UNIVERSITI PUTRA MALAYSIA

ENZYMATIC DIRECTED INTERESTERIFICATION OF PALM OIL FOR PRODUCTION OF HIGHLY UNSATURATED PALM FRACTION

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FBSB 2017 27

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

May 2017

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

ENZYMATIC DIRECTED INTERESTERIFICATION OF PALM OIL FOR PRODUCTION OF HIGHLY UNSATURATED PALM FRACTION

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Chairman : Professor Lai Oi Ming, PhD
Faculty : Biotechnology and Biomolecular Sciences

Palm oil (PO) is a balanced oil with equal parts of unsaturated fatty acids (USAFA) mainly oleic acid (monounsaturated fatty acid, MUFA) and saturated fatty acids (SAFA) mainly palmitic acid. USAFA which include polyunsaturated fatty acids and MUFA are classified as good fat that can reduce the risk of coronary heart disease. The ultimate objective of this study was to produce a highly unsaturated palm fraction by enzymatic directed interesterification (EDIE) of PO and fractionation of the interesterified PO (EDIE PO). EDIE is a process whereby randomization of fatty acids on the triacylglycerol (TAG) molecules of oil takes place at a temperature lower than the melting temperature of its highest melting TAGs, normally the trisaturated (S_3) TAGs. The EDIE of oil will eventually result in a structured fat with a high concentration of triunsaturated (U_3) and S_3 TAGs. Since the S_3 TAGs were formed simultaneously with the U_3 TAGs, the second objective of this project was to obtain a highly saturated palm fraction. The first part of the study focused on the optimization of the EDIE processing parameters namely enzyme load, reaction temperature and reaction time using Response Surface Methodology in order to obtain EDIE PO having the highest U_3 TAGs concentration, with a tolerable if not the lowest amount of by-products (free FAs, monoacylglycerol, and diacylglycerol). The optimum reaction parameters were: enzyme load of 10%, reaction temperature of 30 °C and reaction time of 18 hours. EDIE of PO was able to significantly ($p<0.05$) increase the percentage of U_3 TAG in PO, i.e., from 4.3% before EDIE to 27.6% after EDIE, and S_3 TAGs, i.e., from 5.2% before EDIE to 31.9% after EDIE. Differential Scanning Calorimetry melting profile showed that the EDIE PO had two well-separated high-melting and low-melting endothermic peaks, indicating ease of fractionation. The EDIE PO was subsequently subjected to fractionation at 5, 10, 15, 20 and 25 °C to obtain the highly unsaturated palm fraction (palm olein, EDIE POo) and its co-product, a highly saturated palm fraction (palm stearin, EDIE POs). The U_3 TAGs and USAFA content of the EDIE POo, and S_3 TAGs and SAFA content of the EDIE POs fractions varied depending on the

fractionation temperatures. The lower the fractionation temperature, the higher the U_3 TAGs and consequently the higher the USAFA content of the EDIE POo. Reducing the fractionation temperature from 25 °C to 5 °C resulted in the increase of the U_3 TAGs and USAFA content of the EDIE POo from 34.6% to 43.0% and 66.7% to 75.0%, respectively. The S_3 TAGs and SAFA content of the EDIE POs were increased from 50.1% to 92.2% and 82.8% to 97.8%**,** respectively, when fractionation temperature increase from 5 °C to 25 °C. In conclusion, EDIE of PO followed by fractionation of the EDIE PO were able to produce a highly unsaturated fraction, EDIE POo and yielded a highly saturated fraction, EDIE POs. The highly unsaturated EDIE POo could be used as a healthy cooking oil. The highly saturated EDIE POs could be used as a *trans* fats alternative.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

INTERESTERIFIKASI TERARAH BERENZIM KE ATAS MINYAK KELAPA SAWIT BAGI PENGHASILAN PECAHAN MINYAK KELAPA SAWIT KAYA ASID LEMAK TAK TEPU

