prof. Dr. Krerkchai Thongnoo)

Dean of Graduate School

Thesis Title	Optimization of Monoacylglycerol (MAG) Production from Crude Palm Oil (CPO) and Recovery of Carotenoids from MAG-riched CPO
Author	Mr. Norholis Majid
Major Program	Biotechnology
Academic Year	2009

ABSTRACT

Recovery of carotenoids from crude palm oil (CPO) by glycerolysis reaction followed by column chromatography was conducted. Triacylglycerol (TAG) in CPO was converted to more polar component such as free fatty acid (FFA), diacylglycerol (DAG) and monoacylglycerol (MAG). Consequently, the non-polar carotenoids could be easier recovered in column chromatography step. Firstly the conversion of TAG to MAG by glycerolysis reaction using immobilized lipase PS on Accurel EP-100 (IM-PS) was optimized. The reaction was performed at 45°C in the batch process. The effects of organic solvents, immobilized lipase amount, molar ratio of glycerol to CPO, CPO concentration on the initial production rate and yield of MAG were investigated. After screening a list of different solvents and their mixtures, tert-butanol/hexane mixture at the ratio of 1:1 (v/v) was the suitable organic solvents for MAG production. The optimum conditions for MAG production were found to be 40% (w/v) IM-PS based on solvent volume, 8:1 molar ratio of glycerol to crude palm oil with 4% of water content in glycerol, and 10% (w/v) of CPO in tert-butanol/hexane mixture. Under these conditions, the maximum yield of MAG 74.26% was obtained at 24 h with the initial production rate of 42 mg MAG/mL/h. Then, the MAG product was subjected to column chromatography and the carotenoids was successfully recovered and concentrated by a single stage process on a synthetic porous polymer Diaion HP-20 column (11.5 cm length, 1 cm I.D.). Carotenoids CPO content was 83% recovered and concentrated to about 11,055.02 ppm, which was about 22 times of the original concentration in CPO. The optimum conditions for carotenoids recovery were column temperature 50°C and one gram of sample loading with ethanol as first and hexane as second eluting solvent.

ACKNOWLEGEMENT

I would like to express my thanks to Allah S.W.T for the blessing and willing give me the opportunity to complete this thesis, my advisor Asst. Prof. Dr. Benjamas Cheirsilp for her willingness to accept me as her master student and for her guidance, advice, patience and frequent discussions during my time in Prince of Songkla University.

Special thanks to Assoc. Prof. Dr. Aran H-Kittikun, Head of Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University and Asst. Prof. Dr. Somchai Krairak, Department of Biology, Faculty of Science, King Mongkut's Institute of Technology Ladkrabang for their helpful suggestion, comment and dedicating valuable time for thesis examination. I would like to thank to Asst. Prof. Saowaluck Jitbundjerkul as well as Dr. Masuda Satoru for providing me Diaion HP-20 resin.

I would like to express my warmest thanks to Prof. Dr. Liliek Sulistyowati as a coordinator of Double Degree Program between Faculty of Agriculture, Brawijaya University, Indonesia and Faculty of Agro-Industry, Prince of Songkla University, Thailand, to give me opportunity to follow this program.

I would like to appreciate Ministry of National Educational Indonesian under Double Degree Master Program for the scholarship, Graduate School of Prince of Songkla University and the Biodiversity Research and Training Program, for provide the budget for thesis experiment.

Special thanks to Double Degree Master students of Prince of Songkla University-Brawijaya University for all the things. There was so many things that I can learn during my time study.

Most importantly, this thesis dedicated to my mother. Hope God Almighty always give bless to our family.

Norholis Majid

CONTENTS

Pages

Contents ………………………………………………………………………. v

List of Tables …………………………………………………………………. vii

List of Figures ………………………………………………………………… viii

List of Abbreviations ………………………………………………………… xi

Chapter 1 Introduction ……………………………………………………….. 1

Literature Review …………………………………………….... 3

Objectives …………………………………………………….. 27

Scope of this study ……………………………………………. 27

Chapter 2 Materials and Methods ……………………………………………. 28

Materials ……………………………………………………… 28

Methods ……………………………………………………….. 29

Chapter 3 Results and Discussion 34

1. Monoacylglycerol production from crude palm oil by glycerolysis reaction in organic solvents using immobilized lipase ……………………………………... 34

1.1 Initial glycerolysis condition ………………………... 34

1.2 Effect of organic solvent ……………………………. 35

1.3 Effect of immobilized lipase amount ……………….. 37

1.4 Effect of molar ratio of glycerol to CPO …………… 39

1.5 Effect of CPO loading in solvent mixture …………... 42

2. Study of carotenoids recovery by column chromatography from CPO and MAG-rich CPO …………………………… 45

2.1 Comparison study of normal phase chromatography and reverse phase chromatography for carotenoids recovery ………………………………………….. 46

2.2 Optimization of carotenoids recovery process ……… 52

2.2.1 Effect of temperature …………………………….. 52

CONTENTS (CONTINUED)

	Page
2.2.2 Effect of sample loading …………………	55
2.2.3 Increasing the number of fraction ………..	59
Chapter 4 Conclusion ………………………………………………………...	61
References …………………………………………………………………...	62
Appendix …………………………………………………………………….	68
Vitae …………………………………………………………………………	89

LIST OF TABLES

Table		Page
1	Fatty acid components of palm oil ……………………………………	4
2	Minor components of crude palm oil …………………………………	5
3	Various types and composition of carotenes in palm oil ……………..	7
4	Specific lipase for the production of lipid modifications …………….	12
5	MAG content after a glycerolysis reaction in various solvents and the properties of the solvents screened ………………………………...	17
6	Effect of CPO loading on the recovery of palm carotene …………….	24
7	Carotene recovery on the isopropanol-hexane and ethanol-hexane system ………………………………………………………………...	25
8	Absorption data for common food carotenoids ………………………	26
9	Product compositions from glycerolysis reactions of CPO at 24 h …..	34
10	MAG content after a glycerolysis reaction in several solvents ………	36
11	The carotenoids concentration in crude palm oil, MAG-rich CPO and hydrolyzed CPO ………………………………………………………	46
12	Recovery of palm carotenoids in each column ……………………….	50
13	Effect of temperature in the recovery on carotenoids from MAG-rich CPO with the ethanol-hexane system ……………………………...	55
14	Effect of the sample loading on the recovery of carotenoids from MAG-rich CPO with the ethanol-hexane system …………………….	59
15	Recovery of palm carotenoids with the ethanol-hexane system ……...	60

LIST OF FIGURES

Figure		Page
1	The predominant geometrical isomers of β-carotene: all-*trans*-β-carotene, 9-*cis*-β-carotene, 13-*cis*-β-carotene, and 15-*cis*-β-carotene.	8
2	Lipase catalyzed reaction …………………………………………	13
3	Reactions of synthesis of MAG are including glycerolysis, esterification, hydrolysis and ethanolysis ……………………………	15
4	Effect of IM-PS loading on MAG production ………………………	18
5	Effect of glycerol/oil molar ratio on glycerolysis of sunflower oil in tert-butanol ………………………………………………………..	20
6	Effect of palm olein concentration on MAG production by IM-PS …	21
7	Effect of organic solvents on MAG production from CPO by IM-PS	37
8	Correlation between the log P value and MAG content ……………..	37
9	Effect of IM-PS amount on MAG production (column) and initial rate (line) from CPO ………………………………………………	39
10	Time course of glycerolysis of CPO in the tert-butanol/hexane 1:1 (v/v) mixture ………………………………………………………	39
11	Effect of molar ratio of glycerol to CPO on MAG product (column) and initial rate (line) ………………………………………………	41
12	Time course of glycerolysis of CPO in the tert-butanol/hexane 1:1 (v/v) mixture ………………………………………………………	41
13	Effect of CPO loading on MAG product (column) and initial rate (line) ………………………………………………………………	44
14	Time course of glycerolysis of CPO in the tert-butanol/hexane 1:1 (v/v) mixture ………………………………………………………	44
15	Oil content and carotenoids content from original CPO chromatography on Diaion HP-20 ………………………………….	51
16	Oil content and carotenoids content from MAG-rich CPO chromatography on Diaion HP-20 …………………………………..	51

LIST OF FIGURES (CONTINUED)

Figure		Page
17	The Diaion HP-20 column chromatography for separation of carotenoids from MAG-rich CPO. Initial solvent was ethanol, second solvent was hexane. Each fraction was 1.5 mL, column temperature 40°C, flow rate 0.5 mL/min and sample loading 1 g …..	53
18	The Diaion HP-20 column chromatography for separation of carotenoids from MAG-rich CPO. Initial solvent was ethanol, second solvent was hexane. Each fraction was 1.5 mL, column temperature 50°C, flow rate 0.5 mL/min and sample loading 1 g …..	54
19	The Diaion HP-20 column chromatography for separation of carotenoids from MAG-rich CPO. Initial solvent was ethanol, second solvent was hexane. Each fraction was 1.5 mL, column temperature 60°C, flow rate 0.5 mL/min and sample loading 1 g …..	54
20	Effect of temperature on carotenoids and oil recovery from MAG-rich CPO on Diaion HP-20 column chromatography. Initial solvent was ethanol, second solvent was hexane. Each fraction was 1.5 mL, column temperature 40-60ºC, flow rate 0.5 mL/min and sample loading 1 g …………………………………………………………..	54
21	The Diaion HP-20 column chromatography for separation of carotenoids from MAG-rich CPO. Initial solvent was ethanol, second solvent was hexane. Each fraction was 1.5 mL, column temperature 50°C, flow rate 0.5 mL/min and sample loading 0.5 g ...	56
22	The Diaion HP-20 column chromatography for separation of carotenoids from MAG-rich CPO. Initial solvent was ethanol, second solvent was hexane. Each fraction was 1.5 mL, column temperature 50°C, flow rate 0.5 mL/min and sample loading 1 g …..	57
23	The Diaion HP-20 column chromatography for separation of carotenoids from MAG-rich CPO. Initial solvent was ethanol, second solvent was hexane. Each fraction was 1.5 mL, column temperature 50°C, flow rate 0.5 mL/min and sample loading 2 g …..	57

LIST OF FIGURES (CONTINUED)

Figure		Page
24	The Diaion HP-20 column chromatography for separation of carotenoids from MAG-rich CPO. Initial solvent was ethanol, second solvent was hexane. Each fraction was 1.5 mL, column temperature 50°C, flow rate 0.5 mL/min and sample loading 3 g …..	58
25	The Diaion HP-20 column chromatography for separation of carotenoids from MAG-rich CPO. Initial solvent was ethanol, second solvent was hexane. Each fraction was 1.5 mL, column temperature 50°C, flow rate 0.5 mL/min and sample loading 4 g …..	58
26	Effect of temperature on carotenoids and oil recovery from MAG-rich CPO on Diaion HP-20 column chromatography. Initial solvent was ethanol, second solvent was hexane. Each fraction was 1.5 mL, column temperature 50°C, flow rate 0.5 mL/min and sample loading 0.5 - 4 g ………………………………………………………………	59
27	The Diaion HP-20 column chromatography for separation of carotenoids from MAG-rich CPO. Initial solvent was ethanol, second solvent was hexane. Each fraction was 1.5 mL, column temperature 50°C, flow rate 0.5 mL/min and sample loading 1 g …..	60

LIST OF ABBREVIATION

CPO	Crude Palm Oil
DAG	Diacylglycerol
FAAE	Fatty Acid Alkyl Ester
FFA	Free Fatty Acid
IM-PS	Immobilized lipase from *Pseudomonas* sp.
MAG	Monoacylglycerol
TAG	Triacylglycerol
TLC/FID	Thin Layer Chromatography/Flame Ionization Detector
ppm	part per million

CHAPTER 1

INTRODUCTION

Introduction

Carotenoids are mainly present in vegetables, fruits and edible oil and display several important biological actions (provitamin A, antioxidant and anticarcinogenic activities and immunomodulation) (Granado *et al.,* 2001). The main source of carotenoids is palm oil. Palm oil has a greater carotenoids concentration than any other oil or fat. It is contains a high concentration of natural carotenoids of 500 – 700 ppm (Wei *et al.,* 2005). The major carotenoids of palm oil are α- and β-carotene; together they constitute more than 90% of the total carotenoids in palm oil. Carotenes, in particular β-carotene and, to a lesser extent, α-carotene, are known for their provitamin A activities. Palm oil has 15 times more retinol equivalent than carrots and 300 times more than tomatoes. Palm oil is the most abundant vegetables oils in South East Asia, especially in Thailand, Malaysia and Indonesia. It is mainly used as frying oil as well as others application such us raw material for margarine, cocoa butter substitute (CBS) and cosmetics. At present, most of the carotenoids in the crude palm oil (CPO) are destroyed in order to obtain light coloring oil (Fernandez *et al.,* 2000; Mortensen, 2005; Wei *et al.,* 2005). This happen because lighter colored oil is preferred by consumer. Moreover, this represents a loss of potential source of natural carotenoids. The importance of carotenoid is well documented and various methods of extraction and recovery from CPO have been developed. These include extraction by saponification, adsorption, precipitation, selective solvent extraction, and transesterification followed by molecular distillation (Chuang and Brunner, 2006). However, only the transesterification followed by molecular distillation process has been further developed into a commercial-scale process (Ooi *et al.,* 1991).

Hydrolysis and transesterification are first steps methods prior to other step such as distillation, solvent extraction, chromatography and nano-filtration to obtained high concentration of carotenoids (Darnoko and Cheryan, 2006). The aims of those methods are to convert triacylglycerol (TAG) to free fatty acid (FFA)/fatty acid alkyl ester (FAAE) and glycerol. FFA and FAAE are less-polar than glycerol but

higher-polar than carotenoids. Thus, carotenoids will dissolve in non-polar fraction. In the same basic principle, another way to concentrate the carotenoids in crude palm oil is glycerolysis reaction. TAG will be reacted with glycerol to produce monoacylglycerol (MAG) as main product besides of FFA and diacylglycerol (DAG). The carotenoids are concentrated in TAG and MAG as valuable product is also obtained. MAG is more-polar than DAG, FFA, FAAE and TAG, respectively (Kaewthong, 2004). The mixture of the products would be separated later by column chromatography. Thus, this method would also be a method to recover the carotenoids in crude palm oil. The glycerolysis reaction could be catalyzed either by chemical or enzymatic reaction. The benefits of enzymatic glycerolysis are using relatively low temperature (45°C) and using immobilized lipase as catalyst instead of inorganic alkaline catalyst. Glycerolysis of oils and fats by employing inorganic alkaline have drawback that is dark-colored by products with an undesirable flavor are formed. The separation of the products of reaction could be done simply by column chromatography as described by Kaewthong (2004) and Watanabe *et al.* (2004). Silica gel column was employed to separate the product by various solvents. The nature of carotenoids as well as TAG would be eluted first then followed by FFA, DAG and the last MAG. The carotenoids would be dissolved and concentrated in most-non polar component (TAG). The significant of this research was to improve the carotenoids recovery process by mean of glycerolysis reaction of crude palm oil. This new approach will not only recover the carotenoids but also produce high value-added product of monoacylglycerols.

Literature Review

1. Palm oil

Palm oil is one the world's most important vegetables oils. It is used mainly for edible purposes and has become an important raw material for many applications during the past few decades. Increasing interest in the chemistry and biotechnology of palm oil has emerged in recent years, which is mainly attributed to the fact that oleo-chemicals are derived from renewable sources (Lim *et al.,* 2005).

The oil palm produces two distinct oils. They are oils from the mesocarp usually refers to common "palm oil" and oils from the fruit kernel. The fatty acid composition of these two kinds of oils is different. Palm oil is light yellow to orange-red in color depending on the amount of carotenoids present (Szulczewska-Remi *et al.,* 2005). The composition of palm oil mainly palmitic and oleic acid, meanwhile, kernel oil mainly composed of lauric acid. In palm oil, each of the saturated palmitic acid and the monounsaturated oleic acid accounts for about 40% wt of the overall free fatty acids (FFAs), depending on harvest conditions and locations of cultivation. The fatty acid component of palm oil is showed in Table 1.

Crude palm oil is a semisolid material at room temperature and has melting point of about 36°C. It may be fractionated into a liquid oil fraction (65-70%) known as palm olein (melting point 18-20°C) and a solid fraction (30-35%) known as stearin (melting point 48-50°C) (Szulczewska-Remi *et al.,* 2005). Crude palm oil contains approximately 1% of minor components: carotenoids, vitamin E (tocopherols and tocotrienols), sterols, phospholipids, glycolipids, terpenic and aliphatic hydrocarbon and other impurities (Table 2). The most important are carotenoids and vitamin E, both of which possess important physiological properties (Choo *et al.,* 1985).

2. Carotenoids

The carotenoids, whose name is derived from the fact that they constitute the major pigment in the carrot root, *Daucus carota*, are undoubtedly among the most widespread and important pigments in living organism. They are

present in numerous vegetables oils, including maize (corn) oil, groundnut oil, soy-bean oil, rapeseed oil, linseed oil, olive oil, barley oil, sunflower-seed oil and cotton oil. The concentration of carotenoids in these vegetables oils is generally low (Choo, 1994).

Carotenoids have been proven to be beneficial to human health apart from having pro-vitamin A which prevents xeropthlamia, a night blindness disease. Carotenoids also play an important role as anti-oxidant by scavenging free radicals and as singlet oxygen quencher. Carotenoids are also found to be capable of inhibiting growth of certain cancer cells such as the colon cancer (Wei *et al.,* 2005).

Table 1. Fatty acid components of palm oil

Common name	Systematic name	Percentage of total weight
	Saturated acids	
Lauric	N-dodecanoic	< 1
Myristic	N-tetradecanoic	1 – 6
Palmitic	N-hexadecanoic	32 - 47
Stearic	N-octadecanoic	1 – 6
Arachidic	N-eicosanoic	< 1
	Monounsaturated acids	
Palmitoleic	N-hexadec-9-enoic	< 1
Oleic	N-octadec-9-enoic	40 – 52
Gadoleic	N-eiocos-9-enoic	< 1
	Polyunsaturated acids	
Linoleic	N-octadec-9,12-dienoic	5 – 7

Source: Lim *et al.* (2005)

Table 2. Minor components of crude palm oil

Components	Concentration (ppm)
Carotenoids	500 – 700
Tocopherol and tocotrienols	600 – 1,000
Sterols	326 – 527
Phospholipids	5 – 130
Triterpene alcohol	40 – 80
Methyl sterol	40 – 80
Squalene	200 – 500
Aliphatic alcohols	100 – 200
Aliphatic hydrocarbons	50

Source: Choo *et al.* (1985)

Carotenoids can be divided into two main groups: a) hydrocarbons, which are termed “carotenes” and b) oxygen containing derivatives, which are termed “xanthophylls”. The hydroxyl derivatives can exist in the free stated or esterified with fatty acids such as linoleic acid (Ambrogi *et al.,* 2003). Xanthophylls members are including cryptoxanthin, lutein, neoxanthin, violaxanthin and zeaxanthin. The xanthophylls family tends to be more-polar since they have hydroxyl groups and tentatively identified in red palm oil when a more polar solvent mixture, i.e. diethyl ether/petroleum ether was used for extraction (Ping and Gwendoline, 2006). However, carotenes such as α- and β-carotenes are the biggest proportion in carotenoids. More than 90% of carotenoids are composed by α- and β-carotenes (Wei *et al.,* 2005).

The commercially available carotenoids mainly β-carotene supplements are produced by synthetic means. Globally, there is a rising trend that people are going for natural products including vitamins and carotenes. The synthetic carotenoids especially β-carotene takes as a supplementation is unlike natural carotenoid which is a mixture of carotenoids compounds, many of which may not be readily metabolized rapidly (Goh, 1996). Moreover, Krishnaiah *et al.* (2007) said that natural antioxidants from plant are preferred over synthetic antioxidants, which are

found to impose side effect. Carotenoids concentrates from plant are preferred nowadays.

Palm oil is the richest plant source of carotenoids in terms of retinol equivalent with 500 – 700 ppm (Fernandez *et al.,* 2000; Han *et al.,* 2006; Markom *et al.,* 2001; Wei *et al.,* 2005). However, these carotenoids are destroyed by conventional refining. In light of the importance of natural carotenoids, various methods have been investigated to recover the carotenoids from palm oil before refining. These include saponification, adsorption, solvent extraction and transesterification of palm oil followed by molecular distillation. A total of 11 types of carotenes have been identified in palm oil as shown in Table 3.

Structure of carotenoids is isoprenoid compounds, biosynthesized by tail to tail linkage of two C_{20} geranyl-geranyl diphosphate molecules. This produces the parent C_{40} carbon skeleton from which all individual variations are derived. This skeleton can be modified by: cyclization at one end or both ends of the molecule to give different end groups, changes in hydrogenation levels, or addition of oxygen containing functional groups (Dutta *et al.,* 2005). In the higher plant, carotenoids have at least five different roles in photosynthesis: 1) light harvesting, 2) chlorophyll triplet quenching, 3) singlet oxygen scavenging, 4) excess energy dissipation, and 5) structure stabilization and assembly (Croce *et al.,* 1999).

Carotenoids that contain one more oxygen atoms are known as xanthophylls, the parent hydrocarbon as carotene. The long system of alternating double and single bond constitutes a conjugated system in which the π electron are effectively delocalized over the entire length of the polyene chain. This feature is responsible for the molecular shape, chemical reactivity and light absorbing properties, and hence color of carotenoids. At least seven conjugated double bonds are needed for the carotenoid to impart color. Each double bond in the polyene chain of a carotenoid can exist in two configuration, *trans* or *cis* geometrical isomers. Most carotenoids occur in nature predominantly or entirely in the all *trans* form. The geometrical isomers may well have different physiological properties (Ping *et al.,* 2002).

Table 3. Various types and composition of carotenes in palm oil

Types of carotenes	Composition (%)
Phytoene	1.27
Cis-β-carotene	0.68
Phytofluene	0.06
β-carotene	56.02
α-carotene	35.16
Cis-α-carotene	2.49
ζ-carotene	0.69
γ-carotene	0.33
δ-carotene	0.83
Neurosporene	0.29
β-zecarotene	0.74
α-zecarotene	0.23
Lycopene	1.30

Source: Wei *et al.* (2005)

Degradation of carotenoids in food is complex in nature as various factors such as nature and composition of foods, processing treatments, packaging and storage conditions, activity of lipoxygenase and other enzymes, and coupled oxidation with lipids are considered to play a vital role. Knowledge of carotenoid degradation is fragmentary. The polyene chain is the cause of instability of carotenoids including their susceptibility to oxidation, cleavage and geometric isomerization (Hu *et al.,* 2006). Heat, light and acids promote isomerization of *trans*-carotenoids to the *cis*-form. Oxidation depends on the available oxygen, the carotenoids involved, and their physical conditions. It is generally accepted that the initial stage of oxidation involves epoxidation and formation of apocarotenals. Subsequent fragmentation results in a series of low molecular weight compounds similar to those obtained in fatty acid oxidation, which contribute to the desirable flavor in wine and tea but can be responsible for the off flavor of dehydrated carrots and sweet potato flakes. Figure 1 described the structure of carotenes.

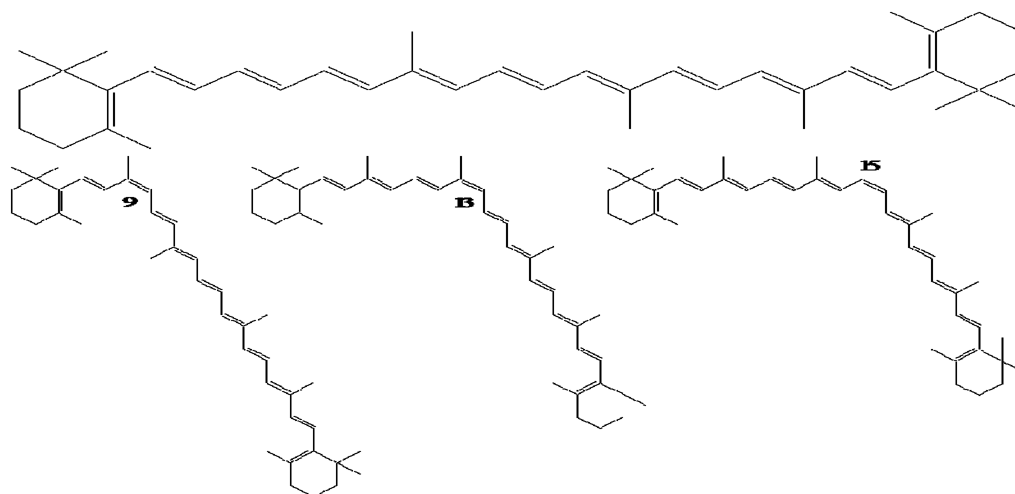


Figure 1. The predominant geometrical isomers of β-carotene: all-*trans*-β-carotene, 9-*cis*-β-carotene, 13-*cis*-β-carotene, and 15-*cis*-β-carotene.

