Novel solutions for lipid-based functional food ingredient development

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NOVEL SOLUTIONS FOR LIPID-BASED FUNCTIONAL FOOD INGREDIENT DEVELOPMENT LEI QIONG 2015

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List of Abbreviations

℃	Degree centigrade
ARA	Arachidonic acid
CLO	Cod liver oil
cm^{-1}	Wave number
DAG	Diacylglycerol
DCC	*N*,*N'*-dicyclohexylcarbodiimide
dd	Doublet of doublets
DEPT	Distortionless enhancement by polarization transfer
DHA	Docosahexaenoic acid
DMAP	4-Dimethyaminopyridine
DNA	Deoxyribonucleic acid
EA	Ethyl acetate
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EPA	Eicosapentaenoic acid
FAA	Fatty alcohol acetate
FAEE	Fatty acid ethyl ester
FAME	Fatty acid methyl esters
FDA	Food and Drug Administration

FFA	Free fatty acid
FOSHU	Foods for Specified Health Use
FT-IR	Fourier transform infrared
FUFOSE	Functional Food Science in Europe
GC-MS	Gas chromatography–mass spectrometry
h	Hour
HMQC	Hetero-nuclear multiple quantum correlation
Hz	Hertz
IY	Interesterification yield
J	Coupling constant
LC-ESI-MS	Liquid chromatography-electrospray ionization-tandem mass spectrometry
LCFA	Long chain fatty acid
LCMFAA	Long chain monounsaturated fatty alcohol acetate
LCMFAC	Long chain monounsaturated fatty acid
LCMFAEE	Long chain monounsaturated fatty acid ethyl ester
LCMFAL	Long chain monounsaturated fatty alcohol
LP	Liquid phase
m	Multiplet

MAG	Monoacylglycerol
MCFA	Medium chain fatty acid
min	Minute
MUFA	Monounsaturated fatty acid
NMR	Nuclear magnetic resonance
ppm	Parts per million
PUFA	Polyunsaturated fatty acid
s	Singlet
SCFA	Short chain fatty acid
SFA	Saturated fatty acid
SP	Solid phase
t	Triplet
TAG	Triacylglycerol
TLC	Thin layer chromatography
TMS	Tetramethylsilane
δ	Chemical shift (ppm)

Abstract

Lipids are important functional food ingredients in promoting human health. Aiming at providing novel solutions for the development of lipid-based functional food ingredient, this thesis discusses the design of novel structured lipid and the improvement of natural oil utilization methodology and processing technology. Firstly, new triacylglycerol cluster is designed as potential fat substitute that can reduce calorie intake and avoid side effects, and the molecular probe has been synthesized and characterized for metabolism study. Secondly, jojoba oil is enzymatically interesterified with ethyl acetate to convert the enriched source of beneficial long chain monounsaturated fatty acid and fatty alcohol to functional ethyl esters and acetates. Lastly, a novel method to enrich omega-3 fatty acids on the basis of two simple food technologies is proposed and efficiently doubles the omega-3 content in cod liver oil. The study provides efficient and economical solutions for the development of lipid-based functional food ingredients, contributing to address human health problems.

Chapter 1

General Introduction

1.1 Functional Food

1.1.1 History

Conventionally, food plays a role of providing nutritional support for individuals in the form of fats, proteins, carbohydrates, vitamins, minerals, etc. Given the increasing demand of global consumers for healthier diets, the functions of food need to go beyond the supply of energy, basic nutrition and pleasure, i.e., enjoyable aroma, color, and taste, and to provide health benefits.[1-2] Over the last few decades, the health-promoting effects of foods and food ingredients have been substantiated with abundance evidence and drawn considerable attention in both scientific community and industrial circles.[3]

In the 1970's, Japanese scientists coined the term 'functional foods', which linked the consumption of certain foods or food products with improved health benefits and disease prevention.[2-3] Systematic and large-scale scientific research and regulatory oversight on the development of functional foods was also initiated in Japan and funded by the Japanese government in the early 1980's, with advances in chemical identification of bioactive compounds, analysis on physiological regulation function by food, processing and formulation of functional foods as well as illumination of the molecular mechanisms involved in the modulation of metabolic disorders.[3] To establish a new category of foods for potential health enhancing benefits as one of the national efforts to reduce the escalating cost of health care, in 1991, the Japanese government established 'Foods for

Specified Health Use' (FOSHU) policy to regulate the production and marketing of functional foods.[2-3] Since then, foods that intend to be used for health improvement and for which specific health-promoting claims are allowed to be displayed, has been included into the food categories within the Nutrition Improvement Law as 'foods for special dietary use',[4] and over 1100 FOSHU products has come onto the Japanese market by early 2014.[5]

Table 1.1. Regulatory bodies governing the health claims of functional foods in different countries around the world.

Country	Establishment date	Regulatory body for nutrition health claims
United States	1906	Food and Drug Administration (FDA)
Sweden	1961	Swedish Nutrition Foundation
Australia & New Zealand	1991	Food Standards Australia New Zealand
Canada	1996	Health Canada
Netherlands	1998	Voedingscentrum
Brazil	1999	National Health Surveillance Agency
United Kingdom	2000	Food Standards Agency
Japan	2001	Ministry of Health, Labor and Welfare
European Union	2002	European Food Safety Authority
France	2010	French Agency for Food, Environmental and Occupational Health & Safety
China	2013	China Food and Drug Administration

Such initiative in Japan has evoked a worldwide enthusiasm for functional foods market, a number of countries have established regulatory bodies and developed policies to govern the manufacture and marketing, especially the health claims, of functional food (Table 1.1).[2-3]