Oleh

NOOR LIDA BINTI HABI MAT DIAN

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Minyak kelapa sawit (PO) adalah minyak yang mempunyai keseimbangan dalam kandungan asid lemak tak tepu (USAFA) terutamanya asid oleik (asid lemak monotaktepu, MUFA) dan asid lemak tepu (SAFA) terutamanya asid palmitik. USAFA yang merangkumi asid lemak politaktepu dan MUFA dikelaskan sebagai lemak baik, yang boleh mengurangkan risiko sakit jantung koronari. Objektif utama kajian ini adalah untuk menghasilkan pecahan PO yang kaya dengan USAFA melalui proses interesterifikasi terarah berenzim (EDIE) ke atas PO diikuti dengan pemeringkatan ke atas PO yang telah diesterifikasi. EDIE adalah proses di mana perawakan asid lemak di dalam molekul triasilgliserida (TAG) minyak berlaku pada suhu lebih rendah daripada suhu lebur TAG yang mempunyai suhu takat lebur yang tertinggi, biasanya TAG tritepu (S_3). EDIE yang dilakukan ke atas PO menghasilkan PO terinterester (EDIE PO) yang kaya dengan TAG tritaktepu (U_3) dan S_3. Oleh kerana berlaku pembentukan TAG S_3 serentak dengan pembentukan TAG U_3, maka projek ini juga berobjektif untuk mendapatkan pecahan PO pejal yang tinggi SAFA. Bahagian pertama kajian ini tertumpu kepada mengoptimakan parameter pemprosesan EDIE, iaitu jumlah enzim, suhu tindak balas dan masa tindak balas, menggunakan “Kaedah Tindakbalas Permukaan” bagi mendapatkan EDIE PO yang mempunyai kandungan TAG U_3 yang tertinggi dan kandungan produk sampingan (asid lemak bebas, monoasilgliserida dan diasilgliserida) yang rendah. Parameter optimum reaksi ialah jumlah enzim sebanyak 10%; suhu tindak balas pada 30 °C dan masa tindak balas selama 18 jam. EDIE ke atas PO telah berjaya dengan ketara ($p<0.05$) meningkatkan kandungan TAG U_3 dan TAG S_3 di dalam PO. Kandungan TAG U_3 dan TAG S_3 setiap satunya meningkat daripada 4.3% sebelum EDIE kepada 27.6% selepas EDIE, dan daripada 5.2% sebelum EDIE kepada 31.9% selepas EDIE. Profil peleburan “Differential Scanning Calorimetry” menunjukkan bahawa EDIE PO mempunyai dua puncak peleburan yang ketara berbeza iaitu puncak peleburan bertakat lebut rendah dan puncak peleburan bertakat lebur tinggi,

yang menunjukkan kemudahan untuk pemeringkatan. Seterusnya, proses pemeringkatan pada suhu 5, 10, 20, 15 dan 25 °C dilakukan ke atas EDIE PO bagi mendapatkan pecahan lembut (EDIE POo) yang kaya dengan USAFA. Hasil sampingan daripada proses pemeringkatan EDIE PO adalah pecahan pejal (EDIE POs) yang kaya dengan SAFA. Kandungan TAG U_3 dan USAFA di dalam EDIE POo dan kandungan TAG S_3 dan SAFA di dalam EDIE POs berbeza mengikut suhu proses pemeringkatan. Semakin rendah suhu pemeringkatan, semakin tinggi kandungan TAG U_3 dan USAFA di dalam EDIE POo. Penurunan suhu pemeringkatan daripada 25 °C kepada 5 °C mengakibatkan peningkatan kandungan TAG U_3 dan USAFA di dalam EDIE POo masing-masing daripada 34.6% kepada 43.0% dan 66.7% kepada 75.0%. Kandungan TAG S_3 dan SAFA di dalam EDIE POs pula masing-masing meningkat daripada 50.1% kepada 92.2% dan 82.8% kepada 97.8%**,** apabila suhu pemeringkatan ditingkatkan daripada 5 °C kepada 25 °C. Kesimpulannya, EDIE ke atas PO diikuti dengan pemeringkatan ke atas EDIE PO berjaya menghasilkan pecahan lembut (EDIE POo) yang kaya dengan USAFA (terutamanya asid oleik) dan pecahan pejal (EDIE POs) yang kaya SAFA (terutamanya asid palmitik). EDIE POo boleh digunakan sebagai minyak masak atau minyak salad yang menyihatkan, manakala EDIE POs boleh digunakan sebagai alternatif kepada lemak *trans*.