Source: Schierle *et al.* (2004)

2.1 Analysis of carotenoids

The properties of carotenoids briefly outlined above indicate a number of points to be considered when designing a carotenoids recovery and analysis:

1) Carotenoids are lipophilic pigments and thus are sometimes denoted as lipochromes or chromolipids. Their hydrophobicity and fat-solubility have two primary consequences: a) they are transported by lipoproteins within organisms and thus their distribution is linked to the lipid profile, b) their insolubility in water and poor solubility in many organic liquids place considerable demands on proper selection of extraction and pre-concentration agents and impose severe limitations on the composition of HPLC mobile phase.

2) Carotenoids exhibit pronounced photo- and thermal sensitivity. Irradiation and an increase temperature lead mainly to *trans-cis* isomerization which causes bending of the originally rod-like molecule and may also cause cleavage of the molecule, especially in the presence of air and catalyst.

3) Due to the presence of a long system of conjugated double bonds, carotenoids are intensively colored and thus strongly absorb in the visible region, between 400 and 500 nm, usually exhibiting three absorption bands or two bands plus a shoulder.

4) Spectroscopic techniques are extremely important for direct analyses and characterization.

5) The conjugated double-bond system of carotenoids causes not only their spectral activity, but also their electro-activity (Ladislav *et al.,* 2005).

The total carotenoids in palm oil are usually determined by ultraviolet-visible spectroscopy at 446 nanometers as ppm of β-carotene. However, because of their complex compositions, various analytical methods have been employed to determine their profile. Earlier studies used column chromatography with different absorbents. More recently, reverse-phase high-performance liquid chromatography (HPLC) has proved to have several advantages for separating carotenoids in the oil (Choo, 1994).

3. Lipase

Lipases (EC 3.1.1.3), also known as glycerol ester hydrolases, belong to the hydrolase enzyme class and were originally employed for the hydrolysis of ester bonds. Lipases catalyze esterification and alcoholysis reactions to produce various esters of important commercial values. Under physiological conditions, this enzyme catalyzes hydrolysis of oils and fats and produces hydrolysis products. The biological role of lipase is metabolism of lipids. Many kinds of esters of short-fatty acids and alcohols are important in the food industry as flavor and aroma constituents. Methyl and ethyl esters of long-chain fatty acids are valuable oleo-chemicals that may be used as replacements for diesel fuel (Chen and Wang, 1997). Free fatty acid (FFA) products from enzymatic hydrolysis reactions regarded as food-grade are obtained from lipases-catalyzed reaction. Moreover, crude palm oil conversion product not only obtained esters and free fatty acid, but also obtained carotenoids and others phytonutrients which are very valuable product. In addition to plants and animals, in which these enzymes are widespread, many microorganisms (naturally or genetically engineered) are also capable of actively producing these enzymes both in endogenous and exogenous form (Balcao *et al*., 1996).

The advantages of using lipase as catalyst instead of chemicals are specificity of enzymes, mild conditions under which they function, biodegradable and

could reduce environmental loading. Chemical catalysts randomize fatty acids in triacylglycerol mixture and fail to yield products with desired physiochemical characteristic. Among attractions in replacing the current chemical technology with enzyme biotechnology are energy savings and minimization of thermal degradation (You and Baharin, 2006).

Hydrolysis procedure by lipase from *Candida rugosa* is an alternate method to increase the carotene recovery from crude palm oil and crude palm olein prior to column chromatography. The objective of hydrolyzing the palm oil was to produce more polar FFA-rich oil in order to enhance non-polar carotene bind to the non-polar adsorbent (You *et al.,* 2002). On the other hand, carotene binding to non-polar solvent in solvent-solvent extraction is also enhanced.

Commonly, enzyme works in aqueous solution. Thus, it is not surprisingly that all studies about enzymology have used water as the reaction medium. But, there are numerous advantages of conduction enzymatic conversions in organic solvent instead of water: i) high solubility of most organic compounds in non aqueous media, ii) ability to carried out new reactions impossible in water because of kinetic or thermodynamic restriction, iii) greater stability of enzymes, iv) relative ease of product recovery from organic solvents as compared to water, and v) the insolubility of enzymes in organic media, which permits their easy recovery and reuse and thus eliminates the need for immobilization (Zaks and Klibanov, 1985).

Zaks and Klibanov (1985) have already done this experiment since two decades ago. They have used three kinds of different lipases namely porcine pancreatic, yeast and mold. They found that the lipases acted as catalysts in a number of nearly anhydrous organic solvents. Various transesterification reactions catalyzed by porcine pancreatic lipases in hexane were obeying Michalis-Menten kinetics.

Recent development in enzyme-mediated reactions in organic solvents has enabled their direct applicable to industrial process. Due to high water content in the reaction mixture, equilibrium of an enzymatic reaction is shifted toward hydrolysis. In contrast, enzymatic reactions in organic solvents containing small amount of water result in shift of equilibrium toward synthesis and are recognized as effective system for several industrial processes. The catalytic property of enzymes in

organic solvent has been applied to interesterification, esterification, peptization and other reactions (Isono *et al.,* 1995).

The absence of water in organic solvents is often immediately conducive to new enzymatic reactions. For instance, in water numerous lipases, esterases and proteases catalyze the hydrolysis of the esters to the corresponding acids and alcohols. In anhydrous solvents, this process obviously cannot occur. However, adding alternative nucleophiles, such as alcohols, amines and thiols, lead to transesterification, aminolysis and thiotransesterification, respectively—reactions suppressed in aqueous solution. Moreover, the synthesis of esters from their constituent acids and alcohols (the reverse of hydrolysis) become thermodynamically favorable (Klibanov, 2001). Moreover, some advantages in using enzymes in organic media as opposed to aqueous media could be summarized as follows: (i) shifting thermodynamic equilibrium to favor synthesis over hydrolysis, (ii) reduction of water-dependent side reactions, (iii) elimination of microbial contamination and (iv) suitable for reactions with substrates insoluble and/or unstable in water.

The reactions catalyzed by lipase are including hydrolysis, ester synthesis and transesterification. They catalyze hydrolysis in an aqueous system, but also esterification (reverse reaction of hydrolysis) in a microaqueous system, where water content is very low. The reaction of the lipids modification could be described by Figure 2.

Regiospecificity is one of the major advantages of using lipase technology for the modification of oils and fats to produce high-value added products, such as cocoa butter equivalents, human milk fat substitutes and other specific structured lipids (Xu, 2000). Regiospecificity is another word to explain positional specificity of the lipase. There are two groups of regiospecificity: 1,3-positional-specific and non-positional-specific. It should be noted that the positional specificity of lipase is not strictly divided into the two categories, but it varies widely in the range of very distinctly 1,3-positional-specific to very weakly specific or completely non-positional-specific. However, the rate reaction on the ester linkage with different fatty acid is different. It is usually determined by comparing the relative hydrolysis rate of various fatty acid esters with different acylgroups. In fact, the specificity spectrum diverges significantly, which indicates that lipases seem not to have strict

fatty acid specificity. Xu (2000) has summarized fatty acid specificity and regio-specificity in Table 4.

There some problems about lipase application. First, there is the widespread belief that enzymes require aqueous media for activity. For lipase, this would be an obstacle since their substrates are generally poorly soluble in water. However, the work of Zaks and Klibanov (1985) has shown that this belief is highly misplaced. Lipase and a number of other enzymes are active in organic media. The second major problem that is perceived is the instability of enzymes. This is easily manipulated by the use of organic media and/or enzyme immobilization (Gandhi, 1997).

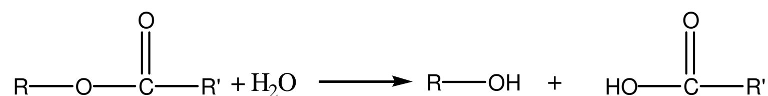
Table 4. Specific lipase for the production of lipid modifications

Lipase source	Fatty acid specifity	Regiospecificity (sn)
Aspergillus niger	S, M, L	1, 3 >> 2
Candida lipolytica	S, M, L	1, 3 > 2
Humicola laguginosa	S, M, L	1, 3 >> 2
Mucor javanicus	M, L >> S	1, 3 > 2
Rhizomucor miehei	S > M, L	1, 3 >> 2
Pancreatic	S > M, L	1, 3
Pre-gastric	S, M >> L	1, 3
Penicillium camembertii	MAG, DAG > TAG	
Penicillium roquefortii	S, M >> L	1, 3
Rhizopus delemar	M, L >> S	1, 3 >> 2
Rhizopus javanicus	M, L > S	1, 3 > 2
Rhizopus japonicus	S, M, L	1, 3 > 2
Rhizopus niveus	M, L > S	1, 3 > 2
Rhizopus oryzae	M, L > S	1, 3 >>> 2
Pseudomonas fluorescens	M, L > S	1, 3 > 2
Pseudomonas sp.	S, M, L	1, 3 > 2
Rhizopus arrhizus	S, M > L	1, 3

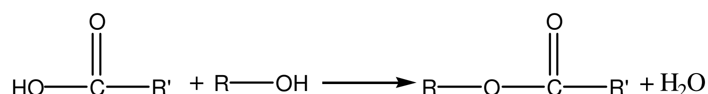
Abbreviations: MAG, monoacylglycerols; DAG, diacylglycerols; TAG, triacylglycerols; L, long-chain fatty acids; M, medium-chain fatty acids; S, short-chain fatty acids.

Source: Xu (2000)

1. Hydrolysis

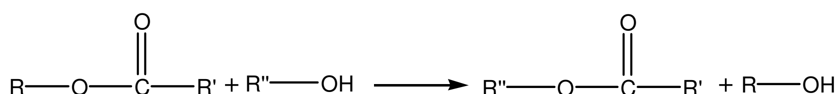


2. Ester synthesis

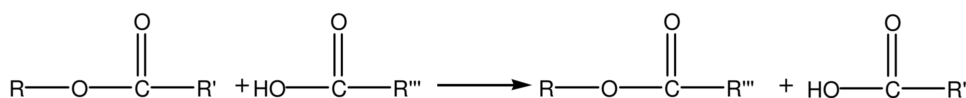


3. Transesterification

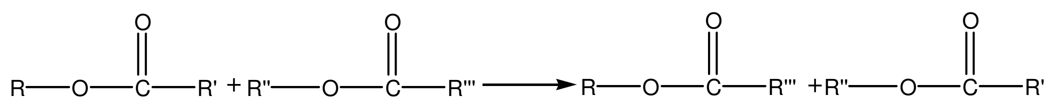
3.1 Alcoholysis



3.2 Acidolysis



3.3 Interesterification



3.4 Aminolysis

Figure 2. Lipase catalyzed reaction.

Source: Gandhi (1997)

4. Monoacylglycerols

Monoacylglycerols (MAG) are the most widely used as emulsifier in food, pharmaceutical and cosmetic industries. Beside bulk application in the food and dairy industry, some other applications for special MAG are for medicine, therapy and hair care additive. There are two kinds of monoacylglycerol, 1-monoacylglycerol (1-MAG) and 2-monoacylglycerol (2-MAG). Most of the emulsifiers used for various

food products in the world are 1-MAG. It stabilizes and facilitates the formation the water in oil emulsions and therefore, they are essential ingredients of margarine and low calorie spreads (Kaewthong *et al.,* 2005; Pawongrat *et al.,* 2007; Watanabe *et al.,* 2004).

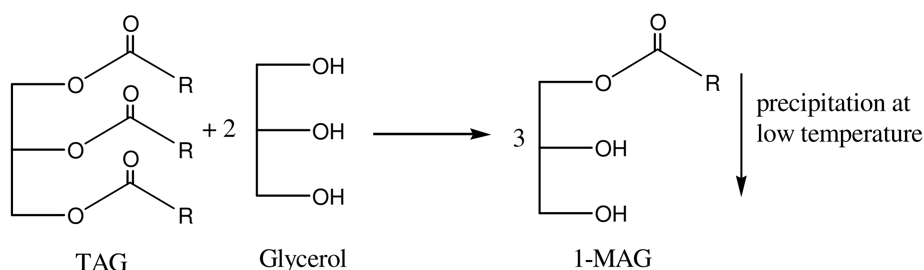
Monoacylglycerols are currently produced industrially by chemical glycerolysis of oils and fats at high temperatures of 210-240°C employing inorganic alkaline catalysts under nitrogen atmosphere, but the process cannot be applied to synthesis MAGs of unstable fatty acids including conjugated linoleic acid (CLA) (Watanabe *et al.,* 2004). Moreover, the product produced by this strategy has several drawbacks (e.g., low yield, dark color, burnt taste). Molecular distillation is necessary because MAG need to be highly pure in the food industry, since they have better emulsifying properties than a mixture of different acylglycerols (Bornscheuer, 1995).

The syntheses of MAGs are enzymatic hydrolysis using 1,3-regiospecific lipases, esterification of fatty acids or transesterification of fatty esters with glycerol, glycerolysis and ethanolysis, but these reactions were conducted in organic solvents. Meanwhile, organic solvent-free systems are attractive from the viewpoint of industrial production of MAGs. Many different reaction systems for batch or continuous production have been used, and several aspects such as type, purity, and immobilization of the biocatalyst, temperature, and solvent system have been investigated in detail. The reactions of MAG synthesis are described in Figure 3.

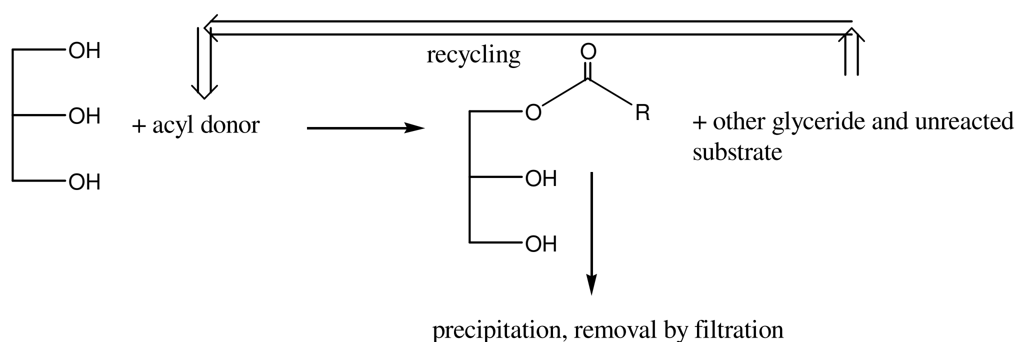
The problem of the low solubility of the hydrophilic glycerol in non-polar organic solvents was overcome easily by prior adsorption of glycerol onto a solid support (e.g., silica gel, charcoal, celite) creating a dry powder. These glycerol preparations were suspended in an organic solvent (e.g., t-butylmethylether, diethylether-hexane) and an acyl donor together with a 1,3-selective lipase (e.g., *Rhizopus delemar, Rhizomucor miehei, Chromobacterium viscosum*) were added, thus mimicking an artificial liquid-liquid interphase. After the reaction was completed, both the enzyme and the solid support were removed simultaneously by simple filtration while the esterification products remained in solution. Under optimized conditions, the MAG content achieved 70%, but higher yields were found to be impossible, because at high concentration, 1(3)-MAG served as a better substrate than glycerol. For a higher yield, a continuous separation system based on compartmental

separation of the two steps of the process (synthesis and isolation) was developed. Unreacted substrates and unwanted by-products (e.g., 2-MAG, DAG, TAG) were completely recycled and 1(3)-MAG was frozen at lower temperatures (Figure 3.b). Nonspecific lipases were used successfully (Bornscheuer, 1995).

a. synthesis of 1-MAG by solid phase-glycerolysis



b. synthesis of 1-MAG by esterification of glycerol



c. synthesis of 2-MAG by deacylation of TAG with 1,3-position-specific lipase

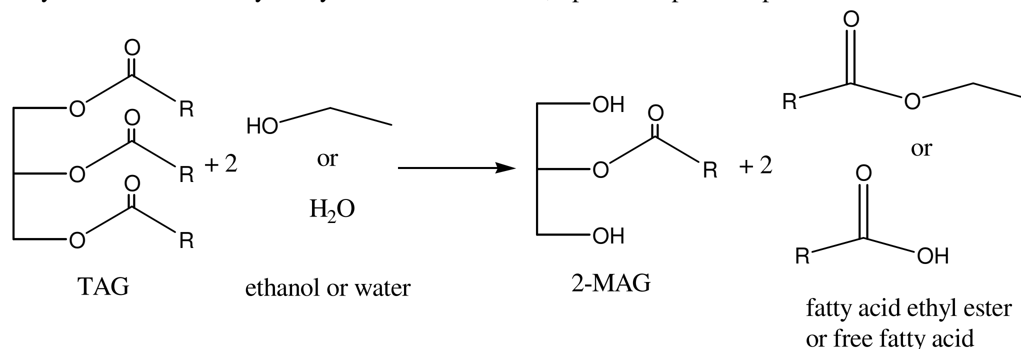


Figure 3. Reactions of synthesis of MAG are including glycerolysis, esterification, hydrolysis and ethanolysis.

Source: Bornscheuer (1995)

4.1 The parameters affecting monoacylglycerol production

4.1.1 Source of lipases

Various commercial lipases were tested in immobilized form for monoacylglycerol production by Rosu *et al*. (1997). The enzymes could be recovered easily from the reaction mixture and recycled to reduce the cost of the catalyst by using immobilized enzyme of *Pseudomonas* sp., *Chromobacterium viscosum* and *Pseudomonas pseudoalkali*. The enzymes were used in the production of monoacylglycerol by solid-phase glycerolysis of olive oil. *Pseudomonas* sp. in $CaCO_3$ solid support material was proved produce 90% MAG (wt% in the glycerol-free reaction mixture after 72 h of reaction time) until fifth use.

Higher MAG production was reported by Bornscheuer (1995). With lipase from *Chromobacterium viscosum* 99% MAG content was achieved after incubation for 120 h at 8°C (first 8 h at 25°C) with triolein as triacylglycerol model. Meanwhile, nine commercial lipase from *Pseudomonas* sp. (lipase PS), *Pseudomonas fluorescens* (lipase AK), *Candida rugosa* (lipase AY), *Rhizopus delemar* (lipase D), *Mucor javanicus* (lipase M), *Rhizopus oryzae* (lipase F), *C. rugosa* (lipase OF), *Alcaligenes* sp. (lipase PL) and *Chromobacterium viscosum* (lipase LP) were screened for production of MAG from palm olein by continuous production in the packed-bed reactor and continuous stirred-tank reactor. The highest conversion was achieved by *Pseudomonas* sp. lipase immobilized in Accurel EP100 support (Kaewthong *et al.,* 2005).

4.1.2 Organic solvents

Organic solvents have been used extensively in lipase-catalyzed reaction. The use of organic solvents can improve the poor solubility in water of substrates or other reaction components of a hydrophobic nature. However, organic solvents produce various physiochemical effects on enzyme molecules and the effects differ depending upon the kinds of organic solvents and enzyme used. Kaewthong and H-Kittikun (2004) tested glycerolysis of palm oil by immobilized lipase from *Pseudomonas* sp. in acetone, hexane and isooctane and combination of these solvents. It was found that acetone/isooctane mixture (3:1, v/v) gave the highest yield of MAG (27.29%) at 24 h. Meanwhile, Elfman-Borjesson and Harrod (1999) used 48% isooctane to produced 17.4% yield of MAG from rapeseed oil using Lipozyme IM.

Methyl *tert*-butyl ether (MTBE) was employed by Soumanou *et al.* (1998) to produce 2-MAG. Triglyceride in MTBE was reacted with ethanol and purified by crystallization and obtained in up to 71.8% yield. Petroleum ether and acetone are also employed, but these solvent gave no improvement yield.

These research results were agreed by several authors. The lipases show good stability and activity in hydrophobic solvents for MAG synthesis by glycerolysis (Damstrup *et al*., 2005; Kristensen *et al.,* 2005). Moreover, Yang *et al*. (2005a) used *tert*-butanol to improve the low homogeneity in the enzymatic glycerolysis system. Recent research result conducted by Damstrup *et al*. (2005) show correlation among MAG yield and log P value of the solvents. The maximum MAG contents were obtained in solvents with log P values in the range of 0.3 – 1 and having tertiary alcohol structure. The low log P value of *tert*-butanol (0.35) indicates both hydrophilic and hydrophobic characteristics. This is in accordance with the expectation that a solvent should have both water-like and octanol-like properties in order to be a suitable solvent for both oil and glycerol. Table 7 shows the research result from Damstrup *et al*. (2005).

Table 5. MAG content after a glycerolysis reaction in various solvents and the properties of the solvents screened

Solvent	MAG content	Log P value	Melting point (^{o}C)	Boiling point (^{o}C)
Solvent free	0.0 ± 0.00			
Chloroform	0.0 ± 0.00	1.97	-63.41	61.17
n-Heptane	1.1 ± 0.02	4.50	-90.55	98.40
n-Hexane	1.4 ± 0.03	4.00	-95.35	68.73
Iso-octane	1.5 ± 0.17	5.15	-107.00	99.20
Acetonitrile	2.0 ± 0.07	-0.34	-43.82	81.65
Toluene	2.9 ± 0.20	2.73	-94.95	110.63
2-Butanone	5.4 ± 0.10	0.29	-86.64	79.59
Acetone	11.5 ± 0.73	-0.24	-94.70	56.05
Isopropanol	18.0 ± 0.31	0.05	-88.50	82.40
Ethanol	21.0 ± 0.18	-0.30	-114.14	78.29
3-Pentanone	29.4 ± 0.26	0.82	-39.00	101.00
tert-Pentanol	64.9 ± 1.12	0.89	-9.10	102.40
tert-Butanol	83.6 ± 0.14	0.35	25.69	82.40

Source: Damstrup *et al.* (2005)

4.1.3 Immobilized lipase amounts

The amount of immobilized lipase was affected on the MAG production. When increasing the amount of immobilized lipase in the reaction mixture the MAG production was also increased. Fifty-percent immobilized lipase on palm oil is the maximum amount of catalyst on production of MAG. Above this amount, there is no benefit came (Kaewthong and H-Kittikun, 2004). For tuna oil, upon increasing the amount of immobilized lipase in the reaction mixture, the MAG production also increased. However, no benefit came from increasing immobilized lipase above 30% wt of tuna oil. (Pawongrat *et al.*, 2007). Figure 4 shows the effect of IM-PS loading on MAG production.

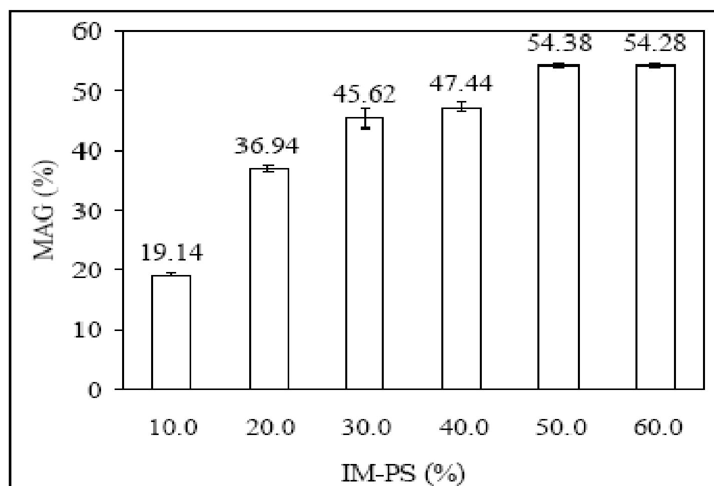


Figure 4. Effect of IM-PS loading on MAG production

Source: Kaewthong and H-Kittikun, 2004

Yang *et al.* (2005b) studied the effect of enzyme loading on enzymatic production of MAG containing polyunsaturated acids. Glycerolysis of sunflower oil was conducted at the Novozyme 435 loads of 5, 10, 15, 20 and 30% weight based on glycerol and oil. Enzyme load more than 10% resulted in little increase of MAG yield. In the used conditions, 10-15% enzyme load is enough for the maximum reaction performance.

Barouh *et al.* (2008) studied about enzymatic production of conjugated linoleic acid monoacylglycerols from dehydrated isomerized castor bean oil. They suggested the operation at low enzyme concentration (around 2%) for economic reasons, since five percent and above of enzyme concentration did not result significant increasing of MAG content.