1.1.2 Definitions

Defining 'functional foods' has been a struggle from the first day the term was generated because it is indeed a wide variety of food products with various components, some of which are classified as nutrients, affecting one or more body functions relevant to either the maintenance of healthy state and/or to the reduction in disease risk.[4, 6] There are as many definitions of functional foods as the number of authors referring to it, which vary from simple statements such as:

- 'foods and food components that may provide benefits beyond basic nutrition';[7]
- 'foods or food products marketed with the message of the benefit to health';[8] or
- 'everyday food transformed into a potential lifesaver by the addition of a magical ingredient';[9]

to very elaborate definitions such as:

- 'food and drink products derived from naturally occurring substances consumed as part of the daily diet and possessing particular physiological benefits when ingested';[10]
- 'similar in appearance to, or may be, a conventional food, consumed as part of a usual

diet, which is demonstrated to have physiological benefits and/or to reduce the risk of chronic disease beyond basic nutritional functions'.[11]

According to the European Commission Concerted Action on 'Functional Food Science in Europe' (FUFOSE), the definition of functional foods in the consensus document on 'Scientific Concepts of Functional Foods in Europe' is:

> A food can be regarded as 'functional' if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and well-being and/or reduction of risk of disease. Functional foods must remain foods and they must demonstrate their effects in amounts that can normally be expected to be consumed in the diet: they are not pills or capsules, but part of a normal food pattern.
>
> A functional food can be a natural food, a food to which a component has been added, or a food from which a component has been removed by technological or biotechnological means. It can also be a food where the nature of one or more components has been modified, or a food in which the bioavailability of one or more components has been modified, or any combination of these possibilities. A functional food might be functional for all members of a population or for particular groups of the population, which might be defined, for example, by age

or by genetic constitution.[12]

Despite the bewildering array of definitions, there is no doubt that 'functional foods' has become a quite unique concept requires (i) that the base product is a conventional or everyday food, (ii) that it contains or is fortified with an ingredient, a micronutrient or a naturally occurring compound with one or more beneficial effects on health, well-being or disease risk reduction, (iii) that these effects go beyond normal and adequate nutritional effects, and (iv) that these effects has been scientifically evaluated and permitted to claim the specific beneficial health effects.[4] It is noteworthy that functional foods are still foods, not drugs, as they have no therapeutic effects. Meanwhile, their role regarding disease, in most cases, will be in disease risk reduction rather than prevention.[4]

1.1.3 Classifications

The category of functional foods varies according to different principles in the literature. It can be classified by the diseases it is expected to reduce risk in or alleviate, such as cardiovascular disease, osteoporosis, colon cancer, diabetes, Alzheimer's disease.[13] According to the category of its specific bio-active ingredients, functional food can be sorted as antioxidants, phytochemicals, lipids, dietary fiber, minerals, probiotics, prebiotics, synbiotics, etc.[14] Based on the physiological effects, it can be classified as immunology, cholesterol-lowering action, digestibility, anti-tumor activity.[15] Physicochemical and organoleptic properties may also serve as the classification standard,

e.g. solubility, color, moisture and texture.[16] Lizhe Wang and Torsten Bohn classified functional foods according to the processes used in its production, for instance, size-classification, sorting, filtration, centrifugation, de-foaming, de-dusting, flotation, mixing, dispersion, freezing, osmosis, encapsulation, chromatography.[17] However, in most cases, food groups were preferred for the classification of functional foods, namely oils and fats, carbohydrates, peptides, meat, seafood, dietary fiber, fruits and vegetables, dairy and probiotic products, soy food products, beverages, miscellaneous.[18]

1.1.4 Market

Over the past two decades, the global market of functional foods has witnessed dramatic expansion in size, outpacing the overall food and beverage industry by some distance.[19] According to Leatherhead Food Research (Surrey, UK), where the term 'functional foods' was limited to 'food and drinks which make specific functional health claims and excludes products such as energy drinks',[20] the revenue of global functional foods market for the year 2013 was $44 billion, indicating an increase of 26.7% in value terms compared with 2009. When a more general definition was applied, along with Research and Markets (Dublin, Ireland), the global functional foods market has topped $168 billion in 2013, and it is forecasted to exceed $305 billion by 2020 with an annual average growth rate of about 8.5%.[21-22]

According to Leatherhead data, Japan accounted for 40% of the whole market of

functional foods in 2013 with $17.5 billion sales worth, increased 25% over 2009. Meanwhile, the United States market has grown by more than 29% since 2009 and accounted for 38% of the global market value in 2013 with $16.5 billion sales worth. Fueled by the rising popularity of wholegrain-fortified breakfast cereals and probiotic dairy foods, the United States has experienced spectacular level of growth and would surpass Japan to be the largest functional foods market in the world.[23] The European market was suffered from both strict regulations for functional foods and the economic downturn, resulting in a 20% share of global market value in 2013.[23]

Apart from these three leading regional markets for functional foods, Asia-Pacific, namely China, Australia, India, Malaysia and Korea, has shown vast expansion potential and is advancing rapidly.[24] Particularly, in China, the ever-growing awareness and demands of consumers for functional foods is a boost for such business, and local support of strong fine chemical industry also contributes to the high-speed market growth.[24-25] The value sales of packaged functional foods market in China reached $24.6 billion in 2012, with a compound annual growth rate of 21.8% from 2007 to 2012.[25] Based on the prediction of Euromonitor International, the impressive growth would remain and the market value would approach $55 billion by 2017.[26]

The main driving force of such growth is the increasing consumer awareness of the health benefits that functional foods can provide, and the growing desire of the

population to maintain an active lifestyle. Hence, the global market for functional foods will continue to be one of the most dynamic and growing segments within food industry.