ACKNOWLEDGEMENTS

First and foremost, great thanks to Allah S.W.T for giving me life, strength and opportunity to complete my study.

My sincere appreciation and deepest gratitude go to my supervisors, Prof. Dr. Lai Oi Ming, Prof. Dr. Tan Chin Ping and Dr. Miskandar Mat Sahri for the invaluable guidance and encouragements throughout my studies at Universiti Putra Malaysia (UPM). My sincere appreciation also dedicated to Dr. Lee Yee Ying for providing me the real help and support in completing my thesis. To all, thank you for sharing the knowledge, wisdom and ideas.

My acknowledgment also goes to Malaysian Palm Oil Board for giving me the opportunity to pursue my study, providing the financial support and for the laboratory facilities made available in MPOB, without which pursuing this research would not have been possible. Special gratitude to Isham Ismail, Adrina Malek and Gan Lee Yin for tirelessly assisting me in my experiments and analysis.

To my best friends forever, Che' Maimon Che' Ha, Zukarinah Kamarudin, Fatah Yah Abd. Manaf and Rohaya Halim, thank you for all the support, encouragement and understanding. Thank you for being my real friends.

To my mother Rokayah Osman, siblings, nieces and nephews, thank you for the continuous encouragement and constant prayers.

To my father Habi Mat Dian Zainal Abidin, this is for you too. You had always proud of me. You are no longer with me, but you left your spirit with me, the spirit that inspired me. May Allah's blessings be upon you and grants you Jannah. Ameen.

To the loves of my life, husband Nik Suhaimi Nik Jaafar, daughter Nik Nurazahara Suhada Nik Suhaimi and son Nik Zharif Aimran Nik Suhaimi, thank you for the unconditional love, for being there for me through thick and thin and when everything seems to feel so wrong. Thank you for believing in me.

Thank you very much.

I certify that a Thesis Examination Committee has met on 17 May 2017 to conduct the final examination of NOOR LIDA BINTI HABI MAT DIAN on her thesis entitled "ENZYMATIC DIRECTED INTERESTERIFICATION OF PALM OIL FOR PRODUCTION OF HIGHLY UNSATURATED PALM FRACTION" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

$-\Delta H_f$	Total crystallization enthalpy
$-\Delta H_{i°C}$	Partial crystallization enthalpy
°C	Degree celcius
%	Percent
ΔH_f	Total melting enthalpy
$\Delta H_{i°C}$	Partial melting enthalpy
1,2-DAGs	1,2- diacylglycerol
2-MAGs	2-monoacylglycerol
2,3-DAGs	2,3-diacylglcyerol
ANOVA	Analysis of variance
AOCS	American Oil and Chemist Society
CCD	Central Composite Design
CIE	Chemical interesterification
CPKO	Crude palm kernel oil
CPO	Crude palm oil
CVD	Cardiovascular disease
DHA	Docosahexaenoic acid
DIE	Directed interesterification
DSC	Differential Scanning Calorimetry
EDIE	Enzymatic directed interesterification
EDIE PO	Enzymatic directed interesterified palm oil
EIE	Enzymatic interesterification
ELCFA	Essential long-chain fatty acids
ELSD	Evaporative Light Scattering Detector
EPA	Eicosapentaenoic acid
FA	Fatty acid
FAC	FA composition
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
FFA	Free fatty acid
g	Gram
HDL	High density lipoprotein