4.1.4 Molar ratio of glycerol to triacylglycerol

Theoretically, 3 mol MAGs were obtained when 1 mol TAG and 2 mol glycerol were used as substrates for glycerolysis. Elfman-Borjesson and Harrod (1999) reported that glycerol to triglyceride ratio had a significant effect on the yield of monoacylglycerol. They found that 17.4% MAG was achieved when glycerol/rapeseed oil molar ratio of 6:1 was used. A yield of 60% monopalmitoylglycerol was obtained when the molar ratio glycerol to palmitate 10:5 by esterification reaction. This yield was greatly influenced by the reaction temperature and molar ratio of substrate (Kwon *et al.,* 1995). Pawongrat *et al*. (2007) reported that mole ratio of glycerol/tuna oil 3:1 gave 24.6% MAG, meanwhile Kaewthong and H-Kittikun used mole ratio glycerol/palm oil 8:1 gave 56% MAG yield.

According to Yang *et al.* (2005b), the increase of glycerol amount will increase the theoretical equilibration value. On the other hand, the glycerol amount will definitely affect the system polarity so as to influence the system stability and homogeneity. The experiments by Yang *et al.* (2005b) were conducted in the following molar substrate ratios between glycerol and oil sunflower oil: 2.5:1, 3.5:1, 4.5:1 and 5.5:1. Less glycerol certainly produced less MAGs since the maximum yield was also reduced. However, more glycerol did not produce more MAGs as well since 4.5:1 and 5.5:1 (mol/mol) glycerol/oil had little difference in MAG yield. This is definitely not due to the equilibrium problem. An optimal ratio must exist considering both effects of glycerol on the reaction equilibrium and the system homogeneity. In their study, 4.5:1 (mol/mol) glycerol/oil was taken for the batch reaction system. Figure 5 shows effect of glycerol/oil molar ratio according to Yang *et al* (2005b). Meanwhile, Barouh *et al.* (2008) using response surface method methodology to obtain optimum parameter in their research. They found that 11:1 glycerol/TAG molar ratio is the optimum ratio. However, in the continuous process by Noureddini *et*

al. (2004) suggested only 2.5 molar ratio of glycerol/TAG since in the higher molar ratio the formation of MAG was independent from the ratio. Coteron *et al.* (1998) conducted study about reaction of olive oil and glycerol over immobilized lipase. They found similar product distribution for glycerol/oil mole ratios of 3:1 and 6:1.

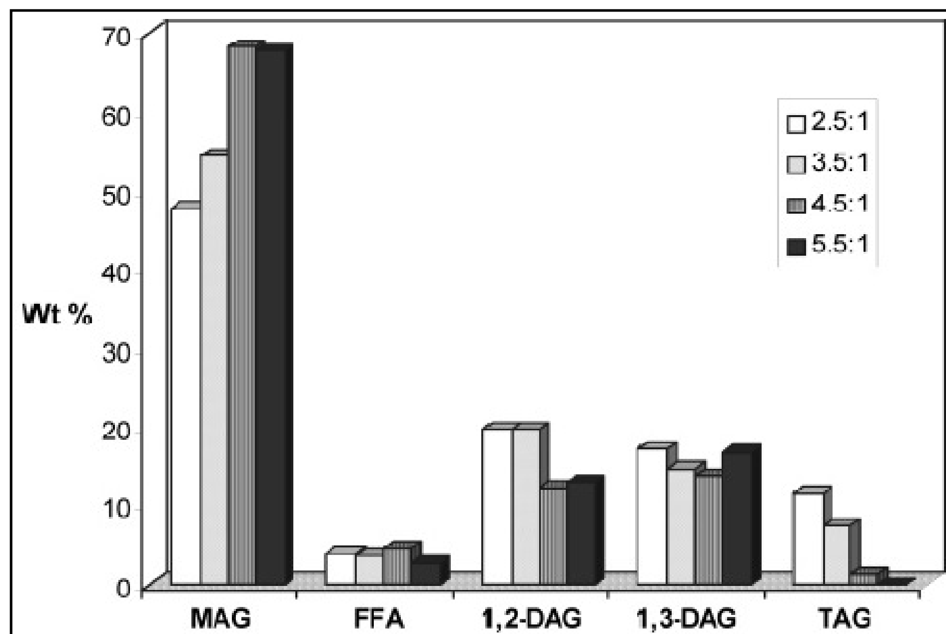


Figure 5. Effect of glycerol/oil molar ratio on glycerolysis of sunflower oil in tert-butanol

Source: Yang *et al* (2005b)

4.1.5 Oil concentration

Oil concentration can affect the production yield of MAG. In a solvent system, the concentration of substrate (the oil) will eventually affect the reaction rate based on Michaelis-Menten kinetics, even though solvent can help create a homogeneous system. According to Pawongrat *et al.* (2007), 10% w/v of tuna oil in MTBE was the best amount of substrate gave 22.1% of MAG yield. When the concentration of tuna oil was below 10% w/v, the yield of MAG was decreased; and also when the concentration above 10%, the MAG yield was decreased too. Similar result was obtained by Kaewthong and H-Kittikun (2004). At the concentration of 10% (w/v) palm olein in acetone/isooctane mixture (3:1, v/v) gave 43.68% of MAG

yield. When the concentration of palm olein was lower than 10% (w/v), the yield of MAG was slightly decreased. It may be due to two reasons: (i) the solvent inhibits the enzyme activity at the low substrate concentration, and (ii) addition of solvent decreases the amount of available substrate at the interface between the solvent and glycerol and hence decreases the MAG yield. Figure 6 shows effect of palm olein concentration on MAG production by IM-PS.

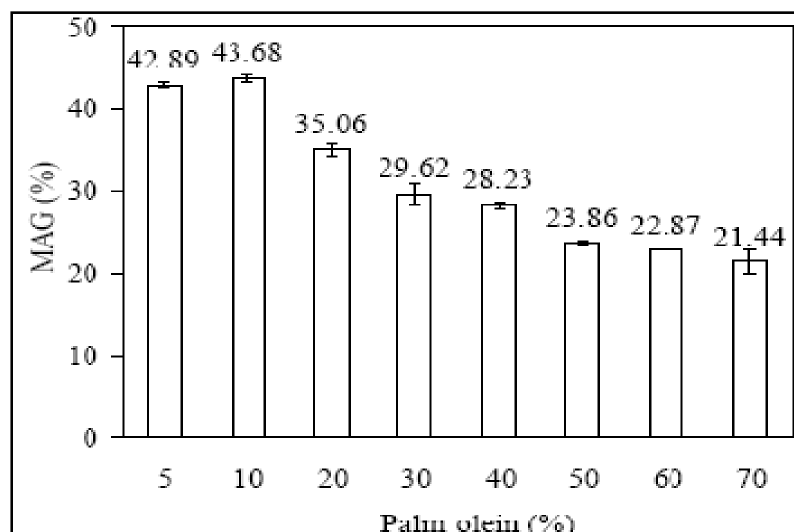


Figure 6. Effect of palm olein concentration on MAG production by IM-PS

Source: Kaewthong and H-Kittikun (2004)

Yang *et al.* (2005b) said that the solvent medium in one way can help improve the system homogeneity and stability as well as reduce the viscosity and mass transfer limitation. On the other hand, the medium will reduce the concentration of substrates thus reduce reaction rates. Yang *et al.* (2005) evaluated this effect. They used 25, 30, 40 and 50% w/v of sunflower oil in *tert*-butanol. Forty percent of oil concentration was the best concentration that gave higher MAG yield; meanwhile, 25%, 30% and 50% had little difference and gave lower MAG yields.

5. Separation of product

5.1 Chromatography for carotenoids separation

Carotenoids could be separated by several methods. Chromatography is the common method to separate and to identify the carotenoids from its matrices such as vegetable oils and other plant tissues. Since in the sample typically contain both non-polar carotenes and the more polar xanthophylls, this separation should be able to cope with this polarity range. The kinds of chromatography for carotenoids isolation and analysis include thin-layer chromatography (TLC), open column chromatography (OCC) and high–performance liquid chromatography (HPLC). For identification and structure elucidation, visible spectrophotometry, NMR and mass spectrometry are the most recommend (Ren and Zhan, 2008; Rodriguez-Amaya and Kimura, 2004). OCC has the advantage of using common laboratory equipment (recording UV-Visible equipment) and does not require a constant supply of carotenoid standards since separated fractions are directly quantified spectrophotometrically by using published coefficient absorption.

At present, most of the carotenoids in the edible oil are destroyed and discarded in the refining process. Carotenoids extraction by adsorption without a chemical conversion of palm oil has been reported, but was not commercially available. Moreover, the current study of carotenoid recovery is desirable to develop a separation technique for carotene extraction from crude palm oil (CPO) that maintains an edible-oil quality. Baharin *et al.* (1998) used Diaion HP-20 resin to adsorb carotene from crude palm oil. Diaion HP 20 is a synthetic highly porous resin made from a styrene-divinyl benzene copolymer. This adsorbent has a broad nonpolar and nonionic surface on many small cavities, with a specific surface are of 511 m^2/g.

Other column supports are alumina and silica used for normal phase chromatography. Separation on these stationary phases depends upon polarity. Separation on magnesium oxide (MgO, also known as magnesia) depends upon the number and arrangement of double bonds in the molecule; in this case, the most strongly adsorbed carotenoids are those with the most extensive conjugated polyene system. Besides, acyclic carotenoids are more strongly adsorbed than cyclic ones with

the same number of double bonds. Basic adsorbents such as $Ca(OH)_2$ and $ZnCO_3$, are useful for the separation of geometrical isomers (Melendez-Martinez *et al*., 2007).

Several researchs show good result in separation of carotenoid by normal-phase chromatography. Ren and Zhang (2008) were succeeded to characterize the carotenoids composition of *Potamogeton crispus* L. (a submersed herbaceous perennial plant) by an open column chromatography of Magnesium oxide-Diatomaceous Earth. This is a normal phase chromatography with three mobile phase system that are petroleum ether/acetone (96:4, v/v), petroleum ether/acetone/methanol/ (85:15:1, v/v/v) and petroleum ether/acetone/methanol/ (85:15:2, v/v/v). By several identification and further spectrophotometry and HPLC analysis, it was found that carotenoids compositions from this plant are neoxanthin, violaxanthin, lutein and β-carotene.

Kimura and Rodriguez-Amaya (2002) reported scheme for obtaining standards of carotenoids by normal-phase OCC. The purpose of their work was to establish a procedure for isolating carotenoid standards by OCC and to use these standards to determine the carotenoid composition by HPLC since the purity of some commercial carotenoids is not sufficient for a chromatographic standard. They found 90-97% purity for β-carotene.

The carotenoid composition of pigmented yeasts isolated in Brazil was studied by Maldonade *et al*. (2008). Open column, thin layer and high performance liquid chromatography were used to separate, identify and quantify the carotenoids. Chromatography on a MgO:Hyflosupercel (1:2) open column was carried out with the following mobile phases: 1%, 2%, 5% ethyl acetate in petroleum ether; 2%, 5%, 8%, 10% to 100% of acetone in petroleum ether; and 2%, 5%, 10% to 50% water in acetone. The major pigments found in these yeasts were torulene and β-carotene.

Generally speaking, the factors that affected the separation and recovery of carotenoid are amount of sample loading, types of stationary and mobile phase, temperature and retention of sample in the column. Baharin *et al.* (1998) suggested that carotene recovery depends mainly on two factors: (i) competitive adsorption between the oil and carotene on the resin surface, and (ii) the adsorption capacity of the resin for carotene in the presence of the solvent.

Baharin *et al.* (1998) varied the amount of crude palm oil (CPO) loading on HP-20 column. CPO loading was examined from 10 to 60 g. The higher carotene recovery (85%) obtained at 10 g CPO loading and dropped rapidly with 60 g load to 22%. On the other hand, the high oil recovery (89-96%) did not depend upon CPO loading. Clearly, carotene recovery was decreasing with increasing CPO loading. Table 8 shows the result from their research about the effect of sample loading. Wan *et al.* (2008) were investigated separation of another antioxidant (tocopherols) from soybean distillate by low-pressure glass column packed with silica gel. They also study about effect of sample loading to the purity and recovery of tocopherols in their system. The separation efficiency was improved when the loading amount increased from 1 to 2 mL. Nevertheless, it dropped when the loading amount was more than 2 mL, which was caused by overload resulting in an overlap of neighboring peaks and a decrease in separation efficiency.

Table 6. Effect of CPO loading on the recovery of palm carotene

			Carotene	
CPO loading (g)	Fraction	Oil recovery (%)	Recovery (%)	Concentration (ppm)
10	Isopropanol	88.9	14.8	144
	Hexane	11.1	85.2	6,645
20	Isopropanol	96.3	35.4	249
	Hexane	3.7	64.6	11,983
30	Isopropanol	94.7	42.2	310
	Hexane	5.3	57.8	7,630
40	Isopropanol	96.3	65.2	401
	Hexane	3.7	34.8	5,529
50	Isopropanol	92.3	73.5	447
	Hexane	7.7	26.5	1,919
60	Isopropanol	93.9	78.2	503
	Hexane	6.1	21.8	2,154

Source: Baharin *et al.* (1998)

Types and of stationary phase influenced the mobile phase used. Normal-phase chromatography choose non-polar solvent as initial solvent, meanwhile reversed-phase chromatography choose more polar solvent as initial solvent. Isopropanol and hexane was used by You *et al.* (2002) to recover of carotene from hydrolyzed palm oil. They used reversed-phase chromatography. Meanwhile, Baharin *et al.* (1998) studied the effect of isopropanol and ethanol as first solvent before

hexane for carotene recovery. They found that carotene recovery on isopropanol is higher than on ethanol. Table 9 shows the carotene recovery on the isopropanol and ethanol.

Table 7. Carotene recovery on the isopropanol-hexane and ethanol-hexane system

Fraction	Oil quantity (g)	Carotene		
		Content (mg)	Recovery (%)	Conc. (ppm)
Isopropanol	30.0	12.03	60.6	410
Hexane	0.2	7.83	39.4	39,150
Isopropanol	28.2	9.19	47.3	326
Hexane	1.6	10.24	52.7	6,400

Source: Baharin *et al*. (1998)

5.2 Separation of monoacylglycerol from other products

The separation of monoacylglycerol (MAG) from other glycerolysis products could be done by silica gel column chromatography. The principle of the separation is based on hydrophilic and hydrophobic interaction among products, stationary phase and mobile phase. In normal-phase chromatography, stationary phase is hydrophilic, initial solvents are hydrophobic, thus TAG, FFA, and carotenoids will elute first. Then, increasing the polarity of solvents will elute the less non-polar components (DAG and MAG).

Several eluent systems have been employed in this job. Two or more solvent system was used. Kaewthong (2004) used hexane and 5% ethanol in the system. Most of the triacylglycerol (TAG) and free fatty acid (FFA) were eluted with hexane. Diacylglycerols (DAG) were eluted with 5% ethanol in hexane at early fractions and then MAG was eluted. Meanwhile, Watanabe *et al.* (2004) used n-hexane and ethyl acetate mixture to separate FFA, DAG and MAG from esterification reaction. First, DAG and FFA were eluted with n-hexane/ethyl acetate (80:20, v/v), then MAG was eluted with n-hexane/ethyl acetate (50:50, v/v).

Crystallization is also employed in separation of MAG. The principle of the method is 1-MAG nature has higher melting point than TAG (i.e. 1-MAG solidifies easier than TAGs). Therefore, by lowering the reaction temperature at less than 10°C, the formed MAG precipitates, and the MAG could be removed from the product.

5.3 Identification and quantification of carotenoids

The chromatographic behavior and the Ultra Violet/Visible (UV/Vis) absorption spectrum provide clues for the identification of carotenoids. Both the wavelengths of maximum absorption (λ_{max}) and the shape of the spectrum (spectral final structure) are the characteristic of the chromophore. Besides that, carotenoids in solution obey the Lambert-Beer law, that is, their absorbance is directly proportional to the concentration. Therefore, carotenoids are identified and quantified spectrophotometrically. The absorption or extinction coefficient ($A^{1\%}_{1cm}$) of a carotenoid (absorbance at a given wavelength of a 1% solution in 1 cm light-path spectrophotometer cuvette), is used in the calculation of the concentration (Rodriguez-Amaya and Kimura, 2004). Table 10 lists the λ_{max} and $A^{1\%}_{1cm}$ of the carotenoids commonly found in foods.

Table 8. Absorption data for common food carotenoids

Carotenoids	Solvent	λ_{max} (nm)			% III/II	$A^{1\%}_{1cm}$
α-carotene	Hexane	442	445	473	55	2710 $_{445}$
β-carotene	Ethanol	(425)	450	478	25	2620 $_{450}$
	Hexane	(425)	446	477	25	2450 $_{446}$
α-cryptoxanthin/Zeinoxanthin	Hexane	421	445	475	60	2636 $_{445}$
Lutein	Ethanol	422	445	474	60	2550 $_{445}$
Lycopene	Petroleum ether	444	470	502	65	3450 $_{470}$
Zeaxanthin	Acetone	(430)	452	479		2340 $_{452}$
	Petroleum ether	(424)	449	476	25	2348 $_{449}$

Parentheses indicate a shoulder.
%III/II is the ratio of the height of the longest-wavelength absorption peak, designated III, to that of the middle absorption peak, designated II, taking the minimum between the two peaks as the baseline, multiplied by 100.
$A^{1\%}_{1cm}$ values are taken from middle absorption peak.
Source: Rodriguez-Amaya and Kimura (2004)

Objectives

1) To recover carotenoids and produce monoacylglycerols from crude palm oil by glycerolysis reaction followed by column chromatography.

2) To optimize the condition for MAG production by glycerolysis reaction using immobilized lipase and carotenoids recovery by column chromatography.

Scope of this study

This study covered the recovery of carotenoids from crude palm oil by glycerolysis reaction following by column chromatography. The reaction conditions including types of solvent, amount of immobilized enzyme, molar ratio of glycerol to crude palm oil and crude palm oil concentration in solvent were optimized to obtain highest monoacylglycerol production with minimum reaction time. Subsequently, carotenoids recovery was performed by column chromatography. The types of stationary phases, solvent types and amount of sample loading were determined.

CHAPTER 2

MATERIALS AND METHODS

Materials

1. Materials

Lipase PS (*Pseudomonas* sp.) was a gift from Amano Pharmaceutical Co. Ltd., Nagoya, Japan. Microporous polypropylene powder, Accurel EP-100 (particle size <200 μm) was a gift from Akzo Nobel (Obernburg, Germany). The crude palm oil was obtained from palm oil mill near Hat Yai, Thailand. Chromatography grade silica gel was obtained from Wako Chemicals (Osaka, Japan). Synthetic highly porous resin Diaion HP-20 (a styrene-divinyl benzene copolymer) was obtained from Mitsubishi Chemical Company (Tokyo, Japan).

2. Chemicals

Company	Chemicals
Carlo Erba Reagenti Co. Ltd.,	Glycerol
	Sodium dihydrogen phosphate
	Sodium hydrogen diphosphate
Fluka Chemie GmbH, Germany	Cupric acetate
	Pyridine
	Potassium hydroxide
Labscan Asia Co. Ltd., Thailand	Acetone
	Benzene
	Chloroform
	Ethanol
	Hexane
	Isooctane
	Methanol
	Tert-butanol
Labscan Asia Co. Ltd., Thailand	Phosphoric acid
Merck KgaA, Germany	Acetid Acid
	Phenolphtalein

3. Instrument

Instrument	Model	Company
Balance	BP210S	Sartorius AG, Germany
Balance	BP2100S	Sartorius AG, Germany
Centrifuge	MPW-52	Arlab, Poland
Evaporator	SB-651	Tokyo Rikakikai Co. Ltd. Japan
Fraction Collector	2110	Bio-Rad, U.S.A
Hot Plate Stirrer	HS-115	HL Instrument, Thailand
IATROSCAN	MK5	Iatron Laboratories Inc., Japan
Oven	ED 115	Binder GmbH, Germany
Peristaltic Pump	BT00-300T	Boading Longer Precision Pump, Co. Ltd., China
pH meter	420A	Orion Research Inc, USA
Sonicator	Transsonic 460/H	Elma, Germany
Spectrophotometer	Libra S22	Biochrom, UK
Suction pump	A-3S	Tokyo Rikakikai Co. Ltd., Japan
Thermomixer	Comfort	Eppendorf AG, Germany
Vortex mixer	FINEVORTEX	FinePCR, South Korea
Water bath	WB-14	Memmert GmbH, Germany

Methods

A. Analytical methods

1. Measuring hydrolytic activity of free and immobilized lipase

Activity was assayed by modified cupric acetate method (Lee and Rhee, 1993). Cupric acetate solution (5% w/v) was prepared and pH was adjusted to 6.1 by pyridine. For the lipase reaction in two-phase system, 0.1 mL of enzyme solution or 1.0 mg of immobilized enzyme, 0.2 mL of 0.1 M phosphate buffer pH 7 and 0.3 mL of 10% palm oil in iso-octane were mixed and incubated at 1200 rpm at 45°C for 15 min by using thermomixer. The enzyme reaction was stopped by adding 0.06 mL of 6 M HCl.

The upper iso-octane layer of 0.25 mL was taken out and mixed with 0.1 mL cupric acetate solution. Free fatty acid dissolved in iso-octane was determined by measuring the absorbance at 715 nm against the control which contains no free fatty acid. The lipase activity was determined by measuring the amount of fatty acid from the standard curves of palmitic acid. One unit of enzyme activity was defined as the enzyme necessary to release 1 μmol of palmitic acid per minute at the specific conditions. For immobilized yield was calculated using the following formula (Kaewthong *et al.,* 2005):

$$\text{Immobilized yield} = \frac{\text{total immobilized activity (U)}}{\text{total initial soluble enzyme activity (U)}} \times 100$$

2. Analysis of product after glycerolysis

About 1 g of the reaction mixture was separated into oil and glycerol layers by centrifugation (5000 rpm, 5 min). The contents of TAG, DAG, MAG and FFA in the oil layer were measured by thin layer chromatography with flame ionization detector (TLC/FID) (IATROSCAN MK5, Iatron Laboratories, Inc. Tokyo, Japan). The sample (1 μL) was spotted onto the Chromarod SIII quartz rods and developed for 35 min in a mixture of benzene/chloroform/acetic acid (50:20:0.7) as developing solvent. After development and drying, the rods were subjected to scanning with FID. Standards were used to identify the peaks. The peaks areas were normalized and used for evaluation of the reactions (Pawongrat *et al.,* 2007).

3. Carotenoids content

Analysis of the total carotenoids content is determined spectrophotometrically as described by Wei *et al.* (2005). The sample was homogenized and weighed to the nearest ±0.0001 g into a 25 mL volumetric flask. The sample was dissolved with *n*-hexane and diluted to the mark. The solution was transferred into a 1 cm quartz cuvette and the absorbance was measured at 450 nm against *n*-hexane using UV-Visible spectrophotometer. The absorbance of the sample then was calculated by equation from standard series calibration curve using β-carotene as standard compound.

4. Analysis of variance (ANOVA)

All the experiments were conducted in three replicates. Appropriate tests of significance, analysis of variance and confidence of difference at the 5% level were used in the evaluation of data.

B. Experiment

1. Crude palm oil pretreatment by degumming process

The degumming processed was described by You *et al*. (2001). In this process, 0.4 g of phosphoric acid was added to 200 g of melted crude palm oil, maintained at 60°C and mixed for 30 min. After that the mixture was filtered by using filter paper No. 1. This degummed crude palm oil (CPO) was used as the substrate for further study.

2. Immobilization of lipase enzyme

The Accurel EP100 in powdered form (0.5 g) was added to 5.0 mL *Pseudomonas* sp. lipase solution containing approximately 100 U/mL enzymes activity and stirred with a magnetic bar at 100 rpm for 30 min at room temperature. Afterwards, 5.0 mL of 0.1 M phosphate buffer pH 7.0 was added and the suspension was filtered through a Buchner funnel. The immobilized enzyme was washed on filter paper with another 5.0 mL of buffer and dried in vacuum desiccator for 8 h.

3. Glycerolysis reaction

The reaction was carried out in a batch system. Molar ratio glycerol to crude palm oil was 8:1. Composition of reactant containing 0.1 gram crude palm oil in 1 mL of acetone/iso-octane (3:1, v/v), 0.05 g immobilized enzyme (IM-PS) (50% w/w of CPO) and 0.083 g of glycerol (water content 4%, w/w). The temperature was controlled at 45°C. The reaction was mixed by thermomixer at 300 rpm for 24 h. Samples of the reaction mixture was centrifuged to remove IM-PS before analysis

Kaewthong and H-Kittikun, 2004). All of the apparatuses were covered by aluminum foil to protect carotenoids from light and the reaction was performed in the dark room.