1.1.5 Customer awareness

As the concept of functional foods becomes more widespread, consumers around the world are realizing that they can take charge of their own health by choosing healthy diets, and about 80% are already using functional foods to maintain healthy state and reduce disease risk, such as diabetes, obesity, hypertension and high cholesterol.[27] For instance, the 2013 Functional Foods Consumer Survey reveals that 90% of American consumers believe functional foods have certain health benefits beyond basic nutrition (87% in 2011, and 89% in 2009), and 86% of the consumers are willing to learn more about functional foods.[28]

The main information sources of functional foods are product advertisements on news media (such as the Internet, magazines, television), medical sources (primarily physicians), reports in the media, product description on the packaging, friends and family, and diet/health books.[18] While the majority of consumers continue to get information from the media, health professionals are named to be the most believable source of nutrition and health information, with enormous influence on motivating consumers to incorporate functional foods into their diet.

Although the term ‘functional foods’ are becoming well-recognized, previous surveys

and research indicate that both American and European consumers have limited knowledge on the interaction of specific functional food ingredient and its health benefits.[2, 18] Yet, both public health authorities and private companies are finding their ways to deliver more information to the population, for better understanding of specific functional ingredients. Despite the relatively low level of awareness of certain functional food ingredient benefits, the overall awareness of functional foods is certainly thriving, which explains the raise in functional foods consumption.

1.2 Functional Food Ingredients

What on earth are the magical elements that make 'functional foods' functional? Some foods may naturally contain a functional ingredient, such as fruits and vegetables which are high in phytochemicals content. Or, a specific ingredient may be added, increased, decreased, or even removed, to make a food product able to positively affect health. This section focuses on some typical functional food ingredients and their functionality in promoting health.

1.2.1 Oxidation and antioxidants

Oxidation is one of the most important metabolic reactions that support the normal functionality of cells, such as enzymatic reactions, electron transport in mitochondria, signal transduction, activation of nuclear transcription factors, etc.[29] During the oxidation reaction, free radical species are produced, and they are stronger and dangerous oxidants

that may overwhelm the protective enzymes and cause destructive or even lethal cellular effects by oxidizing membrane lipids, enzymes, cellular proteins and DNA, when an excess of such free radicals is formed.[29] It is one of the main reasons that accelerate human ageing and disease, and at the same time, the major cause of chemical spoilage of foods.[29] It was estimated that post-harvest deteriorative oxidation reactions have caused half of total loss in the global fruit and vegetable crops.[29] For example, lipid, one of the major bulk constituents in foods, is easily subjected to oxidation, and results in rancidity, deterioration of the flavor, color, texture and safety of foods by forming toxic compounds, and/or nutritional value loss (e.g., destruction of vitamin A, C, and E). Additionally, lipid oxidation is also believed to be very important in the development of atherosclerosis, coronary heart disease, cancer and human aging.[30-33]

Fortunately, the onset of such oxidation can actually be prevented or delayed by intentionally adding antioxidants to foods and including them in our diet.[29] Halliwell and Guteridge defined food antioxidants as ‘any substance that when present in low concentrations compared to those of an oxidizable substrate significantly delays or prevents oxidation of that substance’.[34] Their major role is to maintain the nutritional value of foods and prevent rancidity and off-flavors which are mainly caused by lipid peroxidation. On the other hand, dietary antioxidants can also decrease the oxidative damages to the DNA and proteins in human gastrointestinal tract and body tissues,

suppressing the development of tumor, cancer, cardiovascular and neurodegenerative diseases.[29] Since the first application of gum guaiac as food antioxidant into animal fats, especially lard, in the 1940's, antioxidants have become an important group of food additives, which has various implications for human well-being and nutritional status.[29]

Chemically, food antioxidants can be classified as phenols, β-diketones, nucleic acid bases, amino acids, peptides and amines, phospholipids, ascorbic acid and reductones, Sulphur and selenium compounds, carotenoids, melanoidines, hydroquinones, organic acids, porphine compounds, protease inhibitors, terpenes, indoles and isothiocyanates.[29] These antioxidants can be found in foods naturally or synthesized intentionally. Natural antioxidants, such as ascorbic acid and tocopherols, could be extracted and included into food formulations to stabilize them. For instance, oat and amaranth oils are rich in tocopherols and squalene, and have been added to other oils for oxidation prevention.[29] Furthermore, extracts from green tea, sage and rosemary can be mixed into various food products to control oxidation. Propyl gallate was synthesized to prevent oils and fats in foods from oxidation.[35] Tertiary butylhydroquinone is used as a synthetic antioxidant to enhance the storage life of unsaturated vegetable oils and edible animal fats.[36] Similarly, butylated hydroxyanisole and butylated hydroxytoluene have been synthesized and added into edible fats and fat-containing foods to prevent or retard food rancidity.[37-38] The conjugated aromatic rings of these two compounds are able to capture free radicals and

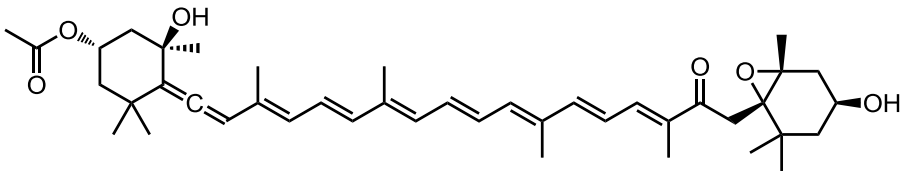
forestall further free radical reactions. Other antioxidant mechanisms have also been well-studied and summarized in Table 1.2.