HMFS	Human milk fat substitutes
HPLC	High Performance Liquid Chromatography
IV	Iodine value
LDL	Low density lipoprotein
MAG	Monoacylglycerol
MFA	Medium chain fatty acid
mg	Miligram
mL	Mililitre
mol	Mole
MUFA	Monounsaturated fatty acid
$Na_2S_2O_3$	Sodium thiosulphate
OLL	1-octadecenoate, 2,3- octadecadienoate sn-glycerol
OLO	1,3- octadecenoate, 2- octadecadienoate sn-glycerol
OOO	Triolein
p-NMR	Pulsed Nuclear Magnetic Resonance
PLL	1-hexadecenoate, 2,3- octadecadienoate sn-glycerol
PLO	1- hexadecenoate, 2- octadecadienoate, 3- octadecenoate sn-glycerol
PLP	1- hexadecenoate, 2- octadecadienoate,3- hexadecenoate sn-glycerol
PMF	Palm mid fraction
PMP	1- hexadecenoate, 2-tetradecanoate, 3-hexadecenoate sn-glycerol
PO	Palm oil
POO	1- hexadecenoate,2,3- octadecenoate sn-glycerol
POo	Palm olein
POs	Palm stearin
POP	1,3- hexadecenoate, 3- octadecenoate sn-glycerol
POS	1- hexadecenoate, 2- octadecenoate, 3-octadecanoic sn-glycerol
PPP	Tripalmitin
PPS	1,2- hexadecenoate, 3- octadecanoic sn-glycerol
PUFA	Polyunsaturated fatty acid
RBD	Refined, bleached and deodorized
RSM	Response surface methodology

S_2U	Disaturated-monounsaturated triacylglycerol
S_3	Trisaturated triacylglycerol
SAFA	Saturated fatty acid
SFC	Solid fat content
SMP	Slip melting point
SOO	1- octadecanoic, 2,3 octadecenoate sn-glycerol
TAG	Tricylglycerol
U_2S	Diunsaturated-monosaturated triacylglycerol
U_3	Triunsaturated triacylglycerol
USAFA	Unsaturated fatty acid
v/v	Volume/volume
vs.	Versus
w/v	Weight/volume
α	Alpha
β	Beta
β’	Beta prime

CHAPTER 1

INTRODUCTION

Dietary fat intake is required for optimal health. Dietary fat supplies the energy for most of our life functions. It provides the essential fatty acid (FA) such as linoleic and linolenic acids that cannot be synthesized in the body and the transport vehicle for fat-soluble vitamins A, D, E and K through the bloodstream. Dietary fats are also important for the functioning of nerves and brain, forming steroid and hormone needed to regulate many bodily processes. Choosing the right types of dietary fats to consume is one of the most important factors in maintaining a healthy life (Hulbert, 2005).

Oils high in unsaturated FA (USAFA) especially oils high in monounsaturated FA (MUFA) such as marine oils containing long-chain MUFA and oils high in oleic acid are considered to be healthy dietary oils. Marine oils containing long-chain MUFA are able to decrease cardiovascular disease risk (Yang *et al.*, 2017; Yang *et al.*, 2016). High oleic oils such as canola and olive with oleic acid content around 60% and 70%, respectively, are able to protect against cardiovascular disease by lowering total blood cholesterol levels, increasing the good high density lipoprotein (HDL) cholesterols and reducing the bad low-density lipoprotein (LDL) cholesterols levels (Crouse *et al.*, 2016; Huang and Sumpio, 2008; Teres *et al.*, 2007; Farinelli *et al.*, 2005). High oleic oils have a protective effect against LDL cholesterols oxidation, which plays an important role in atherogenesis (Teres *et al.*, 2007; Bonanome *et al.*, 1992; Reaven *et al.*, 1991). Artificial *trans* fats (or *trans* FAs) created in partial hydrogenation process to convert liquid vegetable oils to more solid oil is the bad fat. *Trans* FAs raise bad (LDL) cholesterol levels, lower good (HDL) cholesterol levels, and associated with a higher risk of developing heart disease, stroke, cancer, and type II diabetes (Iqbal, 2014).