3.1 The effect of solvent

To select the most suitable solvent for glycerolysis reaction systems, the effect of organic solvents on the catalytic activity of lipase for glycerolysis was examined. Reactant composition and condition were similar except the solvents were different. Acetone (log P = -0.24), isooctane (log P = 5.83), tert-butanol (log P =0.35) and hexane (log P = 3.90) were employed in this experiment. The solvent combinations were: acetone, acetone/isooctane (1:1 v/v), acetone/isooctane (3:1 v/v), tert-butanol/hexane (1:1 v/v), tert-butanol/hexane (3:1, v/v), isooctane, tert-butanol and hexane. The highest yield MAG result from particular solvent was used in the next step of experiment (Kaewthong and H-Kittikun, 2004).

3.2 The effect of immobilized lipase amount

The effect of immobilized lipase amount was also conducted. The amounts of lipase in the reaction mixture were 10, 20, 30, 40, 50 and 60% (w/w of CPO). The highest yield MAG result from particular lipase amount was used in the next step of experiment.

3.3 The effect of molar ratio of glycerol to CPO

The effect of molar ratio of glycerol to CPO was conducted by varying molar ratio of the glycerol. The ratio of glycerol to CPO were: 3:1, 4:1, 5:1, 6:1, 7:1, 8:1 and 9:1. The highest yield MAG result from particular molar ratio was used in the next step of experiment.

3.4 The effect of CPO concentration

The effect of CPO concentration was conducted by varying CPO concentration in the solvent. The concentrations were: 5, 10, 20, 30, 40, 50 and 60% (w/v of solvent). The highest yield MAG result from particular CPO concentration will be used in the next step of experiment.

4. Study of carotenoids recovery by column chromatography

4.1 Study of normal phase chromatography

The reaction product was separated by centrifugation (5000 rpm, 5 min). The oily part (1 g) was diluted with 0.5 mL hexane and was applied to silica gel

60 on column (11.5 cm length, 1 cm I.D.) for normal phase chromatography. The solvents for silica column were petroleum ether, 50% hexane in petroleum ether (v/v), 15% diethyl ether in petroleum ether (v/v) and 30% ethanol in petroleum ether (v/v). Elution was done at flow rate 0.5 mL/min; each fraction was contained 1.5 mL and collected in an Eppendorf tube. The number of fractions was 20 fractions. After that, carotenoids concentration was determined spectrophotometrically. Carotenoids content was determined in each fraction. Carotenoids recovery was determined by dividing the carotenoids content in each fraction by total carotenoids content in all fractions.

Oil content was determined by gravimetrically after removal the solvent in the fume hood for one night, then drying in the oven at 60°C for 3 h (You *et al.*, 2002). The tube was cooled-down in a desiccator prior weighing. Repeating the weighing of the tube was done in the next day in order to get constant weight. Chromatogram of this separation will be plotted and analyzed. All the apparatus was wrapped by aluminum foil and done in the dark room to prevent carotenoids degradation.

4.2 Study of reverse phase chromatography

The reaction product was separated by centrifugation (5000 rpm, 5 min). The oily part (1 g) was diluted with 0.5 mL ethanol and was applied to Diaion HP-20 on column (11.5 cm length, 1 cm I.D.) for reverse phase chromatography. The solvents for Diaion HP20 column were ethanol and hexane. The elution and the rest procedure were same to 4.1 procedures. The highest carotenoids recovery on particular system was used for the next experiment.

4.3 The effect of temperature on carotenoids recovery by column chromatography

The effect of temperature was conducted to the best system from previous experiment. The temperatures used were: 40, 50 and 60°C. The highest carotenoids recovery and concentration was used for the next experiment.

4.4 The effect of sample loading on carotenoids recovery by column chromatography

The effect of sample loading was also conducted by varying sample loading at 0.5, 1.0, 2.0, 3.0 and 4.0 gram of sample.

CHAPTER 3

RESULTS AND DISCUSSION

1. Monoacylglycerol production from crude palm oil by glycerolysis reaction in organic solvents using immobilized lipase

1.1 Initial glycerolysis condition

Many reports studied about enzymatic glycerolysis reaction to produce monoacylglycerol (MAG) (Damstrup *et al*., 2005; Kaewthong and H-Kittikun, 2004). In this study, since crude palm oil (CPO) is abundant raw material in the southern Thailand, the production of MAG from CPO was attempted. Moreover, converting CPO to MAG would be benefit for carotenoids recovery. To study the glycerolysis reaction of CPO by immobilized enzyme (IM-PS, 1.34 ± 0.38 U/mg), the reaction mixture contained 0.1 g CPO in 1 mL of acetone/iso-octane (3:1, v/v), 50% IM-PS (w/v) based on solvent volume and glycerol at 8:1 molar ratio to CPO with 4% (w/w) water content in glycerol. The temperature was controlled at 45°C. The mixture was mixed by thermomixer at 300 rpm for 24 h. The result of this experiment is shown in Table 9.

Table 9. Product compositions from glycerolysis reactions of CPO at 24 h.

Oil Component	Initial Composition (%)	Product Composition (%)
Triacylglycerol (TAG)	93.13 ± 2.32	29.91 ± 1.47
Free fatty acid (FFA)	2.05 ± 1.32	5.41 ± 0.26
Diacylglycerol (DAG)	2.03 ± 0.48	9.26 ± 0.44
Monoacylglycerol (MAG)	2.79 ± 1.26	55.42 ± 2.41

Each value represents mean ± standard deviation
The reaction mixture contained 1 mL of 10% (w/v) CPO in organic solvent, glycerol with 8:1 molar ratio to CPO and 4% (w/w) water content in glycerol. The amount of IM-PS was 50% (w/v) based on solvent volume. The temperature was 45°C, 300 rpm of shaking speed and 24 h of reaction time.

Table 9 clearly shows a change in the oil composition after the reaction. The triacylglycerol (TAG) was reduced about 63% and monoacylglycerol

(MAG) was increased to 55%; meanwhile free fatty acid (FFA) and diacylglycerol (DAG) were also increased. The increase of FFA was due to presence of water in the reaction system. The water is essential in maintaining enzyme structure and stability. In the glycerolysis reaction for MAG production by IM-PS using palm olein as substrate, 55.75% of MAG was obtained with 11.75% remained TAG (Kaewthong and H-Kittikun, 2004). Elfman-Borjesson and Harrod (1999) reported synthesis of MAG by glycerolysis of rapeseed oil in isooctane using Lipozyme IM. The MAG yield of 28% was obtained at 75°C after 20 h incubation. Meanwhile, Damstrup *et al.* (2005) achieved about 60 – 80% of MAG yield from glycerolysis reaction of sunflower oil using lipase Novozym 435 and the mixture of tert-butanol/hexane, tert-pentanol/hexane and tert-butanol/tert-pentanol as solvent system. Therefore, two phase glycerol-oil system needs solvent to overcome poor solubility of glycerol and improve mass transfer. In this study, to increase the MAG yield, solvent optimization was attempted.

1.2 Effect of organic solvents

The effect of organic solvents on MAG production from CPO was examined to select the most suitable solvent for glycerolysis reaction by immobilized lipase. A suitable solvent system to improve the miscibility of substrates will result in a more homogeneous system and enhance the conversion of substrate, reaction rate and the product distribution in favor of MAG formation. The reaction mixture contained 1 mL of 10% (w/v) CPO in organic solvent, glycerol with 8:1 molar ratio to CPO and 4% (w/w) water content in glycerol. The amount of IM-PS was 50% (w/v) based on solvent volume. The organic solvents were acetone, iso-octane, tert-butanol, hexane and combination of these solvents for 24 h reaction time. These solvents have different octanol-water partition coefficient (log P), a parameter to characterize solvent properties in relation to lipids and lipases. The results of glycerolysis reaction of CPO in the solvent system are shown in Figure 7. It was found that tert-butanol gave higher yield of MAG than acetone, hexane, and iso-octane. The nonpolar medium (isooctane and hexane) gave the lowest MAG yield (6.62 and 10.08%, respectively). This might be a coarser and more unstable emulsion of glycerol and CPO in the nonpolar medium, and hence diminish substrates access to the active site

of the enzyme. It has been reported that the tertiary alcohol could improve MAG formation dramatically (Damstrup *et al.,* 2005). The reason might be because tert-butanol have moderate log P value (0.35) compared to hydrophobic solvent (isooctane log P=5.83 and hexane log P=3.90) and hydrophilic solvent (acetone log P=-0.24) (Table 10). Furthermore, the combination of 1:1 (v/v) tert-butanol/hexane gave the highest yield of MAG (77.86%) at 24 h and it was not significance different to that 3:1 (v/v) from tert-butanol/hexane. The 1:1 (v/v) tert-butanol/hexane was chosen because it was cheaper than 3:1 (v/v) tert-butanol/hexane (Table 10). Tert-butanol/hexane (1:1, v/v) have both hydrophilic and hydrophobic characteristics, with predominantly hydrophobic characteristic (log P = 1.82). This phenomenon was accordance with the expectation that a solvent should have both water-like and octanol-like properties in order to be a suitable solvent for both oil and glycerol (Damstrup *et al.,* 2005). However, the significant variations in MAG content between the tert-butanol/hexane system and acetone/isooctane system cannot be explained by polarity variations (Figure 8). It was reported that functional groups played more important role than the polarity in the solvent properties. Tert-butanol/hexane system has tertiary alcohol groups which probably induced the accessibility of the substrate to the active site of the enzyme; meanwhile, acetone/isooctane has not. Damstrup *et al.* (2005) used binary mixture of tert-butanol:tert-pentanol 8:2 v/v as organic medium for continuous glycerolysis and achieved high MAG yields of 47 – 56%. Yang *et al.* (2005b) studied the enzymatic production of MAG in tert-butanol at 40^oC. They obtained MAG yield of 60 – 70% (w/w) in the lipid phase in a stirred tank after 2 h of reaction time. MAG yield up to 70% (w/w) was also obtained in a packed bed reactor with a residence time of only 30 – 40 min.

Table 10. MAG content after a glycerolysis reaction in several solvents

Solvent/solvent mixture	MAG content	Log P value	Solvent Price Index
Acetone	21.20 ± 3.67	-0.24	0.73
Hexane	10.08 ± 0.56	3.90	1.00
Iso-octane	6.62 ± 1.39	5.83	2.99
Tert-butanol	57.31 ± 2.25	0.35	2.49
Acetone/Isooctane, 1:1 (v/v)	32.20 ± 9.85	1.64[d]	2.24
Acetone/Isooctane, 3:1 (v/v)	35.71 ± 8.18	0.55[d]	1.30
Tert-butanol/Hexane, 1:1 (v/v)	77.86 ± 3.66	1.82[d]	1.75
Tert-butanol/Hexane, 3:1 (v/v)	76.68 ± 3.38	1.06[d]	2.12

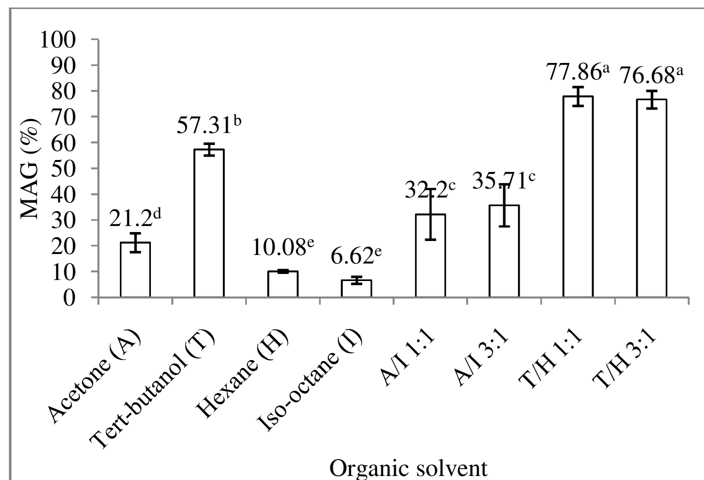


Figure 7. Effect of organic solvents on MAG production from CPO by IM-PS. The reaction mixture contained 1 mL of 10% (w/v) CPO in organic solvent, glycerol with 8:1 molar ratio to CPO and 4% (w/w) water content in glycerol. The amount of IM-PS was 50% (w/v) based on solvent volume. The temperature was 45°C, 300 rpm of shaking speed and 24 h of reaction time. The values with different superscript are significantly different ($p<0.05$).

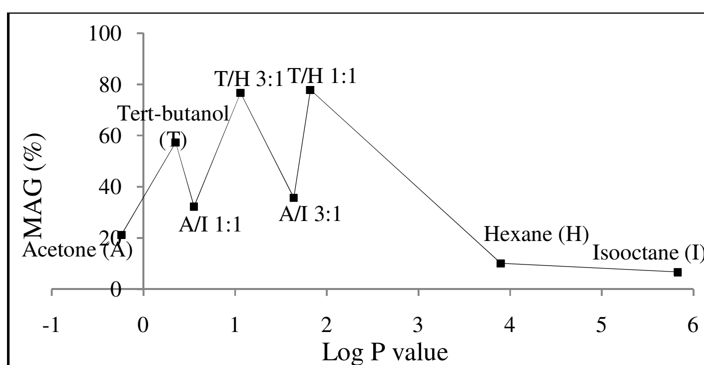


Figure 8. Correlation between the log P value and MAG content. The reaction mixture contained 1 mL of 10% (w/v) CPO in organic solvent, glycerol with 8:1 molar ratio to CPO and 4% (w/w) water content in glycerol. The amount of IM-PS was 50% (w/v) based on solvent volume. The temperature was 45°C, 300 rpm of shaking speed and 24 h of reaction time. Log P value, octanol-water partition coefficient. Higher value of log P means higher hydrophobicity.

1.3 Effect of immobilized lipase amount

The effect of IM-PS amount on MAG production from CPO was investigated to determine the optimal amount. The MAG production was performed with various IM-PS amounts while keeping other parameters constant. The amount of IM-PS was varied at 10, 20, 30, 40, 50 and 60% (w/v) based on solvent volume. The

yield and initial rate of MAG production are shown in Figure 9. The initial rate was defined as the slope of the MAG yield versus time in the initial 6 h of reaction. With increasing the amount of IM-PS in the reaction mixture the MAG yield was also increased. The increased initial MAG production rate was also correlated to the increasing of the amount of IM-PS. This happened due to the increase in concentration of catalyst increases the collision frequency with reactants. However, there was no significant benefit after increasing IM-PS above 40% (w/v) based on solvent volume since the MAG yield and initial production rate was not significantly different at 40, 50 and 60% (w/v) of IM-PS loading. Therefore, the amount of IM-PS at 40% (w/v) based on solvent volume was considered as the most suitable for MAG production from economic point of view. Time course of glycerolysis of CPO at 40% IM-PS is shown in Figure 10. MAG yield was constantly produced at the initial 3 h until reach its maximum at 24 h; meanwhile, TAG decreased very fast at initial 6 h of reaction and slowly declined until 24 h of reaction. FFA and DAG increased at the first 6 h and FFA relatively remained constant while DAG reduced. Kaewthong and H-Kittikun (2004) conducted the glycerolysis of palm olein using immobilized lipase PS in the mixture solvent of acetone/isooctane (3:1, v/v). They found that 50% of immobilized lipase based on palm oil weight was the optimal amount of catalyst for production of MAG from palm olein because more than this amount, there was no significant increase of MAG yield. Yang *et al*. (2005b) studied the effect of Novozym 435 load in the glycerolysis of sunflower oil. They found that the enzyme load more than 10% resulted in small increase in MAG yield; therefore, they suggested that 10 – 15% of enzyme load was enough for the maximum reaction performance. Cheirsilp *et al*. (2009) determined the optimal amount of alginate immobilized lipase from *Pseudomonas* sp. for glycerolysis reaction of palm oil in 2-methyl-2-butanol solvent. They found that increasing the amount of the immobilized lipase the MAG yield was decreased. This might be due to a faster hydrolysis reaction occurring with a higher immobilized lipase loading. However, the initial rate of MAG production did increase with the increasing amounts of immobilized lipase. Thus, immobilized lipase loading at 27 U was chosen for high yield and initial rate of MAG production.

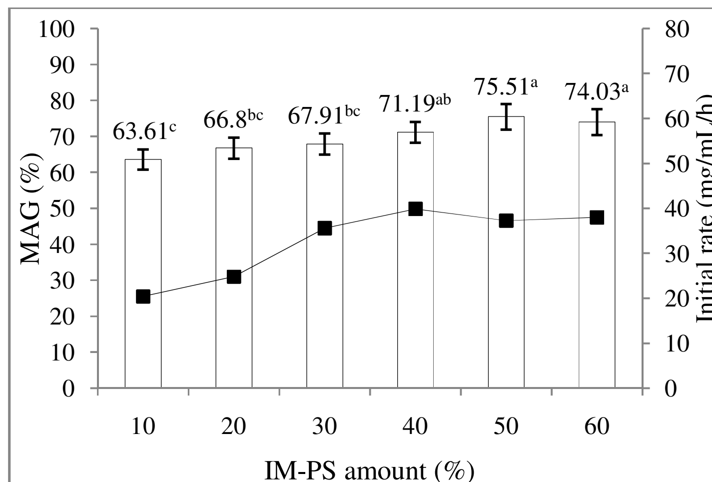


Figure 9. Effect of IM-PS amount on MAG production (column) and initial rate (line) from CPO. The reaction mixture contained 1 mL of 10% (w/v) CPO in tert-butanol/hexane 1:1 (v/v), glycerol with 8:1 molar ratio to CPO and 4% (w/w) water content in glycerol. The amount of IM-PS was 10 – 60% (w/v) based on solvent volume. The temperature was 45°C, 300 rpm of shaking speed and 24 h of reaction time. The values with different superscript are significantly different ($p<0.05$).

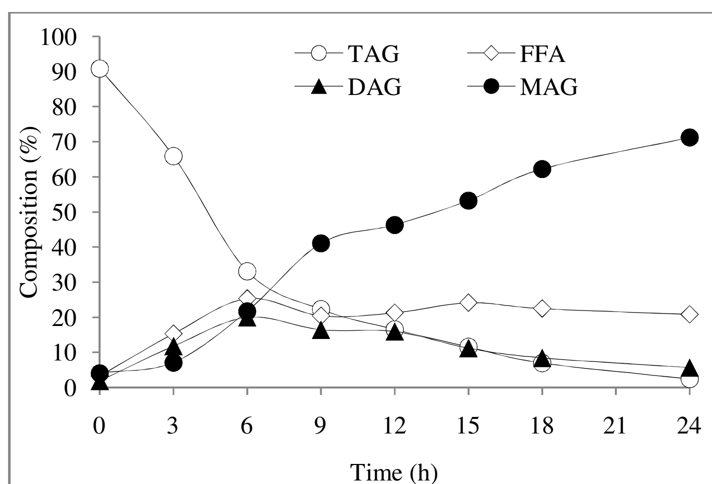


Figure 10. Time course of glycerolysis of CPO in the tert-butanol/hexane 1:1 (v/v) mixture in the optimum conditions: 1 mL of 10% (w/v) CPO in tert-butanol/hexane 1:1 (v/v), glycerol with 8:1 molar ratio to CPO, 4% (w/w) water content in glycerol and the amount of IM-PS was 40% (w/v) based on solvent volume. The temperature was 45°C, 300 rpm of shaking speed and 24 h of reaction time.

1.4 Effect of molar ratio of glycerol to CPO

The effect of molar ratio of glycerol to CPO was determined. Theoretically, 3 mol of MAG would be obtained when 1 mol TAG and 2 mol glycerol

were used as substrates for glycerolysis reaction. However, with increasing the molar ratio of glycerol to CPO, the MAG product was also increased (Figure 11). The initial rate was defined as the slope of the MAG yield versus time in the initial 6 h of reaction. It was found that higher initial concentrations of glycerol lead to a faster initial rate of MAG production. This happened because the increase of glycerol amount could increase the theoretical equilibrium value and the velocity of the reaction. When the molar ratio of glycerol to CPO was increased to 8:1, the highest yield of MAG was obtained. Even though the initial rate of MAG production was achieved maximum level at 9:1 molar ratio of glycerol to CPO, but the MAG yield was decreased. This might be due to the high glycerol concentration having a lower miscibility with CPO. An optimal ratio must exist considering both effects of glycerol on the reaction equilibrium and the system homogeneity. Therefore, the reaction having molar ratio of glycerol to CPO at 8:1 was chosen for further studies. Time course of glycerolysis of CPO at 8:1 molar ratio of glycerol to CPO is shown in Figure 12. MAG yield was constantly produced at the initial 3 h until reach its maximum at 24 h; meanwhile, TAG decreased very fast at initial 6 h of reaction and continue declined until 24 h of reaction. FFA and DAG increased at the first 6 h and relatively remained constant. Elfman-Borjesson and Harrod (1999) conducted the synthesis of MAG by glycerolysis of rapeseed oil using Lipozyme IM. They reported that the ratio of glycerol to triglyceride had a significant effect on the yield of MAG. They found that 17.4% of MAG was achieved when glycerol/rapeseed oil molar ratio of 6:1 was used. In the study of Kwon *et* al. (1995), a yield of 60% monopalmitoylglycerol was obtained when the molar ratio glycerol to palmitate was 10:5 in lipase catalyzed-esterification reaction. This yield was greatly influenced by the reaction temperature and molar ratio of substrate. Pawongrat *et al*. (2007) reported that the mole ratio of glycerol/tuna oil of 3:1 gave 24.6% MAG, meanwhile Kaewthong and H-Kittikun (2004) reported the optimal mole ratio glycerol/palm oil at 8:1 and this ratio gave 56% of MAG yield. Cheirsilp *et al*. (2009) studied the effect of molar ratio of glycerol to palm oil on the MAG production by alginate immobilized lipase. They found that the molar ratio of glycerol to palm oil at 10:1 gave the highest yield and initial rate of MAG production. Yang *et al*. (2005) also studied the effect of molar substrates ratio between glycerol and sunflower oil in the glycerolysis reaction

at 2.5:1, 3.5:1, 4.5:1 and 5.5:1. They found that there was no significant difference of MAG yield from 4.5:1 and 5.5:1 of molar ratio glycerol to oil. Therefore, they chose 4.5:1 molar ratio glycerol to oil.

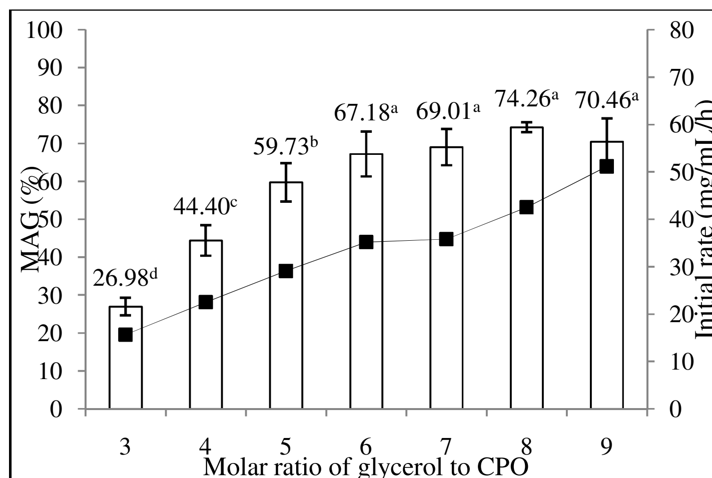


Figure 11. Effect of molar ratio of glycerol to CPO on MAG product (column) and initial rate (line). The reaction mixture contained 1 mL of 10% (w/v) CPO in tert-butanol/hexane 1:1 (v/v), glycerol with 3:1 to 9:1 molar ratio to CPO and 4% (w/w) water content in glycerol. The amount of IM-PS was 40% (w/v) based on solvent volume. The temperature was 45°C, 300 rpm of shaking speed and 24 h of reaction time. The values with different superscript are significantly different ($p<0.05$).