Table 1.2. Different function mechanisms of food antioxidants and their examples.[39]

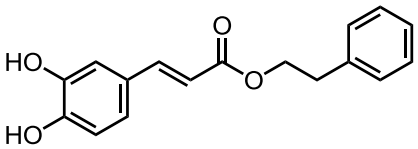
Function mechanism	**Examples**
Inactivate lipid free radicals	phenolic compounds
Prevent the conversion from hydroperoxides to free radicals	phenolic compounds
Promote the activity of proper antioxidants	citric acid and ascorbic acid
Bind heavy metals to form inactive compounds	phosphoric acid and Maillard compounds
Transform singlet oxygen to triplet oxygen	carotenes
Reduce hydroperoxides via non-radical way	proteins and amino acids

Nowadays, new isolation methods for natural antioxidants and novel structure design for synthetic antioxidants are required due to the growing industrial demands. Furthermore, there is a trend to discover innovative antioxidants that can serve as therapeutic agents with minimum side effects and have least biopharmaceutical problems.[40] For instance, fucoxanthin, a characteristic carotenoid-type antioxidant, can be extracted from edible brown seaweeds and has shown a wide range of biological properties and physiological functions, such as insulin resistance improvement, blood glucose level control, anti-obesity, anti-diabetic, anti-inflammatory and anticancer effects (Figure 1.1a).[41] Purified from propolis, caffeic acid phenethyl ester is a phenol-type antioxidant that is effective against oxidative stress, inflammation, infections, anxiety,

neurodegeneration, diabetes and cancer (Figure 1.1b).[42] Such broad spectra of biological pharmacological activities of fucoxanthin and caffeic acid phenethyl ester make them quite promising to be functional ingredients in food industry.



(a) Fucoxanthin



(b) Caffeic acid phenethyl ester

Figure 1.1. Molecular structure of (a) fucoxanthin and (b) caffeic acid phenethyl ester.

1.2.2 Infant Formula and human milk fat substitute

Supplemental infant nutrition has a long history which can date back to the 18th century, before pediatricians recommended a formula involving evaporated milk as alternatives to breastfeeding.[43] Animal milks from cows, mares, donkeys and goats were tried, among which cow's milk became the most popular thanks to its ready availability. Later, cow's milk was found to be of high content in not only protein but also electrolyte (e.g., sodium), which might put a strain on infant kidneys and therefore were not recommended.[43] Although the evaporated milk is easier to digest, since it is in fact denatured proteins, it is nutritionally inadequate for infant growth. As the inadequate mother milk supply becomes far more common and an increasing number of mothers

even choose not to nurse their babies, the needs to find new human milk substitutes is unprecedented. During the 1920's, considerable effort was spent on the development of artificial feeding and society showed high expectations and acceptance towards scientific infant foods that enriched with extra nutritional value.[43] In 1931, Pablum, a mineral- and vitamin-fortified infant cereal, was firstly commercialized in the United States and Canada and turned out to be a major commercial success. Since then, a vigorous research campaign on the study of modern infant formula was started in both academia and industrial circles.[43]

Modern infant formula was defined as industrially produced milk substitutes that were designed for infant consumption.[43] Usually, the design is on the basis of either soy or cow milk, and strives to duplicate the nutritional profile of the natural human breast milk. Since the exact biological and nutritional properties of human breast milk are not completely understood to date, infant formula is just an imperfect approximation. One typical example is that human breast milk can provide potent immunity for a breast-fed infant considering its own immune system is not mature.[43] But still, infant formula is the only substitute for natural human breast milk that is considered nutritionally acceptable for the infants under one year old according to the medical community.[43]

The gross composition of infant formula and milk from human and animals are summarized in Table 1.3. The content of human milk protein is approximately 1.0%,

while that of lactose is 7.0%, providing 40% of total human milk's energy. The major energy source of human breast milk is fat (3.8%), which plays multiple roles in infant nutrition and development, such as energy source and storage, absorption and transportation vehicles for fat-soluble compounds. In fact, fat is the most variable constituent in human milk and becomes the focus of infant formula research.

Table 1.3. Milk compositions from different source (%).[43]

Milk source	Fat	Protein	Lactose	Ash	Total solids
Human	3.80	1.00	7.00	0.20	12.40
Formula	3.80	1.50	7.20	0.30	13.0
Bovine	3.50	3.40	5.00	0.70	12.50

Although the fatty acid compositions vary a lot with diet, season, lactation stage and individual conditions, one general structural pattern is shared in the triacylglycerol (TAG) molecules of human milk fat.[44] Specifically, it is a unique fatty acid distribution where approximately 70% of palmitic acid locates at the sn-2 position while oleic acid and linoleic acid locate at the sn-1 and sn-3 positions.[44-46] Such a unique fatty acid distribution was substantiated to be able to facilitate absorption and metabolism.[45, 47] Because the saturated fatty acid (SFA) palmitic acid would not be hydrolyzed by the pancreatic lipase and stay at the sn-2 position, forming 2-monopalmitin and thereafter micelle with bile salt to be efficiently absorbed into the intestine wall cells.[45] On the contrary, most of the traditional infant formula have not taken this factor into

consideration and have a random fatty acid distribution within TAG molecules. In this case, the long-chain SFA have a good chance to be placed at the sn-2 position and hydrolyzed to form free fatty acids (FFA), leading to the generation of calcium soap which is poorly absorbed in the intestine and possible constipation symptoms.[48-49]