Palm oil (PO) and its liquid fraction palm olein (POo) are considerably high in oleic acid. PO contains about 37.4-44.1% of oleic acid whereas the regular POo which has iodine value (IV) of 56-59 and super POo with IV of 60-67 have approximately 39.8-43.9% and 43.2-49.2% oleic acid, respectively (MPOB, 2009). Despite having significantly lower oleic acid than the commonly known high oleic oils like olive and canola oils, PO and POo have been reported to have a comparable effect on the blood cholesterol levels. Numerous reputable nutrition studies have evidenced that PO and POo diet significantly increases the level of HDL cholesterol, and decreases the total blood cholesterol and LDL cholesterol levels in human subject which have normal blood cholesterol level (Zhang *et al.*, 1997a; Choudhury *et al.*, 1995; Ghafoorunissa *et al.*, 1995; Sundram *et al.*, 1995; Khosla and Hayes 1994; Khosla and Hayes 1992; Sundram *et al.*, 1992; Truswell *et al.*, 1992; Hayes *et al.*, 1991; Marzuki *et al.*, 1991). The non-raising cholesterol effect of PO might be due to the influence of the amount of saturated FAs (SAFA) at the *sn*-2 position of the triacylglycerol (TAG) molecules backbone, as reported by Decker (1996). Despite the high palmitic acid content of around 39.2-45.8%, only 13-14% of the *sn*-2 position in PO TAG molecules is occupied by palmitic acid. More than 85% of *sn*-2 position of PO's TAG molecules are occupied by USAFA, mainly oleic acid (Ng *et al.*, 1992; Padley *et al.*, 1986; Berger, 1983). According to Kubow (1996) and Small (1991), the absorption of FAs dependent not only on its chain length and degree of saturation but also on its positional distribution in the glycerol backbone. The gastric and pancreatic lipases hydrolyze FAs at the *sn*-1 and *sn*-3 positions of dietary TAGs to produce free FAs (FFA) and 2-monoacylglycerol (MAG) (Carriere *et al.*, 1993; Small 1991). The FAs at the *sn*-2 position of dietary TAGs are preferentially absorbed through the intestinal wall while the FAs esterified at the *sn*-1 and *sn*-3 position, especially long-chain SAFAs such as palmitic acid are not well absorbed (Innis *et al.*, 1997; Pronczuk *et al.*, 1994). The FA at *sn*-1 and *sn*-3 position, especially the long chain SAFA are not absorbed because of their melting points that is substantially above body temperatures, causing them to have a strong tendency to form insoluble soaps with divalent cations, such as calcium and magnesium which in turn is excreted in the feces, reducing their absorption by animals (Innis *et al.*, 1997). Since the majority of FA attached at the *sn*-2 position of PO and POo is of oleic acid, most of this oleic acid will be absorbed in the gut and as such PO and POo can behave like a high oleic oil despite their high SAFA content.

The quest for an ideal oil has always been a longstanding concern for the food industry. An ideal oil should have excellent functional properties and yet should not pose adverse health effect to human upon consumption. Over the years, deliberations over healthy oil and beneficial FAs have resulted in recommendations for a diet high in USAFA, i.e., high in MUFA and polyunsaturated FAs (PUFA), and low in SAFA. This declaration by some means had tarnished the image of PO and POo as a good and versatile food oil as despite their high content of MUFA, they also have high content of SAFA. Therefore, it is desirable to be able to convert PO and POo into products that are high in USAFA especially oleic acid and low in SAFA to improve the nutritive and functional values as well to counter the negative perceptions of PO and POo as unhealthy oil having limited functional properties due to their SAFA content.

Oleic acid enhancement of PO and POo could be made possible by various means. Through plant breeding, a new variety of oil palm known as PS12 could produce oil palm fruits that could yield PO having oleic acid content of more than 48% (Isa *et al.*, 2006). Fractionation of POo IV56 produced super POo which has about 50% oleic acid, with IV of more than 62 (MPOB, 2009). Additionally, enzymatic acidolysis of POo IV56 with oleic acid produced POo with the oleic acid content of about 56% (Saw and Siew, 2009). Chemical interesterification (CIE) of POo IV62 with FA methyl ester high in oleic acid (methyl oleate) followed by fractionation produced POo with 57% oleic acid (Ramli *et al.*, 2009). Enzymatic interesterification (EIE) of POo IV62 followed by fractionation yielded POo having oleic acid content of up to 67.0% (Siew and Saw, 2009).