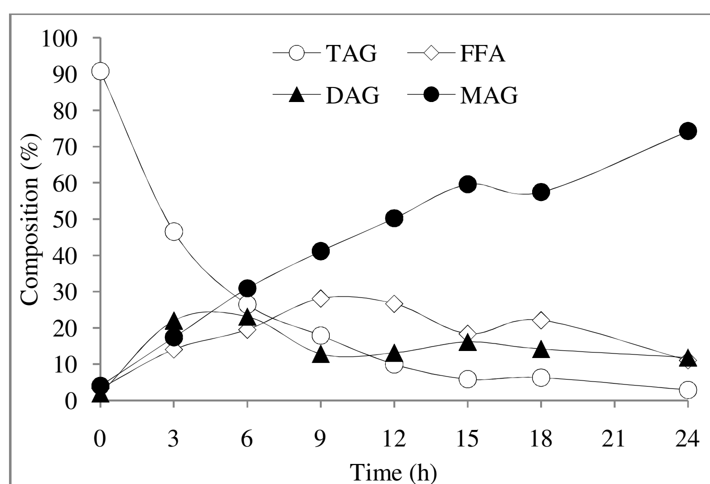


Figure 12. Time course of glycerolysis of CPO in the tert-butanol/hexane 1:1 (v/v) mixture in the optimum conditions: 1 mL of 10% (w/v) CPO in tert-butanol/hexane 1:1 (v/v), glycerol with 8:1 molar ratio to CPO, 4% (w/w) water content in glycerol and the amount of IM-PS was 40% (w/v) based on solvent volume. The temperature was 45°C, 300 rpm of shaking speed and 24 h of reaction time.

1.5 Effect of CPO loading in solvent mixture

The effect of CPO loading on MAG synthesis in solvent mixture was determined in order to select an efficient initial substrate concentration for glycerolysis reaction. The load of CPO in this experiment was varied at 10, 20, 30, 40 and 50% (w/v) in tert-butanol/hexane (1:1, v/v), meanwhile, the amount of IM-PS was constant and glycerol molar ratio to CPO was constant. The result of this experiment is shown in Figure 13. The initial rate was defined as the slope of the MAG yield versus time in the initial 6 h of reaction. In a solvent system, oil concentration affected the production yield of MAG and the reaction rate, even though solvent can create a homogeneous system. Ten percent of CPO loading was the best result and the results were showed in Figure 13. The highest MAG yield was 70.49% with 10% (w/v) CPO in tert-butanol/hexane (1:1, v/v) mixtures. When the concentration of CPO was lower than 10% (w/v), the yield of MAG was decreased. It may be due to two reasons: (1) the solvent inhibits the enzyme activity at the low substrate concentration, and (2) addition based on solvent volume decreases the amount of available substrate at the interface between the solvent and glycerol, thus decrease the MAG yield (Kaewthong and H-Kittikun, 2004). When the CPO concentration was higher than 10%, the systems were more viscous resulting in lower yield of MAG. Moreover, the increasing of CPO would lead to a decreased amount based on solvent volume resulting in a decreasing availability of substrate at the interface between solvent and glycerol. Furthermore, the initial rate of MAG also decreased at high CPO concentration. This might be due to the high viscosity of CPO resulting in low homogeneity. Therefore, 10% CPO loading was selected for the optimum CPO loading in this experiment. Time course of glycerolysis of CPO at 10% of CPO loading is shown in Figure 14. MAG yield was constantly produced at the initial 3 h until reach its maximum at 24 h; meanwhile, TAG decreased very fast at initial 3 h of reaction and slowly declined until 24 h of reaction. FFA and DAG increased at the first 3 h and FFA relatively remained constant while DAG reduced. Moreover, the MAG yield was remained constant even the time was extended until 48 h. Yang *et al.* (2005b) reported that the solvent medium in one way can help improve the system homogeneity and stability as well as reduce the viscosity and mass transfer limitation. On the other hand, the medium will reduce the concentration of substrates

thus reduce reaction rates. They found that 40% of oil concentration was the best concentration that gave higher MAG yield; meanwhile, 25%, 30% and 50% had little difference and gave lower MAG yields. Cheirsilp *et al*. (2009) studied about the optimum initial palm oil concentration in 2-methyl-2-butanol in the palm oil glycerolysis reaction by alginate immobilized lipase. The palm oil concentration was varied at 10, 20, 30, 40, 50 and 60% in 2-methyl-2-butanol and they found that 10% of palm oil concentration gave highest MAG yield.

Damstrup *et al*., (2005) reported MAG yield of 60 – 80% within few hours in a batch reaction system consisting of glycerol, sunflower oil, Novozym 435 lipase and tert-butanol or tert-pentanol solvents. Yang *et al*., (2005b) also reported the similar result. In their study, enzymatic production of MAG in tert-butanol was conducted at 40°C. They reached a MAG content of 60-70% in the lipid phase in a stirred tank after a 2 h reaction. MAG contents up to 70% of lipid phases (molar ratio of glycerol/oil 4:1) were also reached in a packed bed reactor with a residence time only 30-40 min. Elfman-Borjesson and Harrod (1999) reported synthesis of MAG by glycerolysis of rapeseed oil in isooctane using Lipozyme IM. The MAG yield of 28% was obtained at 75°C after 20 h incubation. With the increasing the MAG composition and reducing the TAG composition, the properties of the oil were changed to be more polar oil than original CPO. This property was beneficially for the column chromatography experiment.

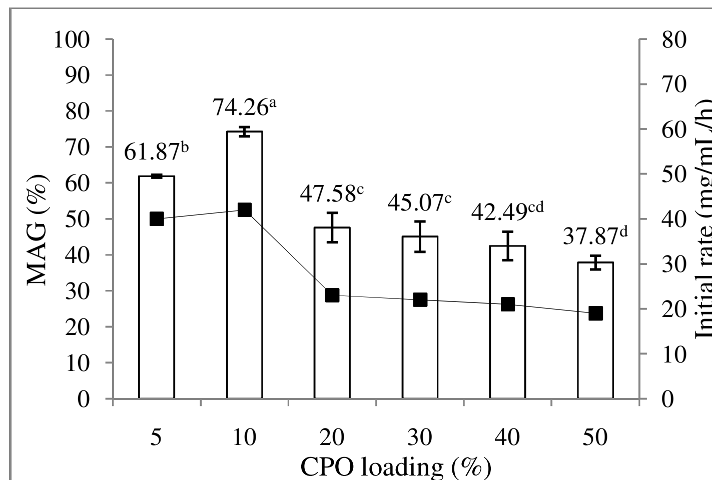


Figure 13. Effect of CPO loading on MAG product (column) and initial rate (line). The reaction mixture contained 1 mL of 5 to 50% (w/v) CPO in tert-butanol/hexane 1:1 (v/v), glycerol with 8:1 molar ratio to CPO and 4% (w/w) water content in glycerol. The amount of IM-PS was 40% (w/v) based on solvent volume. The temperature was 45°C, 300 rpm of shaking speed and 24 h of reaction time. The values with different superscript are significantly different ($p<0.05$).

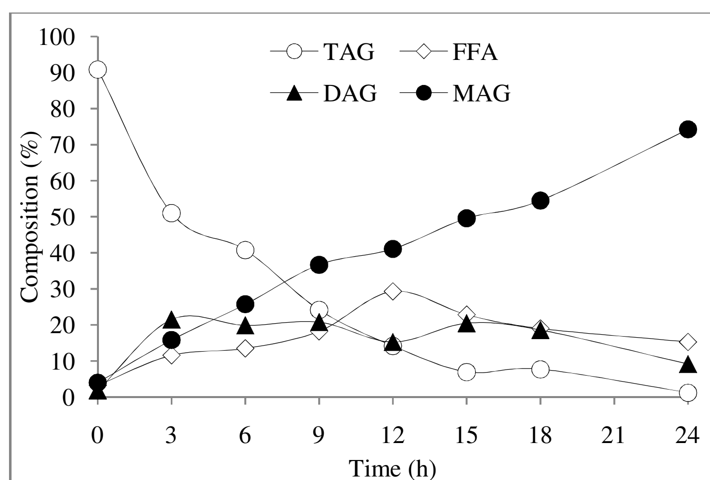


Figure 14. Time course of glycerolysis of CPO in the tert-butanol/hexane 1:1 (v/v) mixture at the optimum conditions: 1 mL of 10% (w/v) CPO in tert-butanol/hexane 1:1 (v/v), glycerol with 8:1 molar ratio to CPO, 4% (w/w) water content in glycerol and the amount of IM-PS was 40% (w/v) based on solvent volume. The temperature was 45°C, 300 rpm of shaking speed and 24 h of reaction time.

2. Study of carotenoids recovery by column chromatography from CPO and MAG-rich CPO

The carotenoids were a minor component of crude palm oil. At present, most of the carotenoids are destroyed during the refining process to obtain light coloring oils. Various methods of carotenoids extraction were saponification, adsorption, precipitation, selective solvent extraction, and transesterification followed by molecular distillation. However, only the transesterification followed by molecular distillation process has been further developed into a commercial-scale process (Ooi *et al.*, 1991). Separation of palm carotenoids from CPO by adsorption chromatography using synthetic polymer adsorbent has been developed (Baharin *et al.*, 1998). In this experiment, carotenoids were obtained without changing the oil form. However, the carotenoids recovery was lower in this approach. An alternate method has been developed to increase the carotenoids recovery from CPO following hydrolysis to produce free fatty acids rich oil which was more polar form in order to enhance carotenoids recovery (You *et al.*, 2002). In the same way, it was thought that producing MAG-rich CPO also enhances carotenoids recovery. Moreover, producing MAG is more advantageous than producing FFA since MAG is more valuable product than FFA. In this study, the total carotenoids were determined spectrophotometrically and the concentrations of carotenoids in the initial crude palm oil and in the MAG-rich CPO were compared with the hydrolyzed CPO (You *et al.* 2002) as shown in Table 11. It was found that carotenoids degradation occurred during the MAG-rich CPO synthesis and hydrolysis reaction. In the hydrolysis reaction, lipase from *Candida rugosa* (1% of weight of oil) was employed, and the reaction was performed at 50°C for 24 h. The amount of CPO was 100 g as well as 100 g of distilled water were added into the CPO. The percentage of carotenoids degradation for MAG synthesis and hydrolysis reaction was 17.86 and 15.56%, respectively. This was probably due to long term exposure to heat and hence oxidation occurred during the process. Lights and thermal are the cause for carotenoids degradation. Carotenoids compounds are consisted of chromophore of conjugated double bound; thus this compound is susceptible to the light and thermal degradation (Melendez-Martinez *et al.*, 2007). However, since the reaction mixtures

were covered by aluminum foil and the reaction was done in the dark room, the main cause of the carotenoids degradation in this experiment might be by heat. Moreover, the initial carotenoids concentration in the CPO would affect the final carotenoids concentration.

Table 11. The carotenoids concentration in CPO, MAG-rich CPO and hydrolyzed CPO

Oil	Carotenoids concentration (ppm)	MAG (%)	FFA (%)
CPO in this research	604.07 ± 63.75	4.07	3.27
MAG-rich CPO	496.19 ± 44.78	74.26	15.32
CPO*	771.40 ± 22.06	n/r	3.08
Hydrolyzed CPO*	651.40 ± 16.12	n/r	94.37

Each value represents mean ± standard deviation
n/r = not reported
*Data from You *et al.* (2002).

2.1 Comparison study of normal phase chromatography and reverse phase chromatography for carotenoids recovery

Two column supports (silica gel and Diaion HP-20) were used in carotenoids recovery from CPO and MAG-rich CPO. Silica gel was used for normal-phase chromatography and Diaion HP-20 for reverse-phase chromatography. The conditions for silica gel chromatography were column temperature 50°C, flow rate 0.5 mL/min, 1.5 mL/fraction, sample loading 1 g and the column were glass tube (11.5 cm length, 1 cm I.D.) with an outer jacket for circulating heated water. One gram of sample was dissolved in petroleum ether and then loaded onto the column bed. The Oil content was defined as the weight of the Oil content in each fraction after evaporation of the solvent. The solvent systems were petroleum ether (9 mL), 50% hexane in petroleum ether (12 mL), 15% diethyl ether in petroleum ether (15 mL) and 30% ethanol in petroleum ether (24 mL). The void volume was 4.03 mL. The silica gel was normal phase chromatography and in this phase, the most non-polar was eluted first. The first elution solvent was high hydrophobicity and the polarity of the solvent was gradually increased. The carotenoids recovery in the silica gel needed broader range based on solvent volume polarity. The petroleum ether fraction was only needed in relatively small amount because the need of a smooth shifting the

polarity properties in the next fraction and the 50% hexane in petroleum ether was suitable in this job. The polarity of petroleum ether and 50% hexane in petroleum ether was similar and the total volume of these two solvent was 21 mL. The gradual change based on solvent volume system was needed for this system; hence 50% hexane in petroleum ether was needed before the medium polar 15% diethyl ether loaded into the column. The 30% of ethanol in petroleum ether was needed to elute the rest of more polar fraction. Hence, in this normal phase chromatography, the carotenoids content in CPO will be eluted prior to MAG product. The conditions for Diaion HP-20 column chromatography were column temperature 50°C, flow rate 0.5 mL/min, 1.5 mL/fraction, sample loading 1 g and the column were glass tube (11.5 cm length, 1 cm I.D.) with an outer jacket for circulating heated water. One gram of sample was dissolved in ethanol and then loaded onto the column bed. The solvent systems were ethanol and hexane. Ethanol was used as primary solvents to elute the oil from the column and hexane was used to elute the carotenoids which had been adsorbed onto the resin (Baharin *et al*., 1998; You *et al*., 2001). This chromatographic adsorption method was a reverse phase system where the stationary phase was nonpolar and highly polarity solvent was used followed by low polarity solvents.

The experiments results of carotenoids recovery on silica gel and on Diaion HP-20 are shown in Table 12. The concentration of carotenoids recovery from CPO and MAG-rich CPO in the petroleum ether fractions of silica gel column were 1,535.78 and 1,683.10 ppm, respectively. Meanwhile, the carotenoids recoveries were 60.75 and 48.10%, respectively. The increase concentration of carotenoids concentration from CPO and MAG-rich CPO samples were 2.5 and 2.9 folds. Although the carotenoids recovery by silica gel column was relatively high (60.75 – 48.10%) at the initial solvent, but the concentration was relatively lower than Diaion HP-20 column. The concentration of carotenoids recovery from CPO and MAG-rich CPO were 2,011.93 and 5,132.79 ppm, respectively. This would happen because larger amount of oil present in the petroleum ether (PE) fraction for carotenoids recovery from CPO in silica column. The carotenoids concentration from MAG-rich CPO in silica column was slightly higher than that from CPO in silica column. This might be due to the fact that the polarity of MAG-rich CPO was higher than CPO. Then the major component of MAG-rich CPO was MAG and it will eluted in the last

fraction of 30% ethanol in petroleum ether, thus the small amount of carotenoids contained oil was eluted first in the petroleum ether fraction. On the other hand, the main component in CPO was TAG; and carotenoids will be diluted together with TAG in petroleum ether fraction because carotenoids are even less-polar than TAG. This is the reason why there was an improvement of carotenoids recovery when CPO was converted to MAG-rich CPO and carotenoids was recovered in silica column. However, by lower ability to separate carotenoids from CPO and MAG-rich CPO was the weakness of this column. However, this column could be used in tocopherol recovery from soybean. Wan *et al.* (2008) studied separation of α, γ and δ-tocopherol from soybean using a low-pressure glass column (500mm x 25mm, I.D., packed with silica gel). Cyclohexane-ethanol 99.07:0.3 (v/v) was employed at flow rate 25 mL/min for α-tocopherol, γ-tocopherol and δ-tocopherol recovery and the recovery were 35.21, 36.25 and 61.25%, respectively.

The concentration of carotenoids from CPO and MAG-rich CPO in the hexane fractions of Diaion HP-20 column were 2,011.93 and 5,132.79 ppm, respectively (Table 12). Meanwhile, the carotenoids recoveries in hexane fractions were 55.06 and 75.70%, respectively. The carotenoids recovery from MAG-rich CPO was 20% higher than that from CPO in Diaion HP-20 column. The increase concentration of carotenoids content from CPO and MAG-rich CPO samples were 3.4 and 10.3 folds. The ethanol fraction of CPO sample contained 83% oil from the total oil quantity, meanwhile the ethanol fraction of MAG-rich CPO contained higher amount of oil (93% oil from the total oil quantity). This higher amount of eluted oil in ethanol fraction means better separation efficiency. This improvement was due to the conversion of nonpolar CPO to polar MAG-rich CPO; therefore the MAG-rich CPO was not adsorbed tightly on Diaion HP-20 resin and easily eluted in ethanol fraction. Moreover, the nonpolar carotenoids could be adsorbed on nonpolar Diaion HP-20 resin and this property enhanced the recovery by column chromatography. The ability of this resin was thought to be due to superior surface area and greater hydrophobicity (Baharin *et al*., 1998). The synthetic adsorbent Diaion HP-20 was three dimensional cross-linked polymers with macro-pores. It did not possess ion exchange or other functional groups; however, it had a large specific surface 511 m^2/g and was able to adsorb a variety of organic substance by means of van der Waals' force. Moreover, it

well suited to the adsorption of large molecule such as carotenoids and the adsorbed substances could be easily eluted from the resin by using suitable solvent (Latip *et al.*, 2000).

You *et al.* (2002) conducted the experiment of recovery of palm carotene from palm oil and hydrolyzed palm oil by Diaion HP-20 resin. They hydrolyzed palm oil with lipase from *Candida rugosa* to produce FFA-rich oil. Isopropanol or ethanol and hexane were used as the first and second eluting solvent, respectively. The result showed that the carotenoids recovery from CPO and hydrolyzed CPO in second fraction (hexane fraction) were 36.79 and 90.12%, respectively. This result suggested that by changing the polarity of palm oil to FFA-rich oil, the adsorption efficiency in the column chromatography was increased.

The objective of changing the CPO to other components such as free fatty acid (FFA) or monoacylglycerol (MAG) was to produce more polar oil in order to enhance the nonpolar carotenoids bind to the nonpolar Diaion HP-20 adsorbent in the column. Hence the carotenoids would be separated from the oil at the initial fractions and increasing the recovery and concentration in the hexane fractions. Figure 15 showed the oil content, oil composition and carotenoids content from original CPO in each fraction of Diaion HP-20 column, meanwhile Figure 16 showed the oil content, oil composition and carotenoids content from MAG-rich CPO in each fraction of the same resin. The results from Figure 16 showed that changing the polarity of the palm oil to more polar oil, MAG-rich CPO, the adsorption efficiency in the column chromatography increased compared to original CPO (Figure 15). Competitive adsorption between CPO and carotenoids on the Diaion HP-20 surface will reduce carotenoids recovery. The increase in the polarity of the oil supported the carotenes which were non-polar, freely to bind tighter to the nonpolar resin without having to compete with the non-polar CPO. However, the carotenoid recovery from MAG-rich oil in this study was not so high (only 75.70%). Thus, further optimization was needed.

Table 12. Recovery of palm carotenoids in each column

Sample/ column	Fraction[a]	Oil Quantity (g)[b]	Carotenoids		
			Content (mg)	Recovery (%)	Concentration (ppm)[c]
CPO/silica gel	PE (9 mL)	0.2277	0.3497	60.75	1,535.78
	50% hexane in PE (12 mL)	0.2838	0.0997	17.33	351.42
	15% diethylether in PE (15 mL)	0.2963	0.0457	7.94	154.26
	30% ethanol in PE (24 mL)	0.1635	0.0896	13.98	547.95
MAG-rich CPO/ silica gel	PE (9 mL)	0.1498	0.2521	48.10	1,683.10
	50% hexane in PE (12 mL)	0.1260	0.0793	15.13	629.61
	15% diethylether in PE (15 mL)	0.1228	0.0799	15.24	650.66
	30% ethanol in PE (24 mL)	0.5611	0.1128	21.53	201.11
CPO/ Diaion	Ethanol (15 mL)	0.7741	0.2496	44.94	322.46
	Hexane (15 mL)	0.1520	0.3058	55.06	2,011.93
MAG-rich CPO/ Diaion	Ethanol (15mL)	0.9042	0.1041	24.29	115.09
	Hexane (15 mL)	0.0632	0.3244	75.70	5,132.79

[a]PE: petroleum ether
[b]Constant weight after evaporation based on solvent volume
[c]Original carotenoids concentration in CPO was about 600 ppm

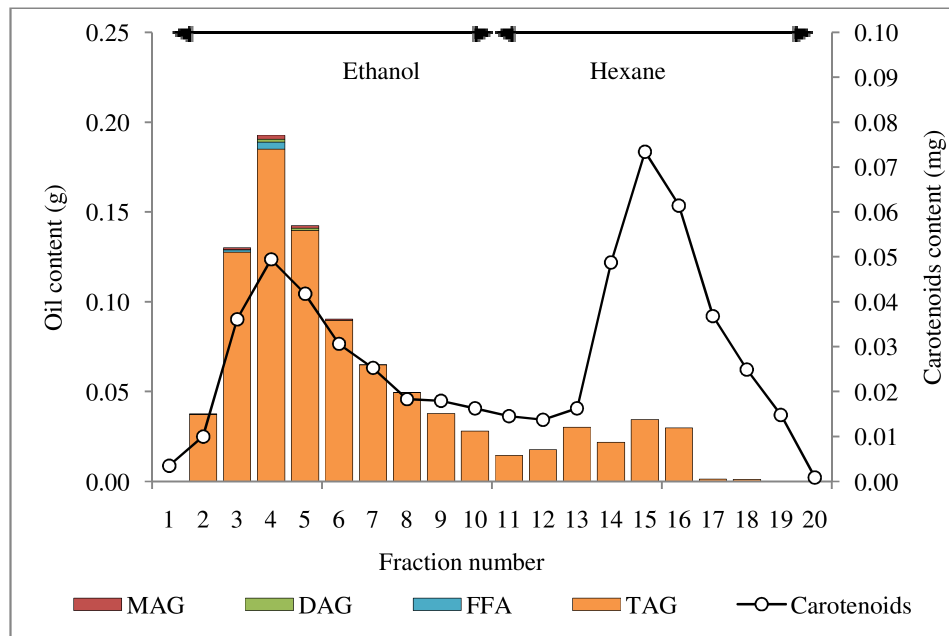


Figure 15. Oil content and carotenoids content from original CPO chromatography on Diaion HP-20. Initial solvent was ethanol, second solvent was hexane. Each fraction was 1.5 mL, column temperature 50°C, flow rate 0.5 mL/min and sample loading 1 g.

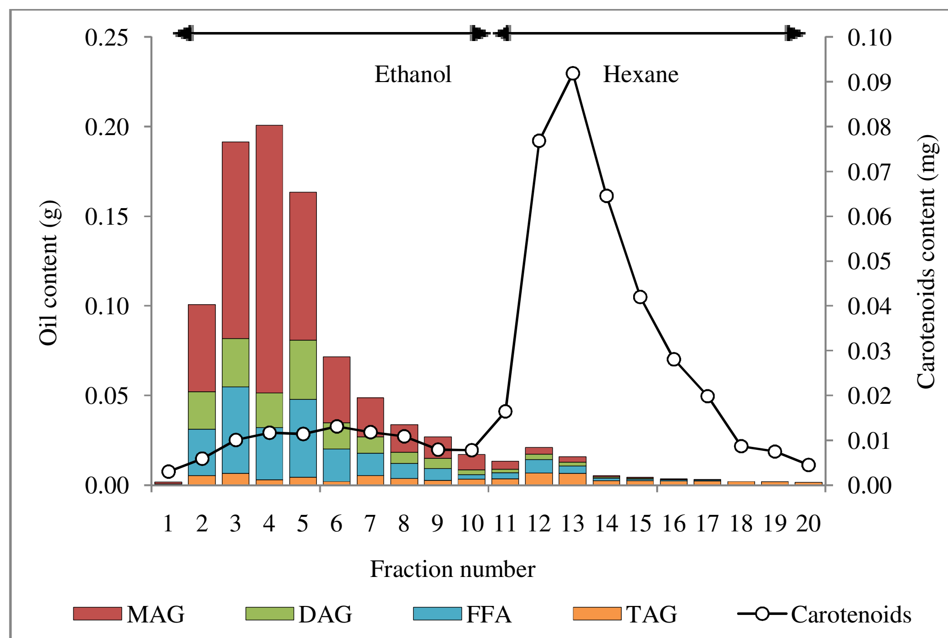


Figure 16. Oil content and carotenoids content from MAG-rich CPO chromatography on Diaion HP-20. Initial solvent was ethanol, second solvent was hexane. Each fraction was 1.5 mL, column temperature 50°C, flow rate 0.5 mL/min and sample loading 1 g.