Considering the advantage of fatty acid distribution in human breast milk fat and the complications induced by traditional infant formulas, imitating the advantageous structure was preferred in the design of novel infant formula.[50-51] Thanks to the regioselectivity of certain lipase types, enzymatic interesterification, transesterification, acidolysis, and re-esterification are developed for the preparation of this structured lipids.[45, 52-53] Furthermore, polyunsaturated fatty acids (PUFA), specifically eicosapentaenoic acid (EPA, C(20:5) ω-3), docosahexaenoic acid (DHA, C(22:6) ω-3), and arachidonic acid (ARA, C(20:4) ω-6), play an important role in bone mineralization and bone mass, as well as the growth and development of infant central nervous system.[44] Hence, researchers are tending to involve these beneficial fatty acids into infant formula design as supplementary nutrients. Till now, the synthesis of structured TAG containing palmitic acids at the sn-2 position and PUFA at the sn-1 and sn-3 positions has been realized in multiple ways.[54-55] For instance, Dilek Turan and his coworkers reported a method where hazelnut oil was firstly enriched with palmitic acid at the sn-2 position via interesterification with ethyl palmitate catalyzed by Novozym 435, followed by

incorporation of DHA and ARA in the presence of Lipozyme RM IM.[54] These products are able to mimic the chemical and physical properties of human breast milk fat and provide additional health benefits brought by PUFA.[44]

1.2.3 Lipid-based films and coatings

Food is easy to deteriorate due to chemical reactions and/or physicochemical changes, which are often induced by mass transfer within the food entity, or between food and the environment medium, as well as microbial spoilage.[56-57] It makes food packaging a principal discipline in food technology which focuses on the protection and preservation of foods and their raw materials.[57] Compounds that should be prevented to migrate include water, flavor and oxygen, among which water is the main object to be controlled.[56] Although the commonly used petrochemical based plastics, such as polyesters, polyolefins and polyamides, are efficient in retarding oxygen and aroma compounds variety in food with good tear and tensile strength and heat sealability, they are completely non-biodegradable, which may result in environmental pollution and pose serious ecological problems.[57] Thus, their use in any shape and form has to be restricted and gradually abandoned as an effort to circumvent waste disposal problems.[58]

In this light, biodegradable barriers are developed based on hydrophobic substances, such as lipids.[59-60] Thanks to their nonpolar nature, such hydrophobic substances are efficient against moisture migration, and can function as barriers to oxygen, aroma, flavor

and oil, enhancing shelf life and improving food quality.[56, 61] It may also physically protect foods and improve food integrity by reducing breakage and bruising. Addition of antimicrobials and antioxidants into lipid-based food packaging is easy thanks to its good compatibility, thus helping enhance its protective functions. Lipid-based food packaging is essentially derived from natural sources and therefore compatible with environment. Additional advantage is that it can act as soil conditioner and fertilizer upon biodegradation or disintegration.[57] Most importantly, lipids are edible which can be an integral component of and to be eaten together with the food product, sometimes providing additional sensory attributes, such as color, gloss, smooth surface.[61] It attracts consumers' interest greatly since it perfectly fulfills their demands for convenient, safe and stable foods and food ingredients, while avoids negative environmental impacts caused by nonbiodegradable packaging waste. Supported by the vast natural sources and expanding synthesis scale, lipid-based food packaging is tomorrow's need, especially for value-added food products.[56, 59]

Numerous studies has been done and the abundant patent, scientific and technical literatures allow us to make a list of all lipid-based substances that can be used as ingredient of edible films and coatings (Table 1.4).[56] A wide range of substances can be incorporated into edible packaging formula according to the target application. The filming and coating efficiency depends on the lipid nature, particularly, its chemical

structure, composition arrangement, hydrophobicity, physical state, and interactions with other components.[61] Most commonly, edible lipids are applied on fresh fruit and confections to retain moisture and slow down aerobic respiration, as well as improve their appearance by providing gloss.

Table 1.4. Lipid-based substances that can be used in edible films and coatings.[56, 62]

Category	Substances	Applications
Oils and fats	Animal fats and plant oils in natural, concentrated, fractionated, hydrogenated and/or reconstituted form.	Fresh vegetables and fruits, confectionery and biscuits.
Waxes	Natural animal or vegetable waxes (e.g., jojoba, carnauba, bees, whales).	Fresh vegetables and fruits, wine cork.
Lacs and varnishes	Native, whitened, dewaxed shellacs and gum lacs; pine, larch, and linseed oil; terpenes from citrus.	Pharmaceutical, food surface and feeling improver, flavor.
Resins	Natural chicle, guarana, olibanum, opopanax, styrax.	Fresh vegetables and fruits.
Essential oils	Mint, camphor and citrus fruit essential oils.	Fish and meat products.
Surfactants	Fatty alcohols, monoacylglycerol (MAG), diacylglycerol (DAG), FFA, fatty acid sucroesters, lecithins.	Fresh vegetables and fruits.

Over the past decade, efforts have also been paid to develop novel formulations that combine different groups of coating material with lipid, to overcome the low mechanical integrity and inconvenient casting conditions required by lipids.[63-65] Proteins and polysaccharides are good candidates due to their fair film-forming properties.[66] Barbara Bravin reported their study on an edible film consisted of corn starch, methylcellulose

and soybean oil and evaluation on its efficiency of extending bakery products shelf-life.[64] Such composition enjoys benefits from both and makes lipid-based films and coatings more attractive for the food industry. Beneficial oils and FFA are also incorporated into lipid-based coating formula as an effort to deliver health-promoting elements into human body, multiplying their functionality.

1.3 Perspective for the thesis

Evidently, lipid species play a prominent role in food industry, of which the application scale and demand keep growing.[67] As a direct response, the annual fats and oils production in 2014 reached 196 million tons, increased by over 50% over the last decade. Approximately 80 % of total lipid production was used for food, such as baking fats, confectionery fats, cooking fats, frying oils, ice cream, mayonnaise, salad oils, shortenings, spreads, etc. Although the consumption varies considerably among under-developed, developing and developed countries, their demands are ineluctably expanding.[67] In addition, highly populated countries, such as China and India, have particularly ascending consumption of fats and oils, and will fuel the demand for years.