Directed interesterification (DIE) is a catalytic process which redistributes FAs on the glycerol backbone of TAG molecules of oil and fats. DIE is carried out at a temperature lower than the melting temperature of the highest melting TAGs of the oils and fats. The reaction is initialized by melting the oils and fats prior to addition of a catalyst to expedite the reaction. Subsequently, the DIE temperature is reduced to a level that is low enough for the higher melting TAGs, normally trisaturated (S_3) TAGs, to crystallize out as they are formed. The high melting TAGs are then withdrawn from the reaction system (liquid phase) as once the crystallized TAGs are in the solid phase they do not take part in the DIE reaction. Theoretically, the reaction will continue to redistribute the residual FAs and thereby continuously form higher melting TAGs to re-establish the equilibrium. As such, continuous crystallization caused the reaction mixture to becomes less saturated. The DIE eventually results in a reaction mixture containing two main types of TAGs, which are S_3 and triunsaturated (U_3) TAGs (Rousseau and Marangoni, 2008). Through DIE, it is possible to convert a liquid oil into a plastic product with the consistency of a margarine or shortening, as reported in United States Patent 4,482,576 (Boot *et al.*, 1984) and United States Patent 4,419,291 (Lathauwer *et al.*, 1983). DIE of lard and tallow significantly increased the level of their S_3 and U_3 TAGs, in simultaneous with the reduction in diunsaturated-monosaturated (U_2S) and disaturated-monounsaturated (S_2U) TAGs (MacKenzie and Stevenson, 2000; Chobanov and Topalova, 1978). In addition, DIE of PO in the presence of sodium-potassium alloy as a catalyst at 30 °C in a nitrogen atmosphere resulted in POo having an IV of more than 95 and softening point below −8 °C (Lago and Hartman, 1986). Separation or fractionation of the DIE lipids resulted in a soft or liquid fraction high in U_3 TAGs and a solid fraction high in S_3 TAGs.

Fractional crystallization or fractionation is one of the most important technique for oils and fats modification. It is a fully reversible modification process. Fractionation is a thermo-mechanical separation process in which oils and fats especially those containing TAGs with a range of melting points, are physically separated into two or more fractions of distinct physical and chemical properties (Kellens *et al.*, 2007; Harris, 2005; Sreenivasan, 1978). Fractionation principally involves the partial crystallization of TAG components followed by physical separation of the solid crystallized higher-melting TAGs from the liquid phase containing the low-melting TAGs (Deffense, 1985). It works based on differences in solubility of the solid TAGs in the liquid phase, which further depends on their molecular weight and degree of unsaturation of the TAGs (Gibon, 2006). Three types of fractionation techniques are available, namely the dry fractionation, solvent fractionation and detergent fractionation. Dry fractionation is the most widely used as it is the simplest, cheapest and safest process (Kellen *et al.*, 2007; Harris, 2005).

The main objective of the current study was to produce a high USAFA especially MUFA or oleic acid palm fraction having a USAFA level of more than 70%, MUFA or oleic acid level of more than 60% and SAFA level of preferably less than 30%. The high USAFA or high MUFA palm fraction was produced *via* a combination of enzymatic directed interesterification (EDIE) of PO followed by fractionation of the EDIE PO. Both the steps above will result in the increase of USAFA and MUFA or oleic acid content in PO. As a solid palm fraction concentrated with S_3 TAGs and highly saturated was the by-product of the fractionation process, it was also the objective of this project to obtain the highly saturated palm fraction from the fractionation of the EDIE PO. Thus, the specific objectives of the present study were as follows:

1. To determine the optimum processing parameters of EDIE of PO to produce EDIE PO containing a high concentration of U_3 TAG, preferably the OOO TAG.
2. To investigate the changes in the physicochemical characteristics of the PO after EDIE process.
3. To fractionate the EDIE PO and subsequently study the physicochemical characteristics of the POo and POs fractions of the EDIE PO.

This thesis is presented in the following lay out:-

Chapter 1 presents the introduction to subjects associated with highly unsaturated oil and potency to produce high oleic palm fraction using modification process namely EDIE and fractionation, problem statement and the objectives of research along with this outline.

Chapter 2 covers the literature review on the topics related to this study.

Chapter 3 discusses on determination of the optimum processing parameters of EDIE of PO using Response Surface Methodology (RSM) to produce EDIE PO containing a high concentration of U_3 TAG, preferably the OOO TAG.

Chapter 4 discusses the changes in the physicochemical characteristics of PO following EDIE.

Chapter 5 discusses the physicochemical characteristics of POo and POs fractions of the EDIE PO. This chapter also discussses the effect of fractionation temperature characteristics of the POo and POs fractions of the EDIE PO.

Chapter 6 presents summary of the discussion, conclusion and recommendation for future work.

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