2.1 Optimization of carotenoids recovery process

2.1.1 Effect of temperature

The effect of temperature on the carotenoids recovery from MAG-rich CPO was examined at 40, 50 and 60°C. The result from this experiment is shown in the Figures 17 – 19. The temperature largely affected chromatographic behavior because the viscosity of sample was depending on temperature. Either lower or higher temperature were not advantageous, because at lower temperatures the viscosity of the MAG-rich CPO was too high, whereas at 75°C and higher will damage the carotenoids. These experiments were carried out based on the following conditions: initial solvent was ethanol, second solvent was hexane, and each fraction was 1.5 mL, flow rate 0.5 mL/min and sample loading 1 g and the column were glass tube (11.5 cm length, 1 cm I.D.) with an outer jacket for circulating heated water. One gram of sample was dissolved in ethanol and then loaded onto the column bed. The carotenoids content in hexane was high at higher temperature (Figure 17 and 18). However, at 60°C, carotenoids content in hexane was decreased. This might be due to the viscosity of MAG-rich CPO at this temperature was low, thus the carotenoids came out earlier in the Oil content in hexane fraction (Figure 19). Table 13 shows the effect of temperature on the recovery of carotenoids from MAG-rich CPO with the ethanol-hexane system. The highest carotenoids recovery reached 79.41% at 50°C in ethanol fraction. The maximum carotenoids concentration was 5,007.59 ppm at 50°C compared to higher or lower temperature as well as the data for carotenoids content. The oil recoveries in ethanol were almost the same at the all examined temperatures (88 to 92%). The oil recovery in ethanol was not affected much by the temperature and at higher temperatures, carotenoids recovery become lower. It could be concluded that the optimal temperature was 50°C for the recovery of carotenoids. Baharin *et al.* (2002) also reported that the temperature largely affected chromatographic behavior. The larger column dimension (3 cm I.D. by 35-cm length) with Diaion HP-20 support, ethanol-hexane system as eluting solvent was used and the temperature was varied at 50, 60 and 70°C. The carotenoids recovery of 50% at 50°C was obtained and it gradually decreased to 40 and 35% at 60 and 70°C, respectively.

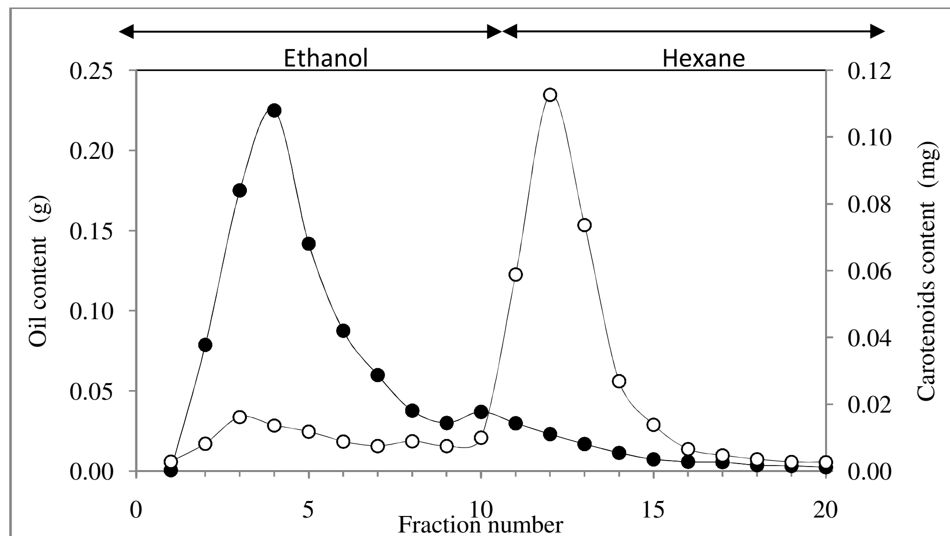


Figure 17. The Diaion HP-20 column chromatography for separation of carotenoids from MAG-rich CPO. Close circle is oil content, open circle is carotenoids content. Initial solvent was ethanol, second solvent was hexane. Each fraction was 1.5 mL, column temperature 40°C, flow rate 0.5 mL/min and sample loading 1 g.

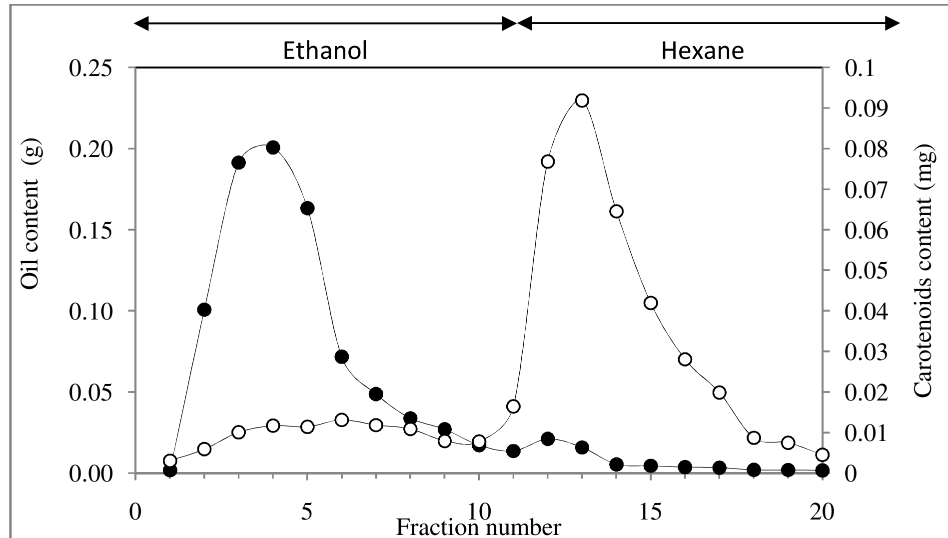


Figure 18. The Diaion HP-20 column chromatography for separation of carotenoids from MAG-rich CPO. Close circle is oil content, open circle is carotenoids content. Initial solvent was ethanol, second solvent was hexane. Each fraction was 1.5 mL, column temperature 50°C, flow rate 0.5 mL/min and sample loading 1 g.

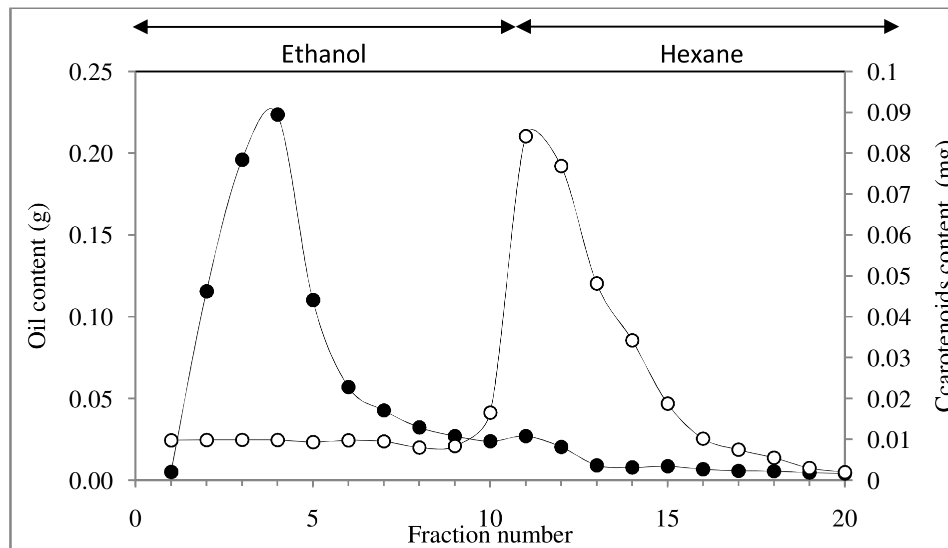


Figure 19. The Diaion HP-20 column chromatography for separation of carotenoids from MAG-rich CPO. Close circle is oil content, open circle is carotenoids content. Initial solvent was ethanol, second solvent was hexane. Each fraction was 1.5 mL, column temperature 60°C, flow rate 0.5 mL/min and sample loading 1 g.

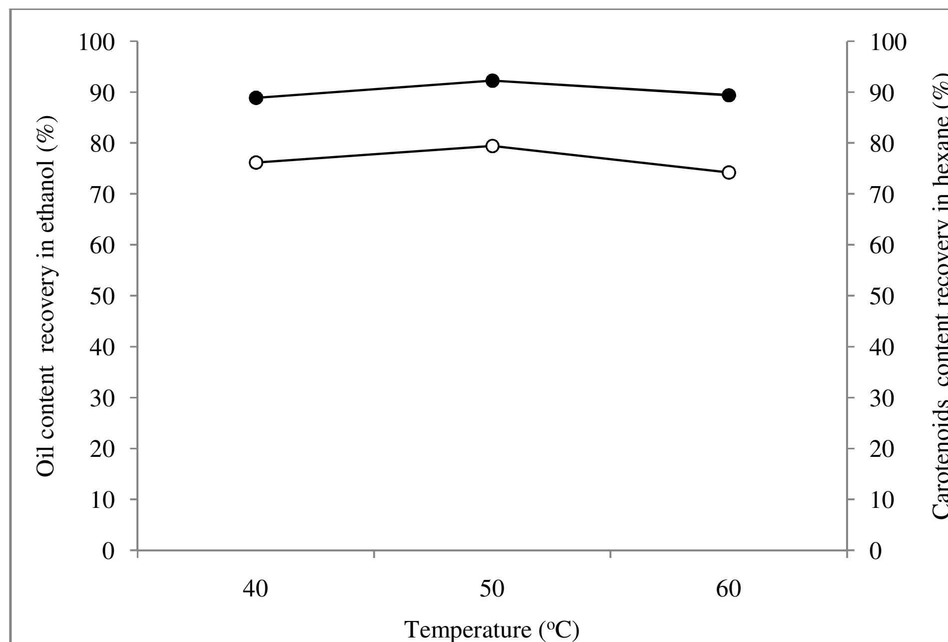


Figure 20. Effect of temperature on carotenoids and oil recovery from MAG-rich CPO on Diaion HP-20 column chromatography. Close circle is oil content, open circle is carotenoids content. Initial solvent was ethanol, second solvent was hexane. Each fraction was 1.5 mL, column temperature 40-60°C, flow rate 0.5 mL/min and sample loading 1 g.

Table 13. Effect of temperature in the recovery on carotenoids from MAG-rich CPO with the ethanol-hexane system

Temperature	Fraction	Oil		Carotenoids		
		Content	Recovery	Content	Recovery	Concentration
^{o}C		(g)	(%)	(mg)	(%)	(ppm)
40	Ethanol	0.8733	88.85	0.0957	23.81	109.56
	Hexane	0.1096	11.15	0.3061	76.18	2,792.97
50	Ethanol	0.8558	92.25	0.0934	20.59	109.11
	Hexane	0.0719	7.75	0.3600	79.41	5,007.59
60	Ethanol	0.8331	89.36	0.1006	25.77	120.75
	Hexane	0.0926	10.64	0.2898	74.23	3,129.70

2.2.2 Effect of sample loading

The effect of sample loading on the Diaion HP-20 column was investigated. The initial solvent was ethanol, second solvent was hexane, and each fraction was 1.5 mL, flow rate 0.5 mL/min and sample loading 1 g and the column were glass tube (11.5 cm length, 1 cm I.D.) with an outer jacket for circulating heated water. The amounts of MAG-rich CPO sample were 0.5, 1, 2, 3 and 4 g. The results are shown in Figure 21 – 25. Figures 21 and 22 show the 0.5 and 1 g sample loading, respectively. The carotenoids were eluted mostly in the hexane fraction, meanwhile, on Figure 23; the carotenoids were eluted in the ethanol fraction and hexane fraction. Moreover, on Figure 24 and 25, the oil content was not completely eluted in the ethanol fraction, thus the oil was also eluted in hexane fraction. Table 14 shows effect of the sample loading on the recovery of carotenoids from MAG-rich CPO with the ethanol-hexane system. The highest carotenoids concentration was at 1 g of MAG-rich CPO sample. Increasing the sample loading decreased the carotenoids concentration in hexane fraction. This happened due to the limiting capacity of the resin. Therefore, the overload of carotenoids was eluted in the ethanol fraction. Figure 26 shows the relationship of sample loading to carotenoids and oil recovery. Carotenoids recovery decreased with increasing sample loading. The high carotenoids recovery was obtained at 0.5 g and 1 g sample loading, respectively and it dropped to 61% at 2 g and 59% at 4 g sample loading. The oil recovery dropped from 93% at 2 g sample loading to 61% at 4 g sample loading. Sample loading on the Diaion HP-20

column was an important condition, because this largely affects carotenoids recovery. The loaded samples on resin were 0.05, 0.11, 0.22, 0.33, and 0.44 g per gram resin for 0.5, 1, 2, 3 and 4 g sample loading, respectively. The adsorption capacity for Oil contents (CPO and its derivatives) on the Diaion HP-20 resin has already been determined, and a 0.2 to 0.5 g loading of Oil contents per gram resin has been recommended (Baharin *et al.*, 1998). However, the optimum loaded sample on resin in this study was slightly lower than recommended value (0.11 g sample/g resin). These results suggest that carotenoids recovery depending mainly on two factors: (i) competitive adsorption between oil and carotenoids on the Diaion HP-20 resin surface. And (ii) the adsorption capacity of the resin for carotenoids in the presence of the ethanol. Baharin *et al*. (2008) loaded Diaion HP-20 resin at 0.05 g per gram resin until 0.3 g per gram resin.

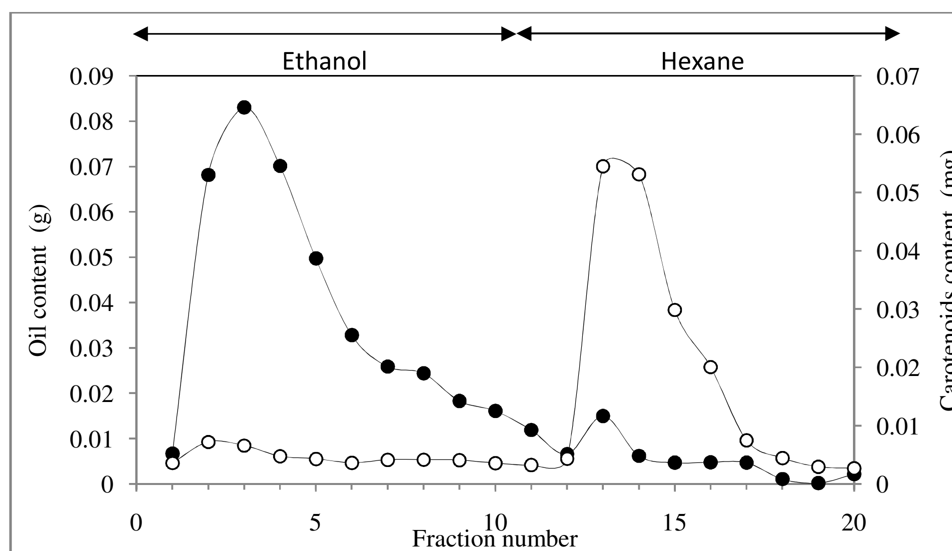


Figure 21. The Diaion HP-20 column chromatography for separation of carotenoids from MAG-rich CPO. Close circle is oil content, open circle is carotenoids content. Initial solvent was ethanol, second solvent was hexane. Each fraction was 1.5 mL, column temperature 50°C, flow rate 0.5 mL/min and sample loading 0.5 g.

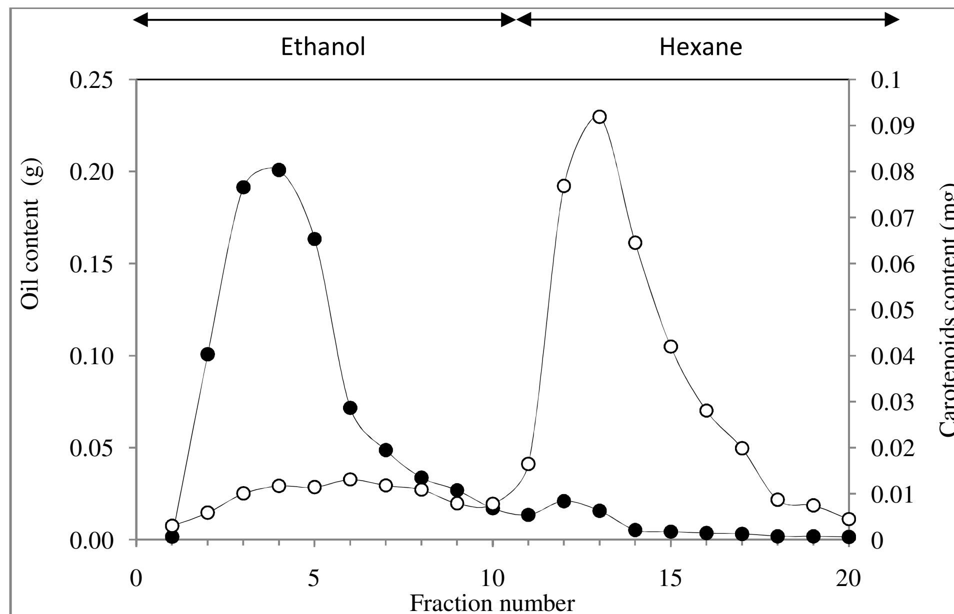


Figure 22. The Diaion HP-20 column chromatography for separation of carotenoids from MAG-rich CPO. Close circle is oil content, open circle is carotenoids content. Initial solvent was ethanol, second solvent was hexane. Each fraction was 1.5 mL, column temperature 50°C, flow rate 0.5 mL/min and sample loading 1 g.

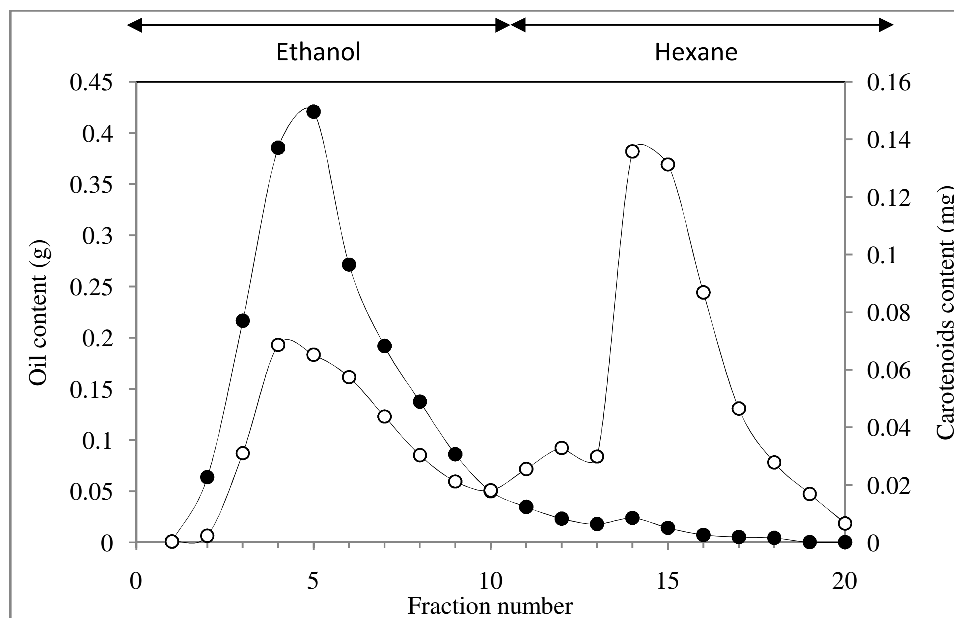


Figure 23. The Diaion HP-20 column chromatography for separation of carotenoids from MAG-rich CPO. Close circle is oil content, open circle is carotenoids content. Initial solvent was ethanol, second solvent was hexane. Each fraction was 1.5 mL, column temperature 50°C, flow rate 0.5 mL/min and sample loading 2 g.

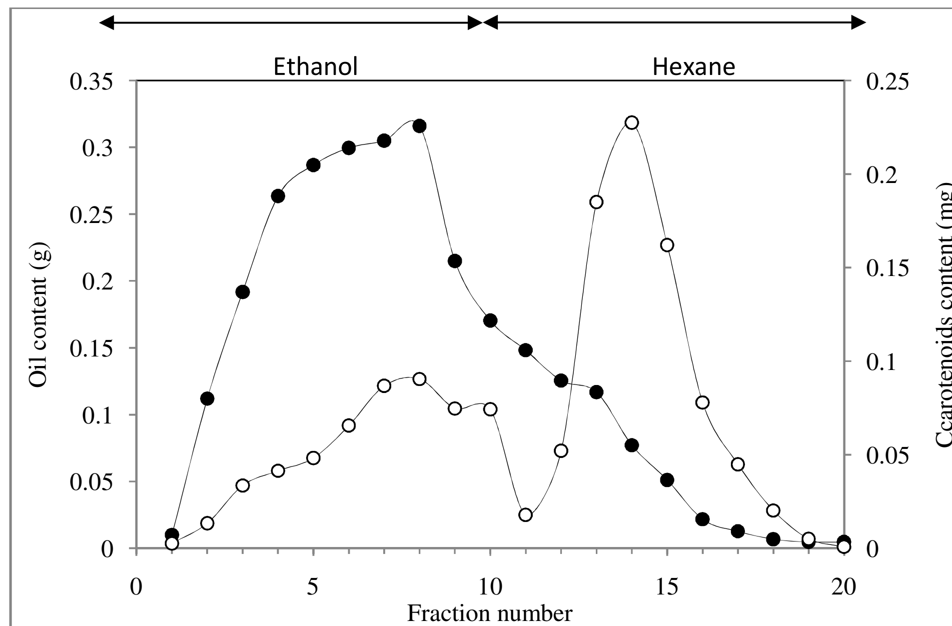


Figure 24. The Diaion HP-20 column chromatography for separation of carotenoids from MAG-rich CPO. Close circle is oil content, open circle is carotenoids content. Initial solvent was ethanol, second solvent was hexane. Each fraction was 1.5 mL, column temperature 50°C, flow rate 0.5 mL/min and sample loading 3 g.

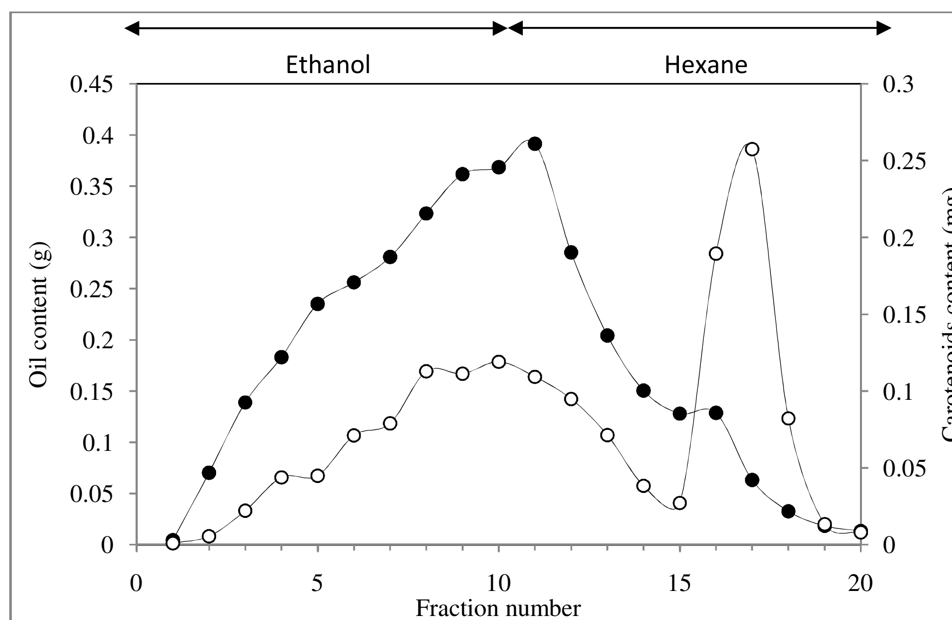


Figure 25. The Diaion HP-20 column chromatography for separation of carotenoids from MAG-rich CPO. Close circle is oil content, open circle is carotenoids content. Initial solvent was ethanol, second solvent was hexane. Each fraction was 1.5 mL, column temperature 50°C, flow rate 0.5 mL/min and sample loading 4 g.