Beyond the conventional applications, lipids are found to be of great potential as functional foods and functional food ingredients in promoting human health. As stated previously, lipids are an important group of compounds in antioxidants, infant formula and edible films and coatings. It is obvious that their origin forms are not always ideal for

such purposes and have to be modified according to the end applications. Thus, adjusting the physical and chemical properties as well as the nutritional profile of lipids is now the new research focus for food scientists.[67]

Specifically, diminishing energy intake and delivering beneficial elements in human diets attract exceptional interests. Although there is already a long list of calorie-zero and calorie-reducing fat substitutes existed, it is followed by another list of side effects brought by these fat substitutes due to their structural difference comparing with TAG. Chapter 2 introduces the design of a cluster of TAG molecules that contain two medium chain fatty acids (MCFA) at the sn-1 and sn-3 positions and one short chain fatty acid (SCFA) at the sn-2 position (shorten as MSM) as potential reduced-calorie fat substitute. A typical structure of such MSM molecules, 1,3-dicapryloyl-2-acetylglycerol, has been successfully synthesized and characterized. Thanks to the same structure as natural fats and oils, our newly designed MSM-structured TAG is expected to avoid side effects that induced by other fat substitutes, while efficiently control obesity and other associated health problems.

New sources of beneficial elements are to be discovered in the preparation of lipid-based functional food ingredients. Chapter 3 describes a novel method to utilize the beneficial long chain monounsaturated fatty acid (LCMFAC) and fatty alcohol (LCMFAL) within jojoba oil for a combined production of their ethyl ester and acetate

accordingly through one simple enzymatic interesterification reaction with ethyl acetate (EA). The interesterified product provide great versatility and potential in a wide range of applications in food industry, such as bio-functional ingredients, edible coatings, emulsifiers, flavor and fragrance agents in both purified form or as mixture.

Evolution in processing technologies is also a research emphasis of lipid-based functional food ingredient development. In Chapter 4, an original solution for the enrichment of omega-3 fatty acids in the form of TAG in cod liver oil (CLO) is proposed. Realized by two simple food techniques, namely solvent winterization and enzymatic interesterification, the method efficiently doubled the content of omega-3 PUFA, providing excellent industrial feasibility and also reducing the production cost drastically.

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Chapter 2

Design, Synthesis and Characterization of 1,3-Dicapryloyl-2-acetylglycerol as Potential Calorie-reduced Fat Substitute

2.1 Introduction

Obesity, which occurs when increase consumption of high-energy food is coupled with reduction in physical activity, has become such a global pandemic problem that it has been claimed as a major contributor to illness.[1-2] In recent decades, vast research has been done and proved that obesity is the cause of many serious public health problems and premature death.[3-4] According to Steven E. Kahn and his collogues, obesity can increase the risk of developing insulin resistance and type 2 diabetes by producing excess amounts of FFA, glycerol, hormones and other factors which contribute to the development of insulin resistance and pancreatic islet β-cells dysfunction, which lead to the failure of blood glucose levels control.[5] Obesity together with the insulin resistance mediated by adipose tissue, increases the risk of cardiovascular diseases in multiple ways, including hypertension, dyslipidemia, glucose dysmetabolism, as well as endothelial dysfunction and increased oxidative stress.[4] Although the molecular mechanisms are still elusive, obesity is widely believed to be a major promoter of gastrointestinal, mammary, esophageal, renal and reproductive cancers.[6-8] Thus, researchers have devoted great efforts and time to the development of new functional foods and functional food ingredients for obesity control.[9-13]

Unavoidable fat consumption and advice in reducing energy intake have stimulated the emergence of fat substitutes, which was defined as ‘food constituents able to replace,

completely or partially, dietary fat in such a manner that certain physical and organoleptic properties of the food product involved are left unaltered as far as possible' by Namal Senanayake and Fereidoon Shahidi.[14] Over years' research, various products have been developed, and they can be categorized as reduced- and zero-calorie fat substitutes on the basis of their practical function, or, as ester- and ether-based fat substitutes according to their chemical structures (Table 2.1).[14-15]

Table 2.1. Examples of current fat substitute products.[14]

Product	Calorie intake	Composition	Applications
Olestra	Zero	Sucrose fatty acid polyester	Salad dressing, mayonnaise, baked and fried foods.
Salatrim	Reduced	TAG of SCFA and LCFA	Coatings, confectionery, dairy and bakery products.
Caprenin	Reduced	TAG of caprylic, capric, and behenic acids	Soft candies and confectionery coatings.
Bohenin	Reduced	TAG of oleic and behenic acids	Tempering aid and antibloom agent in chocolate (coatings).
Captrin	Reduced	TAG of caprylic and capric acids	Flavor carrier.
Sorbestrin	Zero	Sorbitol fatty acid polyesters	Baked and fried foods, formulated products.
Grindsted PGE	Reduced	Polyglycerol esters	Margarine, shortening, confections, frozen desserts, bakery products.
EPG	Reduced	Esterified propoxylated glycerol	Margarine, frozen desserts, salad dressing, baked and fried foods.

(TAG, triacylglycerol; SCFA, short chain fatty acid; LCFA, long chain fatty acid.)

Olestra is the most famous zero-calorie fat substitute to date, since it got marketing approval from FDA in 1996.[14, 16-17] It is produced by connecting six to eight fatty acids, predominantly C(18:0), to a sucrose molecule via ester bonds to generate a mixture of hexa- to octa- fatty acyl esters of sucrose.[14, 18-19] Although its structure is similar to that of TAG molecules (Figure 2.1), olestra differs from TAG due to the absence of ester bonds formed with primary hydroxyl group. It is this unique property that prevents olestra from hydrolyzation by pancreatic lipase and protects it to travel through the gastrointestinal track completely undigested.[14, 18-19] Given the similar appearance, texture and taste characteristics to true fats and oils, olestra can perform as conventional fat for salad dressing, mayonnaise, frying and baking applications as listed in Table 2.1.[14] However, such physical properties of olestra may also cause an elimination of fat-soluble vitamins (e.g., vitamin A, D, E, and K) and carotenoids from the body.[14] Additionally, gastrointestinal disturbances, such as stool softening and abdominal cramping have been found related to olestra consumption, according to the research conducted by the Center for Science in the Public Interest (Washington, DC).[18] Hence, different lipid substitution strategies targeting unconventional area of lipid metabolism pathway need be investigated.