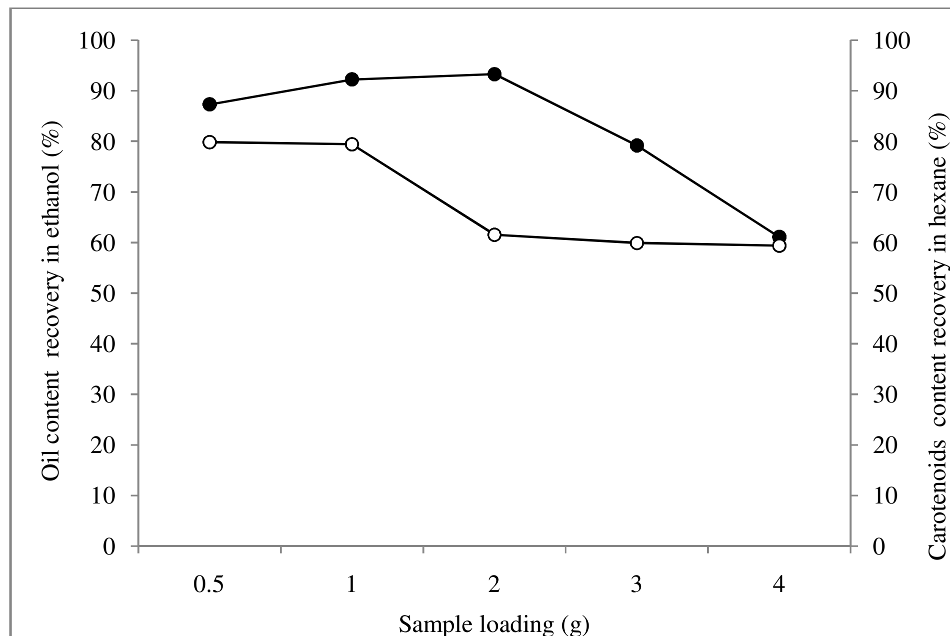


Figure 26. Effect of temperature on carotenoids and oil recovery from MAG-rich CPO on Diaion HP-20 column chromatography. Close circle is oil content, open circle is carotenoids content. Initial solvent was ethanol, second solvent was hexane. Each fraction was 1.5 mL, column temperature 50°C, flow rate 0.5 mL/min and sample loading 0.5-4 g

Table 14. Effect of the sample loading on the recovery of carotenoids from MAG-rich CPO with the ethanol-hexane system

Sample load	Fraction	Oil		Carotenoids		
g		Content (g)	Recovery (%)	Content (mg)	Recovery (%)	Concentration (ppm)
0.5	Ethanol	0.3951	87.31	0.0463	20.22	117.18
	Hexane	0.0574	12.69	0.1828	79.85	3,185.53
1	Ethanol	0.8558	92.25	0.0900	20.59	109.11
	Hexane	0.0719	7.75	0.3600	79.41	5,007.59
2	Ethanol	1.8243	93.29	0.3384	38.54	185.49
	Hexane	0.1312	6.71	0.5401	61.51	4,116.55
3	Ethanol	2.1707	79.19	0.5317	40.10	244.95
	Hexane	0.5703	20.81	0.7941	59.89	1,392.44
4	Ethanol	2.2227	61.08	0.6104	40.66	274.61
	Hexane	1.4162	38.92	0.8911	59.37	629.23

2.2.3 Increasing the number of fraction

The aim of this experiment was to improve carotenoids separation by increasing the number of fraction. Five fractions were added both on the hexane and ethanol fraction. Carotenoids was recovered to 83% and concentrated to 11,055.02 ppm, which was about 22 times of the original concentration in CPO (Table 15). The result of increasing the number of fraction on the separation of carotenoids from MAG-rich CPO was shown in Figure 27. The oil content was eluted in the ethanol fraction efficiently. The optimum conditions were 50°C of column temperature and one gram of sample loading in a Diaion HP-20 column (11.5 cm length, 1 cm I.D.) with ethanol as first and hexane as second eluting solvent.

Table 15. Recovery of palm carotenoids with the ethanol-hexane system

Fraction	Oil		Carotenoids		
	Content (g)	Recovery (%)	Content (mg)	Recovery (%)	Concentration (ppm)
Ethanol	0.9393	95.24	0.1002	16.19	106.64
Hexane	0.0469	4.76	0.5185	83.81	11,055.02

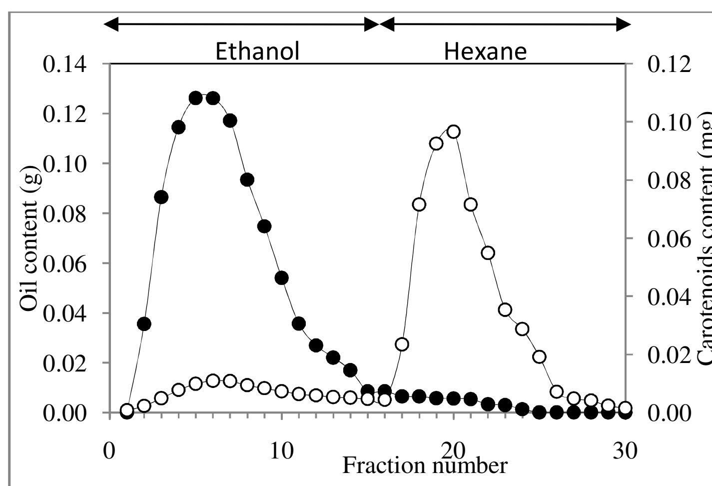


Figure 27. The Diaion HP-20 column chromatography for separation of carotenoids from MAG-rich CPO. Close circle is oil content, open circle is carotenoids content. Initial solvent was ethanol, second solvent was hexane. Each fraction was 1.5 mL, column temperature 50°C, flow rate 0.5 mL/min and sample loading 1 g.

CHAPTER 4

CONCLUSION

Recovery of carotenoids from CPO by glycerolysis reaction followed by column chromatography was conducted. The first step was monoacylglycerol production from CPO by enzymatic glycerolysis reaction and the second step was carotenoids recovery from MAG-rich CPO. The initial glycerolysis conditions were 0.1 g CPO in 1 mL of acetone/iso-octane (3:1, v/v), 50% immobilized enzyme (IM-PS) (w/v) based on solvent volume and glycerol at 8:1 molar ratio to CPO with water content 4% (w/w) in glycerol and the temperature was controlled at 45°C. The 55.42% of MAG yield was obtained. The mixture of tert-butanol/hexane, 3:1 (v/v) was attempted as solvent and 77.86% of MAG yield was achieved. The optimum condition for MAG synthesis by glycerolysis reaction using immobilized lipase was: amount of immobilized lipase was 40% (w/v) based on solvent volume, 8:1 glycerol to CPO molar ratio, 10% of CPO loading and mixture of tert-butanol/hexane, 3:1 (v/v) as solvent. The average 74.26% MAG yield was achieved in this optimum condition.

The carotenoids recovery from MAG-rich CPO was investigated. The comparison of silica gel chromatography and Diaion HP-20 was determined and the result was 79.41% carotenoids was recovered with 5,007.59 ppm of carotenoids concentration in the Diaion HP-20 column (11.5 cm length, 1 cm I.D.) with ethanol as first and hexane as second eluting solvent. This experiment was followed to the effect of temperature and the result found that 50°C showed the best separation. The effect of sample loading was also studied and the best sample loading was 1 g of sample in the column or 0.11 gram sample per gram resin. Finally, carotenoid was recovered to 83.81% with 11,055.02 ppm by using these conditions and increasing the amount of eluting solvent.

REFERENCES

A.O.A.C. 1999. Official Methods of Analysis of AOAC International. Vol. II. 16th Ed. AOAC International. USA.

Ambrogi, A., Cardarelli, D.A. and Eggers, R. 2003. Separation of natural colorants using a combined high pressure extraction-adsorption process. Lat. Am. App. Res. 33: 323-326.

Baharin, B.S., Rahman, K.A., Karim, M.I.A., Oyaizu, T., Tanaka, K., Tanaka, Y. and Takagi, S. 1998. Separation of palm carotene from crude palm oil by adsorption chromatography with a synthetic polymer adsorbent. J. Am. Oil Chem. Soc. 75 (3): 399-344.

Balcao, V.M., Paiva, A.L. and Malcata, F.X. 1996. Bioreactors with immobilized lipases: state of the art. Enzyme Microb. Technol. 18: 392-416.

Barouh, N., Piombo, G., Goli, T., Barea, B., Pina, M., Lago, R. and Villeneuve, P. 2008. Enzymatic production of conjugated linoleic acid monoacylglycerols from dehydrated isomerized castor bean oil. J. Food Lipids. 15: 13-27.

Bezbradica, D., Mijin, D., Siler-Marinkovic, S. and Knezevic, Z. 2007. The effect of substrate polarity on the lipase-catalyzed synthesis of aroma esters in solvent-free system. J. Mol. Cat. B: Enzymatic. 45: 97-101.

Bornscheuer, U.T. 1995. Lipase-catalyzed syntheses of monoacylglycerols. Enzyme Microb. Technol. 17: 578-586.

Cheirsilp, B., Jeamjounkhaw, P. and H-Kittikun, A. 2009. Optimizing an alginate immobilized lipase for monoacylglycerol production by the glycerolysis reaction. J. Mol. Cat. B: Enzymatic. 59: 206-211.

Chen, J.P. and Wang, J.B. 1997. Wax ester synthesis by lipase-catalyzed esterification with fungal cells immobilized on cellulose biomass support particles. Enzyme Microb. Technol. 20: 615-622.

Choo, Y.M. 1994. Palm oil carotenoids. Food Nutr. Bull. 2: 1-13. Available online at: http://www.unu.edu/unupress/food/8F152e/8F152E05.htm [accessed: 24 February 2008]

Choo, Y.M., Goh, S.H. and Ong, S.H. 1985. Minor constituents of palm oil. J. Am. Oil. Chem. Soc. 62: 270-240.

Chuang, M.H. and Brunner, G. 2006. Concentration of minor components in crude palm oil. J. Supercrit. Fluids. 37: 151-156.

Coteron, A., Martinez, M. and Aracil, J. 1998. Reactions of olive oil and glycerol over immobilized lipases. J Am. Oil Chem. Soc. 75: 657-660.

Croce, R., Weiss, S. and Bassi, R. 1999. Carotenoid-binding sites of the major light-harvesting complex II of higher plants. J. Biol. Chem. 274 (42): 29613-296623.

Darnoko, D. and Cheryan, M. 2006. Carotenoids from red palm methyl ester by nanofiltration. J. Am. Oil Chem. Soc. 83: 365-370.

Damstrup, M.L., Jensen, T., Sparso, F.V., Kiil, S.Z., Jensen, A.D. and Xu, X. 2005. Solvent optimization for efficient enzymatic monoacylglycerol production based on a glycerolysis reaction. J. Am. Oil Chem. Soc. 82: 559-564.

Dutta, D., Chaudhuri, U.R. and Chakraborty, R. 2005. Structure, health benefits, antioxidant property and processing and storage of carotenoids. Afr. J. Biotechnol. 4 (13): 1510-1520.

Elfman-Borjesson, I. and Harrod, M. 1999. Synthesis of monoglycerides by glycerolysis of rapeseed oil using immobilized lipase. J. Am. Oil Chem. Soc. 76: 701-707.

Fernandez, X. E., Shier, N.W. and Watkins, B.A. 2000. Effect of alkali saponification, enzymatic hydrolysis and storage time on the total carotenoid concentration of Costa Rican crude palm oil. J. Food Compos. Anal. 13:179-187.

Gandhi, N.N. 1997. Applications of lipase. J. Am. Oil Chem. Soc. 74:621-634.

Goh, S.H. 1996. Carotene controversy. Malaysian Oil Sci. Technol. 5: 17.

Granado, F., Olmedilla, B., Gil-Martinez, E. and Blanco, I. 2001. A fast, reliable and low-cost saponification protocol for analysis of carotenoids in vegetables. J. Food Compos. Anal. 14: 479-489.

Han, N.M., May, C.Y., Ngan, M.A., Hock, C.C. and Hashim, M.A. 2006. Separation of coenzyme Q_{10} in palm oil by supercritical fluid chromatography. Am. J. App. Sci. 3 (7): 1929–1932.

Hu, K.Q., Liu, C., Ernst, H., Krinsky, N.I., Russell, R.M. and Wang, X.D. 2006. The Biochemical characterization of ferret carotene-9',10'-monooxygenase catalyzing cleavage of carotenoids in vitro and in vivo. J. Biol. Chem. 281(28): 19327-19338.

Isono, Y., Nabetani, H. and Nakajima, M. 1995. Lipase-surfactant complex as catalyst in interesterification and esterification in organic media. J. Ferment. Bioeng. 80(2): 170-175.

Kaewthong, W. 2004. Continuous Production of Monoacylglycerols by Glycerolysis of Palm Olein with Immobilized Lipase. Ph.D Dissertation. Prince of Songkla University.

Kaewthong, W and H-Kittikun, A. 2004. Glycerolysis of palm olein by immobilized lipase PS in organic solvents. Enzyme Microb. Technol. 35:218-222.

Kaewthong, W., Sirisansaneeyakul, S., Prasertsan, P. and H-Kittikun, A. 2005. Continuous production of monoacylglycerols by glycerolysis of palm olein with immobilized lipase. Process Biochem. 40: 1525-1530.

Kimura, M. and Rodriguez-Amaya, D.B. 2002. A scheme for obtaining standards and HPLC quantification of leafy vegetable carotenoids. Food Chem. 78: 389-398.

Klibanov, A.M. 2001. Improving enzymes by using them in organic solvents. Nature. 409: 241-246.

Krishnaiah, D., Sarbatly, R. and Bono, A. 2007. Phytochemical antioxidants for health and medicine – A move towards nature. Biotechnol. Mol. Biol. Rev. 1:97-104.

Kristensen, J.B., Xu, X. and Mu, H. 2005. Diacylglycerol synthesis by enzymatic glycerolysis: screening of commercially available lipases. J. Am. Oil Chem. Soc. 82: 329-334.

Kwon, S.J., Han, J.J. and Rhee, J.S. 1995. Production and *in situ* separation of mono- or diacylglycerol catalyzed by lipase in *n*-hexane. Enzyme Microb. Technol. 17: 700-704.

Ladislav, F., Vera, P., Karel, S. and Karel, V. 2005. Reliability of carotenoid analyses. Curr. Anal. Chem. 1: 93-102.

Latip, R.A., Baharin, B.S., Man, Y.B.C. and Rahman, R.A. 2000. Evaluation of different types of synthetic adsorbents for carotene extraction from crude palm oil. J. Am. Oil Chem. Soc. 77: 1277-1282.

Lee, S.Y. and Rhee, J.S. 1993. Production and partial purification of a lipase from *Pseudomonas putida* 3SK. Enzyme Microb. Technol. 15: 617-623.

Lim, T.Y., Li, J.L. and Chen, B.H. 2005. Solubilization of selected free fatty acids in palm oil by biodegradable ethoxylated surfactants. J. Agric. Food Chem. 53: 4476-4483.

Markom, M., Singh, H. and Hasan, H. 2001. Supercritical CO_2 fractionation of crude palm oil. J. Supercrit. Fluids. 20: 45-53.

Maldonade, I.R., Rodriguez-Amaya, D.B. and Scamparini, A.R.P. 2008. Carotenoids of yeasts isolated from the Brazilian ecosystem. Food Chem. 107: 145-150.

Melendez-Martinez, A.J., Vicario, I.M. and Heredia, F.J. 2007. Analysis of carotenoids in orange juice. J. Food Compos. Anal. 20: 638-649.

Mortensen, A. 2005. Analysis of a complex mixture of carotenes from oil palm (*Elais guineensis*) fruit extract. Food Res. Int. 38: 847-853.

Noureddini, H., Harkey, D.W. and Gutsman, M.R. 2004. A Continuous process for the glycerolysis of soybean oil. J. Am. Oil Chem. Soc. 81: 203-207.

Ooi, C.K., Choo, Y.M., Yap, S.C., Basiron, Y. and Ong, A.S.H. 1991. Recovery of carotenoids. US Patent 5019668.

Pawongrat, R., Xu, X. and H-Kittikun, A. 2007. Synthesis of monoacylglycerol rich in polyunsaturated fatty acids from tuna oil with immobilized lipase AK. Food Chem. 104: 251-258.

Ping, B.T.Y. and Gwendoline, E.C.L. 2006. Identification of lutein in crude palm oil and evaluation of carotenoids at various ripening stages of the oil palm fruit. J. Oil Palm Res. 18: 189-197.

Ping, B.T.Y., May, C.Y., Gwendoline, .E.C.L. and Hock, G.S. 2002. Geometrical isomers of the major provitamin A palm carotenes, α- and β-carotenes in the mesocarp oil of fresh and sterilized palm fruits, crude palm oil and palm carotene based products: red palm olein and carotene concentrates. J. Oil Palm Res. 13 (2): 23-32.

Ren, D. and Zhang, S. 2008. Separation and identification of the yellow carotenoids in *Potamogeton crispus* L. Food Chem. 106: 410-414.

Rodriguez-Amaya, D.B. and Kimura, M. 2004. HarvestPlus Handbook for Carotenoid Analysis. HarvestPlus. Washington DC. Available online at: http://www.harvestplus.org/pdfs/tech02.pdf [accessed: 14 January 2008]

Rosu, R., Uozaki Y., Iwasaki, Y. and Yamane, T. 1997. Repeated use of immobilized lipase for monoacylglycerol production by solid-phase glycerolysis of olive oil. J. Am. Oil Chem. Soc. 74: 445-450.

Sangster, J. 1989. Octanol-water partition coefficients of simple organic compounds. J. Phys. Chem. Ref. Data. 18(3): 1111-1227.

Schierle, J., Pietsch B., Ceresa, A. and Fizet, C. 2004. Method for the determination of β-carotene in supplements and raw materials by reversed-phase liquid chromatography: single laboratory validation. J. Am. Oil Anal. Chem. Int. 87(5): 1070-1082.

Soumanou, M.M., Bornscheuer, U.T. and Schmid, R.D. 1998. Two-step enzymatic reaction for the synthesis of pure structured triacylglycerides. J. Am. Oil Chem. Soc. 75: 703-710.

Szulczewska-Remi, A., Nogala-Kalucka, M., Kwiatkowski, J., Lampart-Szczapa, E. and Rudzinska, M. 2005. Preparation of tocochromanol concentrates from red palm oil. J. Food Lipids. 12: 112-123.

Wan, J., Zhang, W., Jiang, B., Guo, Y. and Hu, C. 2008. Separation of individual tocopherols from soybean distillate by low pressure column chromatography. J. Am. Oil Chem. Soc. 85: 331-338.

Watanabe, Y., Yamauchi-Sato, Y., Nagao, T., Yamamoto, T., Ogita, K. and Shimada, Y. 2004. Production of monoacylglycerol of conjugated linoleic acid by esterification followed by dehydration at low temperature using *Penicillium camembertii* lipase. J. Mol. Cat. B: Enzymatic. 27: 249-254.

Wei, P.C., May, C.Y., Ngan, M.A. and Hock, C.C. 2005. Supercritical fluid extraction of palm carotenoids. Am. J. Environ. Sci. 1 (4): 264-269.

Xu, X. 2000. Production of specific-structured triacylglycerols by lipase-catalyzed reactions: a review. Eur. J. Lipid Sci. Technol. 287-303.

Yang, T., Rebsdorf, M., Engelrud, U. and Xu, X. 2005a. Monoacylglycerol synthesis via enzymatic glycerolysis using a simple and efficient reaction system. J. Food Lipids. 12: 299-312.

Yang T., Rebsdorf, M., Engelrud, U. and Xu, X. 2005b. Enzymatic production of monoacylglycerols containing polyunsaturated fatty acids through an efficient glycerolysis system. J. Agric. Food Chem. 53: 1475-1481.

You, L.L. and Baharin, B.S. 2006. Effect of enzymatic hydrolysis on crude palm olein by lipase from *Candida rugosa*. J. Food Lipids. 13: 73-87.

You, L.L., Baharin, B.S. and Man, Y.B.C. 2001. Effect of degumming process on chromatographic separation of carotenes from crude dan degummed palm oil. J. Food Lipids. 8: 27-35.

You, L.L, Baharin, B.S., Quek, S.Y. and Abdullah, M.A. 2002. Recovery of palm carotene from palm oil and hydrolyzed palm oil by adsorption column chromatography. J. Food Lipids. 9: 87-93.

Zaks, A. and Klibanov, A.M. 1985. Enzyme-catalyzed processes in organic solvents. Proc. Natl. Acad. Sci. USA. 82: 3192-3196.

APPENDIX

1. Hydrolytic activity of lipase

Activity was assayed by modified cupric acetate method (Lee and Rhee, 1993). Cupric acetate solution (5% w/v) was prepared and pH was adjusted to 6.1 by pyridine. For the lipase reaction in two-phase system, 0.1 mL of enzyme solution or 1.0 mg of immobilized enzyme, 0.2 mL of 0.1 M phosphate buffer pH 7 and 0.3 mL of 10% palm oil in iso-octane were mixed and incubated at 1200 rpm at 45°C for 15 min by using thermomixer. The enzyme reaction was stopped by adding 0.06 mL of 6 M HCl.

The upper iso-octane layer of 0.25 mL was taken out and mixed with 0.1 mL cupric acetate solution. Free fatty acid dissolved in iso-octane was determined by measuring the absorbance at 715 nm against the control which contains no free fatty acid. The lipase activity was determined by measuring the amount of fatty acid from the standard curves of palmitic acid. One unit of enzyme activity was defined as the enzyme necessary to release 1 μmol of palmitic acid per minute at the specific conditions.

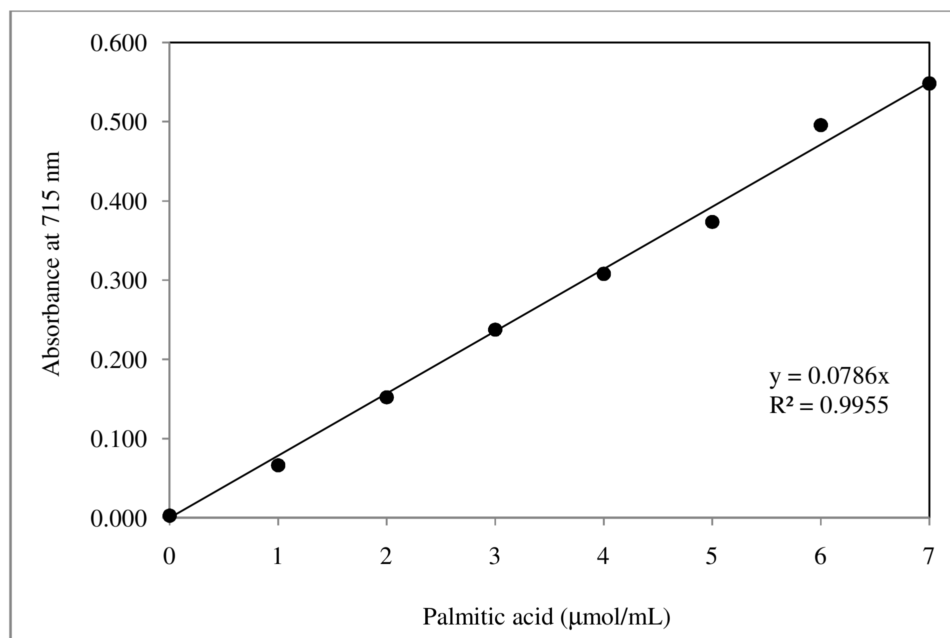


Figure 28. Standard curve of palmitic acid

2. Determination of oil composition by TLC/FID analyzer

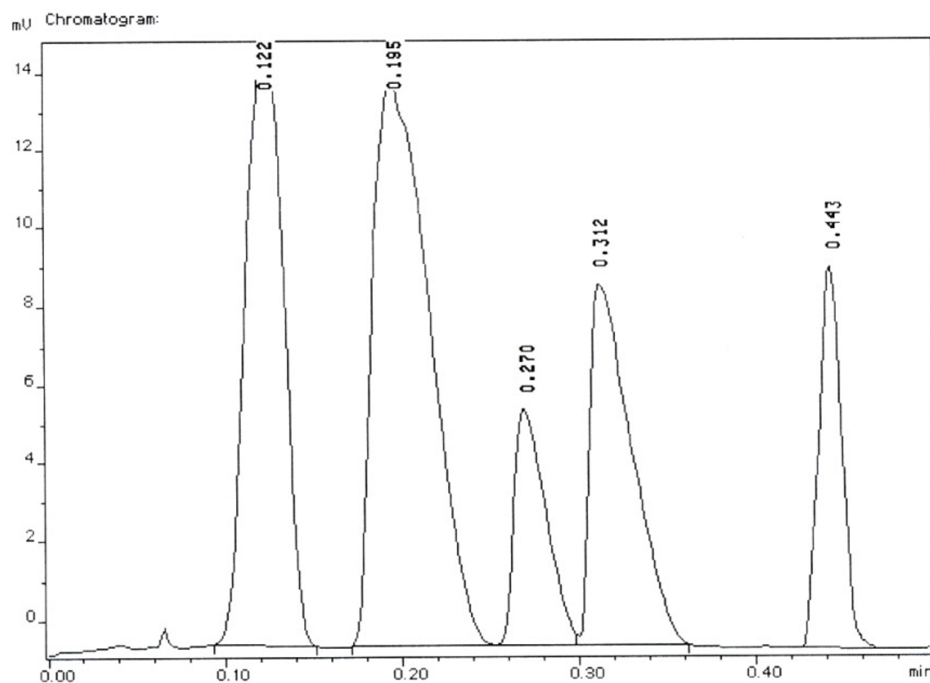


Figure 29. TLC/FID chromatogram of standard oil composition

Table 16. Standard oil composition obtained from TLC/FID analysis

Peak No	Name	Ret. Time (min)	Pk. Start (min)	Pk. End (min)	Area	Height (mV)	Area (%)
1	Triolein	0.123	0.093	0.152	12059	15.26	26.731
2	Oleic acid	0.195	0.172	0.248	16658	14.41	36.923
3	1,3-Diolein	0.270	0.248	0.298	3913	5.99	8.674
4	1,2-Dioleoyl-rac-glycerol	0.313	0.298	0.362	7831	9.07	17.358
5	Monopalmitin	0.443	0.427	0.472	4654	9.67	10.315
Total					45115	54.39	100.00

Condition:

Stationary phase: CHROMAROD S-III

Mobile phase: benzene : chloroform : acetic acid (50:20:0.7)

Gas flow: H_2 150 mL/min, Air 2.0 mL/min

Scanning speed: 30 s/scan

3. Analysis of total carotenoids concentration

Analysis of the total carotenoids concentration is determined spectrophotometrically as described by Wei *et al.* (2005). The sample was homogenized and weighed to the nearest ±0.0001 g into a 25 mL volumetric flask. The sample was dissolved with *n*-hexane and diluted to the mark. The solution was transferred into a 1 cm quartz cuvette and the absorbance was measured at 450 nm against *n*-hexane using UV-Visible spectrophotometer. The absorbance of the sample then was calculated by equation from standard series calibration curve using β-carotene as standard compound.