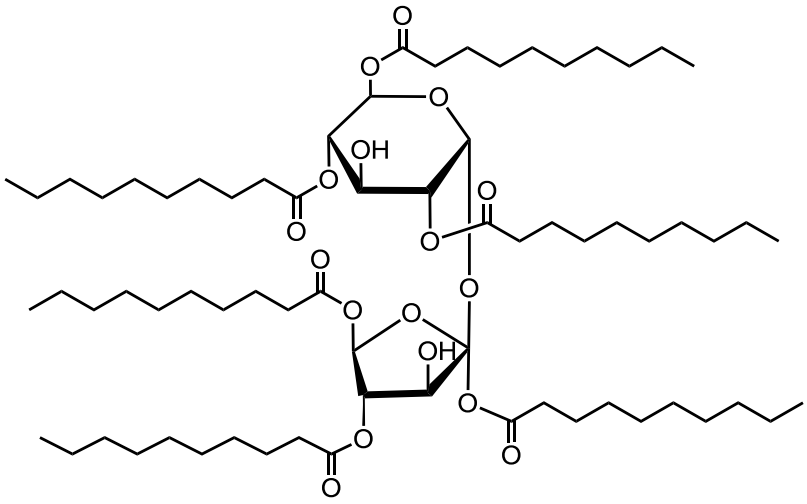

Figure 2.1. Typical molecular structure of olestra.

The current study describes a newly designed cluster of TAG molecules that contain two MCFA at the sn-1 and sn-3 positions and one SCFA at the sn-2 position (shorten as MSM), which is in theory of great potential to replace fat partially or completely as a reduced-calorie fat substitute. A typical structure of such MSM molecules, 1,3-dicapryloyl-2-acetylglycerol, is shown in Figure 2.2.

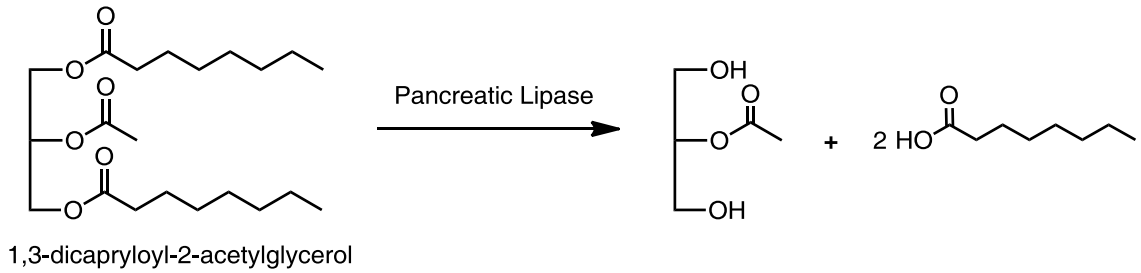

Figure 2.2. Structure of 1,3-dicapryloyl-2-acetylglycerol as a typical molecule of the designed TAG cluster, and its anticipated hydrolysis by pancreatic lipase within human body.

It is well known that normal TAG molecules in food are regioselectively hydrolyzed by the pancreatic lipases in human body, generating one 2-monoacylglycerol (2-MAG) and

two FFA.[20-21] Similar to FFA, 2-MAG could also serve as emulsifiers thanks to its composition of one long hydrophobic chain and two hydrophilic hydroxyl groups.[20, 22] Such emulsifiable nature of FFA and 2-MAG facilitate their formation of globular micelles together with bile salt, which would be absorbed by the intestine cells.[20] Passing through intestinal interface, the FFA and 2-MAG in form of globular micelles would then group into new TAG molecules to continue their succeeding metabolisms.[20] This is the normal metabolism pathway of dietary lipids in human body, and obviously it counts a lot on the emulsifiable characteristic of the hydrolyzed products from the first step of enzymatic digestion.

In the case of our newly designed MSM type TAG molecules, specifically 1,3-dicapryloyl-2-acetylglycerol, it will also be digested by the pancreatic lipase, whereas 2-monoacetin would be produced instead of a regular long chain 2-MAG (Figure 2.2). Considering the fact that it contains great polar portions (two hydroxyl groups) and little nonpolar segment (only two carbons in the aliphatic chain) in its molecular structure, 2-monoacetin might not be a proper emulsifier molecule any more. Consequently, it is hardly miscible with the bile salts, reducing the chances for its absorption into intestine cells. As a result, the FFA inside intestinal cell would hardly build TAG due to shortage of glycerol backbone. When the carbon atoms or hydrogen atoms in the acetyl group of 1,3-dicapryloyl-2-acetylglycerol are substituted with ^{14}C or ^{3}H, such radioactively labeled

molecules can function as molecular probe for both in vitro and in vivo studies on metabolic actions of the designed MSM type TAG. In other words, if the radioactively labeled 2-monoacetin is quickly excreted by the subject when fed with radioactive labeled 1,3-dicapryloyl-2-acetylglycerol in advance, it would indicate that the MSM type TAG could be hardly absorbed by intestinal cells. If the occurrence of this presumptive process could be proved in human body, such MSM structured TAG can be a brand new candidate as calorie-reduced fat substitutes, contributing to obesity control and other associated health problems.