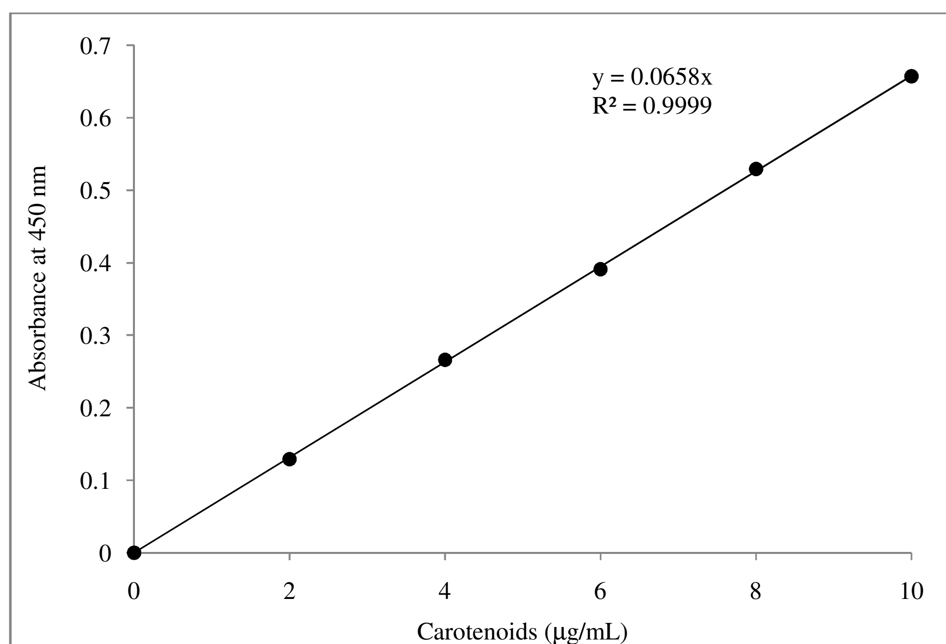


Figure 30. Standard curve of carotenoids concentration

4. Log P calculation based on solvent volume mixture

Log P of the solvent mixture was calculated by following equation:

$$\text{Log } P_{mix} = x_1(\text{Log } P_1) + x_2(\text{Log } P_2)$$

Where x_1 and x_2 represent molar fractions based on solvent volume, while Log P_1 and LogP_2 stand for Log P values based on solvent volume

Solvent:	Acetone	Solvent:	Isooctane
Molecular weight:	58.08	Molecular weight:	114.23
Specific gravity:	0.79	Specific gravity:	0.69

Solvent:	Tert-butanol	Solvent:	Hexane
Molecular weight:	74.12	Molecular weight:	86.18
Specific gravity:	0.78	Specific gravity:	0.66

Table 17. Log P calculation based on solvent volume mixture

1st Solvent	Log P	2nd Solvent	Log P	Mixture (v/v)	Mixture (w/w)	Molar fraction of 1st solvent	Molar fraction of 2nd solvent	Log P_{mix}
Acetone	-0.24	Isooctane	5.83	1:1	0.79:0.69	0.69	0.31	1.64
				3:1	2.37:0.69	0.87	0.13	0.55
Tert-butanol	0.35	Hexane	3.90	1:1	0.78:0.66	0.58	0.42	1.82
				3:1	2.34:0.66	0.80	0.20	1.06

5. Saponification value (AOAC, 1999)

Reagent:

1. 0.5 N Alcoholic potassium hydroxide solution
2. 0.5 N HCl
3. 1% phenolphthalein

Procedure:

Accurately weight 2 g of CPO into 250 mL Erlenmeyer. Pipet 25 mL alcoholic KOH solution into flask. Connect flask with water condenser and boil until CPO is completely saponified (ca 1 h). Cool and titrate with 0.5 N HCl, using phenolphthalein as indicator. Conduct titration duplicate and titrate blank too.

Calculation:

Saponification value, $SV = \frac{(B-A)\times N\times 56.1}{W}$

Where: B = volume of HCl to titrate blank

A = volume of HCl to titrate sample

N = HCl concentration

W = weight of sample

Molecular weight of CPO, $MW = \frac{56.1\times 1000\times 3}{SV}$

Where SV = saponification value

Result:

B (mL)	A (mL)	W (g)	N	SV	MW
14.9	0.6	2.12	0.5	189.2052	889.51
14.9	0.6	2.02	0.5	198.5718	847.55

Average of CPO Molecular Weight = 868.53 ± 29.67

6. Experimental data

Table 18. Effect of organic solvents on MAG production from CPO by IM-PS

Solvent	MAG content (%)	Standard deviation	Mark*
Acetone (A)	21.2	3.67	d
Tert-butanol (T)	57.31	2.25	b
Hexane (H)	10.08	0.56	e
Isooctane (I)	6.62	1.39	e
A:I (1:1, v/v)	32.20	9.85	c
A:I (3:1, v/v)	35.71	8.18	c
T:H (1:1, v/v)	77.86	3.66	a
T:H (3:1, v/v)	76.68	3.38	a

* Series with the same mark mean no significant different (p<0.05)

Table 19. Effect of IM-PS amount on MAG production and initial rate from CPO

IM-PS Loading (%)	MAG yield (%)	Standard deviation	Mark	Initial rate (mg/mL/h)
10	63.61	2.78	c	2.0361
20	66.80	2.93	bc	2.4758
30	67.91	2.93	bc	3.5623
40	71.19	2.92	ab	3.9863
50	75.51	3.58	a	3.7267
60	74.03	3.58	a	3.9248

* Series with the same mark mean no significant different (p<0.05)

Table 20. Time course of glycerolysis of CPO

Composition (%)	Sampling time (h)							
	0	3	6	9	12	15	18	24
TAG	90.81	65.88	33.07	22.2	16.52	11.49	6.93	2.37
FFA	3.27	15.31	25.31	20.39	21.27	24.13	22.47	20.81
DAG	1.85	11.72	19.91	16.38	15.92	11.16	8.39	5.63
MAG	4.07	7.1	21.71	41.03	46.29	53.22	62.21	71.19

The optimum conditions: 1 mL of 10% (w/v) CPO in tert-butanol/hexane 1:1 (v/v), glycerol with 8:1 molar ratio to CPO, 4% (w/w) water content in glycerol and the amount of IM-PS was 40% (w/v) based on solvent volume. The temperature was 45°C, 300 rpm of shaking speed and 24 h of reaction time.

Table 21. Effect of molar ratio of glycerol to CPO on MAG product and initial rate

Molar ratio glycerol to CPO	MAG yield (%)	Standard deviation	Mark	Initial rate (mg/mL/h)
3:1	26.98	2.35	d	1.56
4:1	44.40	4.02	c	2.25
5:1	59.73	5.04	b	2.91
6:1	67.18	5.91	a	3.52
7:1	69.01	4.78	a	3.58
8:1	74.26	1.28	a	4.25
9:1	70.46	6.10	a	5.12

* Series with the same mark mean no significant different ($p<0.05$)

Table 22. Time course of glycerolysis of CPO

Composition (%)	Sampling time (h)							
	0	3	6	9	12	15	18	24
TAG	90.81	46.56	26.48	17.92	9.99	5.88	6.28	2.90
FFA	3.27	14.04	19.50	28.12	26.67	18.37	22.08	11.02
DAG	1.85	22.01	23.13	12.81	13.12	16.15	14.17	11.82
MAG	4.07	17.40	30.90	41.15	50.22	59.60	57.47	74.26

The optimum conditions: 1 mL of 10% (w/v) CPO in tert-butanol/hexane 1:1 (v/v), glycerol with 8:1 molar ratio to CPO, 4% (w/w) water content in glycerol and the amount of IM-PS was 40% (w/v) based on solvent volume. The temperature was 45°C, 300 rpm of shaking speed and 24 h of reaction time.

Table 23. Effect of CPO loading on MAG product and initial rate

CPO load (%)	MAG yield (%)	Standard deviation	Mark	Initial rate (mg/mL/h)
5	61.87	0.40	b	4.05
10	74.26	1.28	a	4.23
20	47.58	4.08	c	2.35
30	45.07	4.19	c	2.22
40	42.29	3.96	cd	2.11
50	37.87	1.89	d	1.91

* Series with the same mark mean no significant different ($p<0.05$)

Table 24. Time course of glycerolysis reaction

Component (%)	Sampling time (h)							
	0	3	6	9	12	15	18	24
TAG	90.81	51.02	40.8	24.21	14.2	7.01	7.75	1.26
FFA	3.27	11.6	13.52	18.29	29.38	22.9	19.07	19.09
DAG	1.85	21.51	19.91	20.77	15.29	20.48	18.63	9.16
MAG	4.07	15.87	25.77	36.74	41.12	49.61	54.55	74.26

The optimum conditions: 1 mL of 10% (w/v) CPO in tert-butanol/hexane 1:1 (v/v), glycerol with 8:1 molar ratio to CPO, 4% (w/w) water content in glycerol and the amount of IM-PS was 40% (w/v) based on solvent volume. The temperature was 45°C, 300 rpm of shaking speed and 24 h of reaction time.

Table 25. Oil content and carotenoids content from original CPO chromatography on Diaion HP-20.

Fraction number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
TAG (g)	0.0002	0.0375	0.1276	0.1851	0.1397	0.0897	0.0651	0.0495	0.0379	0.0281	0.0147	0.0179	0.0302	0.0219	0.0346	0.0299	0.0014	0.0012	0.0001	0.0001
FFA (g)	0.0000	0.0000	0.0015	0.0039	0.0001	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
DAG (g)	0.0000	0.0000	0.0001	0.0016	0.0013	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
MAG (g)	0.0000	0.0001	0.0010	0.0021	0.0014	0.0007	0.0001	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Carotenoids content (mg)	0.0035	0.0100	0.0361	0.0495	0.0418	0.0307	0.0253	0.0184	0.0180	0.0163	0.0146	0.0138	0.0163	0.0488	0.0734	0.0614	0.0368	0.0250	0.0148	0.0009
Oil content (g)	0.0002	0.0376	0.1301	0.1927	0.1424	0.0904	0.0652	0.0495	0.0379	0.0281	0.0147	0.0179	0.0302	0.0219	0.0346	0.0299	0.0014	0.0012	0.0001	0.0001

Initial solvent was ethanol, second solvent was hexane. Each fraction was 1.5 mL, column temperature 50°C, flow rate 0.5 mL/min and sample loading 1 g.

Table 26. Oil content and carotenoids content from MAG-rich CPO chromatography on Diaion HP-20.

Fraction number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
TAG (g)	0.0001	0.0053	0.0066	0.0030	0.0043	0.0020	0.0054	0.0036	0.0026	0.0033	0.0035	0.0066	0.0065	0.0026	0.0026	0.0023	0.0024	0.0019	0.0018	0.0015
FFA (g)	0.0001	0.0259	0.0483	0.0290	0.0435	0.0182	0.0124	0.0085	0.0066	0.0025	0.0034	0.0076	0.0043	0.0013	0.0007	0.0006	0.0004	0.0000	0.0000	0.0000
DAG (g)	0.0005	0.0208	0.0270	0.0194	0.0331	0.0146	0.0089	0.0062	0.0057	0.0027	0.0020	0.0030	0.0019	0.0007	0.0006	0.0004	0.0002	0.0000	0.0000	0.0000
MAG (g)	0.0010	0.0487	0.1096	0.1494	0.0824	0.0368	0.0219	0.0154	0.0120	0.0086	0.0046	0.0038	0.0030	0.0008	0.0005	0.0004	0.0002	0.0000	0.0000	0.0000
Carotenoids content (mg)	0.0030	0.0059	0.0100	0.0117	0.0114	0.0131	0.0118	0.0109	0.0079	0.0078	0.0164	0.0768	0.0919	0.0645	0.0419	0.0280	0.0198	0.0087	0.0075	0.0045
Oil content (g)	0.0016	0.1007	0.1914	0.2008	0.1633	0.0716	0.0487	0.0337	0.0269	0.0171	0.0135	0.0210	0.0157	0.0053	0.0044	0.0036	0.0032	0.0019	0.0018	0.0015

Initial solvent was ethanol, second solvent was hexane. Each fraction was 1.5 mL, column temperature 50°C, flow rate 0.5 mL/min and sample loading 1 g.

Table 27. The Diaion HP-20 column chromatography for separation of carotenoids from MAG-rich CPO. Initial solvent was ethanol, second solvent was hexane. Each fraction was 1.5 mL, column temperature 40°C, flow rate 0.5 mL/min and sample loading 1 g.

Fraction number	Oil content (g)	Carotenoids content (mg)
1	0.0006	0.0028
2	0.0787	0.0082
3	0.1751	0.0162
4	0.2249	0.0137
5	0.1418	0.0119
6	0.0875	0.0089
7	0.0599	0.0075
8	0.0377	0.0089
9	0.0301	0.0075
10	0.0370	0.0100
11	0.0299	0.0588
12	0.0231	0.1126
13	0.0169	0.0736
14	0.0114	0.0269
15	0.0074	0.0139
16	0.0059	0.0066
17	0.0056	0.0047
18	0.0037	0.0036
19	0.0033	0.0027
20	0.0024	0.0026

Table 28. The Diaion HP-20 column chromatography for separation of carotenoids from MAG-rich CPO. Initial solvent was ethanol, second solvent was hexane. Each fraction was 1.5 mL, column temperature 50°C, flow rate 0.5 mL/min and sample loading 1 g.

Fraction number	Oil content (g)	Carotenoids content (mg)
1	0.0016	0.0030
2	0.1007	0.0059
3	0.1914	0.0100
4	0.2008	0.0117
5	0.1633	0.0114
6	0.0716	0.0131
7	0.0487	0.0118
8	0.0337	0.0109
9	0.0269	0.0079
10	0.0171	0.0078
11	0.0135	0.0164
12	0.0210	0.0768
13	0.0157	0.0919
14	0.0053	0.0645
15	0.0044	0.0419
16	0.0036	0.0280
17	0.0032	0.0198
18	0.0019	0.0087
19	0.0018	0.0075
20	0.0015	0.0045

Table 29. The Diaion HP-20 column chromatography for separation of carotenoids from MAG-rich CPO. Initial solvent was ethanol, second solvent was hexane. Each fraction was 1.5 mL, column temperature 60°C, flow rate 0.5 mL/min and sample loading 1 g.

Fraction number	Oil content (g)	Carotenoids content (mg)
1	0.0051	0.0098
2	0.1155	0.0098
3	0.1960	0.0098
4	0.2236	0.0098
5	0.1102	0.0093
6	0.0570	0.0097
7	0.0427	0.0095
8	0.0323	0.0080
9	0.0269	0.0083
10	0.0238	0.0165
11	0.0269	0.0841
12	0.0203	0.0768
13	0.0091	0.0481
14	0.0078	0.0342
15	0.0085	0.0187
16	0.0067	0.0101
17	0.0057	0.0074
18	0.0055	0.0054
19	0.0046	0.0029
20	0.0041	0.0019

Table 30. Effect of temperature on carotenoids and oil recovery from MAG-rich CPO on Diaion HP-20 column chromatography. Initial solvent was ethanol, second solvent was hexane. Each fraction was 1.5 mL, column temperature 40-60°C, flow rate 0.5 mL/min and sample loading 1 g.

Temperature (^{O}C)	Oil recovery in ethanol (%)	Carotenoids recovery in hexane (%)
40	88.85	76.1845
50	92.25	79.4101
60	89.36	74.2341

Table 31. The Diaion HP-20 column chromatography for separation of carotenoids from MAG-rich CPO. Initial solvent was ethanol, second solvent was hexane. Each fraction was 1.5 mL, column temperature 50°C, flow rate 0.5 mL/min and sample loading 0.5 g.

Fraction number	Oil content (g)	Carotenoids content (mg)
1	0.0067	0.0036
2	0.0681	0.0072
3	0.0830	0.0066
4	0.0701	0.0048
5	0.0497	0.0043
6	0.0328	0.0036
7	0.0259	0.0042
8	0.0244	0.0042
9	0.0183	0.0041
10	0.0161	0.0036
11	0.0119	0.0033
12	0.0066	0.0044
13	0.0150	0.0545
14	0.0062	0.0531
15	0.0047	0.0299
16	0.0048	0.0201
17	0.0047	0.0075
18	0.0011	0.0045
19	0.0002	0.0030
20	0.0022	0.0027

Table 32. The Diaion HP-20 column chromatography for separation of carotenoids from MAG-rich CPO. Initial solvent was ethanol, second solvent was hexane. Each fraction was 1.5 mL, column temperature 50°C, flow rate 0.5 mL/min and sample loading 1 g.

Fraction number	Oil content (g)	Carotenoids content (mg)
1	0.0016	0.0030
2	0.1007	0.0059
3	0.1914	0.0100
4	0.2008	0.0117
5	0.1633	0.0114
6	0.0716	0.0131
7	0.0487	0.0118
8	0.0337	0.0109
9	0.0269	0.0079
10	0.0171	0.0078
11	0.0135	0.0164
12	0.0210	0.0768
13	0.0157	0.0919
14	0.0053	0.0645
15	0.0044	0.0419
16	0.0036	0.0280
17	0.0032	0.0198
18	0.0019	0.0087
19	0.0018	0.0075
20	0.0015	0.0045

Table 33. The Diaion HP-20 column chromatography for separation of carotenoids from MAG-rich CPO. Initial solvent was ethanol, second solvent was hexane. Each fraction was 1.5 mL, column temperature 50°C, flow rate 0.5 mL/min and sample loading 2 g.

Fraction number	Oil content (g)	Carotenoids content (mg)
1	0.0008	0.0003
2	0.0637	0.0024
3	0.2166	0.0310
4	0.3855	0.0686
5	0.4209	0.0652
6	0.2714	0.0574
7	0.1918	0.0438
8	0.1376	0.0303
9	0.0861	0.0212
10	0.0499	0.0181
11	0.0347	0.0255
12	0.0232	0.0328
13	0.0179	0.0299
14	0.0239	0.1359
15	0.0142	0.1313
16	0.0074	0.0869
17	0.0052	0.0465
18	0.0043	0.0278
19	0.0002	0.0169
20	0.0002	0.0067

Table 34. The Diaion HP-20 column chromatography for separation of carotenoids from MAG-rich CPO. Initial solvent was ethanol, second solvent was hexane. Each fraction was 1.5 mL, column temperature 50°C, flow rate 0.5 mL/min and sample loading 3 g.

Fraction number	Oil content (g)	Carotenoids content (mg)
1	0.0101	0.0027
2	0.1121	0.0135
3	0.1919	0.0336
4	0.2636	0.0415
5	0.2868	0.0483
6	0.2997	0.0657
7	0.3050	0.0869
8	0.3161	0.0905
9	0.2150	0.0748
10	0.1704	0.0743
11	0.1483	0.0180
12	0.1256	0.0522
13	0.1169	0.1851
14	0.0772	0.2275
15	0.0512	0.1621
16	0.0218	0.0780
17	0.0128	0.0449
18	0.0069	0.0203
19	0.0048	0.0051
20	0.0048	0.0009

Table 35. The Diaion HP-20 column chromatography for separation of carotenoids from MAG-rich CPO. Initial solvent was ethanol, second solvent was hexane. Each fraction was 1.5 mL, column temperature 50°C, flow rate 0.5 mL/min and sample loading 4 g.

Fraction number	Oil content (g)	Carotenoids content (mg)
1	0.0044	0.0010
2	0.0703	0.0055
3	0.1389	0.0221
4	0.1832	0.0438
5	0.2352	0.0449
6	0.2561	0.0711
7	0.2809	0.0789
8	0.3234	0.1128
9	0.3618	0.1112
10	0.3685	0.1190
11	0.3915	0.1092
12	0.2854	0.0948
13	0.2043	0.0714
14	0.1505	0.0383
15	0.1280	0.0271
16	0.1287	0.1894
17	0.0633	0.2574
18	0.0327	0.0822
19	0.0184	0.0133
20	0.0134	0.0080

Table 36. Effect of temperature on carotenoids and oil recovery from MAG-rich CPO on Diaion HP-20 column chromatography. Initial solvent was ethanol, second solvent was hexane. Each fraction was 1.5 mL, column temperature 50°C, flow rate 0.5 mL/min and sample loading 0.5-4 g

Sample loading (g)	Oil recovery in ethanol (%)	Carotenoids recovery in hexane (%)
0.5	87.31	79.85
1.0	92.25	79.41
2.0	93.29	61.51
3.0	79.19	59.89
4.0	61.08	59.37

Table 37. The Diaion HP-20 column chromatography for separation of carotenoids from MAG-rich CPO. Initial solvent was ethanol, second solvent was hexane. Each fraction was 1.5 mL, column temperature 50°C, flow rate 0.5 mL/min and sample loading 1 g.

Fraction number	Oil content (g)	Carotenoids content (mg)
1	0.0001	0.0008
2	0.0356	0.0023
3	0.0865	0.0049
4	0.1146	0.0078
5	0.1263	0.0099
6	0.1262	0.0109
7	0.1172	0.0108
8	0.0935	0.0094
9	0.0748	0.0084
10	0.0541	0.0073
11	0.0357	0.0064
12	0.0270	0.0059
13	0.0221	0.0054
14	0.0170	0.0051
15	0.0086	0.0047
16	0.0086	0.0044
17	0.0065	0.0235
18	0.0065	0.0716
19	0.0058	0.0926
20	0.0057	0.0967
21	0.0054	0.0716
22	0.0034	0.0549
23	0.0030	0.0353
24	0.0014	0.0287
25	0.0001	0.0191
26	0.0001	0.0072
27	0.0001	0.0048
28	0.0001	0.0041
29	0.0001	0.0024
30	0.0001	0.0015

VITAE

Name Mr. Norholis Majid

Student ID 5011020070

Educational Attainment

Degree	Name of Institution	Year of Graduation
Bachelor of Science (Chemistry)	Mulawarman University	2006

Scholarship Awards during Enrollment

Double Degree Program Scholarship under National Education Department, Indonesia

Work – Position and Address

Chemical Analyst on TOTAL E&P, Handil Base, Indonesia

List of Publication and Proceedings

Majid, N., Cheirsilp, B. and H-Kittikun, A. 2008. Monoacylglycerol production from crude palm oil by glycerolysis reaction in organic solvents using immobilized lipase. The 20th Annual Meeting and International Conference of the Thai Society for Biotechnology. Taksila Hotel, Maha Sarakham, Thailand. 14-17 October 2008.