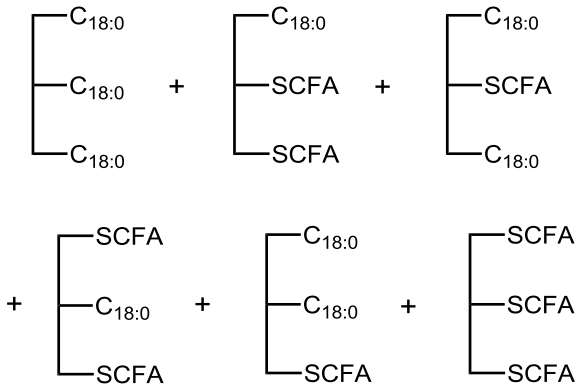

Figure 2.3. Illustration of Salatrim as a mixture of these TAG molecules in various ratios. (SCFA, short chain fatty acid, including C(2:0), C(3:0) and C(4:0)).

Salatrim, claimed as reduced-calorie structured lipids, is also a family of TAG composed by short and long fatty acids available in the market (Figure 2.3).[23-24] However, it is commonly synthesized by interesterification and/or acidolysis reactions of methyl stearate and tributyrin, producing TAG mixtures because of the random nature of these reactions.[25] The unpurified TAG mixture is not able to serve as molecular probe in the current study. On the contrary, our designed reduced-calorie MSM structured TAG can be

prepared with high purity and serve as molecular probe for the metabolism study.

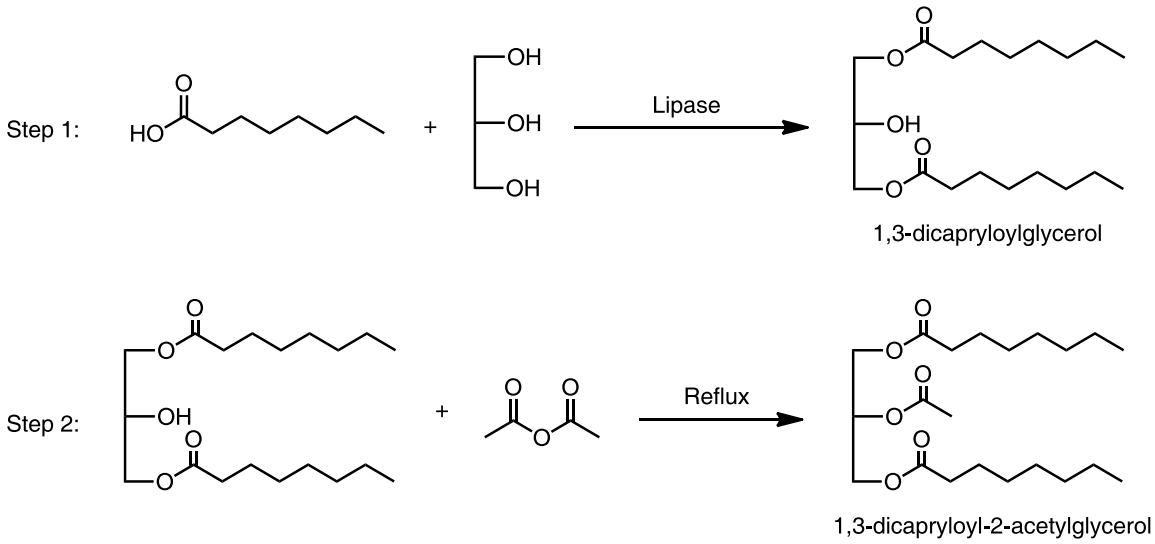

Figure 2.4. Synthesis method for 1,3-dicapryloyl-2-acetylglycerol.

The current study will describe the synthesis method of this MSM structured TAG in two steps of esterification reactions. The first step involves the enzymatic esterification between caprylic acid and glycerol towards the formation of 1,3-dicapryloylglycerol, followed by a second esterification with acetic anhydride to yield 1,3-dicapryloyl-2-acetylglycerol as shown in Figure 2.4. Meanwhile, discussion about the main side reaction is also included. After the confirmation of its calorie-reducing effectiveness, our MSM structured TAG could be a brand new reduced-calorie fat substitute and applied into food industry to refrain the ascending trend of obesity together with other associated health complications. Moreover, thanks to the structural and metabolic similarity between normal fats and our designed MSM type TAG, it would be free of side effects, such as elimination of fat-soluble vitamins and nutrients from the

body and diarrhea caused by other fat substitutes.

2.2 Results and Discussion

2.2.1 Rationale for the synthesis methodology

Regarding the synthesis of such symmetrically structured TAG molecules, two major methodologies have been developed and applied to quite a few researches.[26-27] The first methodology involves 2-MAG as the intermediate product, which can be prepared by enzymatic hydrolyzation of monoacid TAG under the catalysis of lipase with 1,3-regioselectivity. Then the resultant 2-MAG is subjected to an enzymatic esterification with another fatty acid or its ethyl ester, which is also catalyzed by a 1,3-specific lipase. Schmid U. *et al*. studied the synthesis of 1,3-oleoyl-2-palmitoylglycerol via this methodology and optimized the reaction conditions to a net yield of 66.3% with the presence of lipase *Rhizopus delemar* (Amano Pharmaceutical Co. Ltd., Nagoya, Japan).[28-29] Roxana Irimescu and her colleagues also applied this method for the synthesis of 1,3-dicapryloyl-2-oleoylglycerol and 1,3-dicapryloyl-2-docosahexaenoylglycerol and achieved net yield 83.3% and 90.2%, respectively, by using Novozym 435 and Lipozyme RM IM in succession.[30-31] Overall, the method described above can help produce symmetrically structured TAG molecules in high yield with high purity, and it is mostly applicable to the case of MCFA and LCFA, since the FFA generated in first step would not affect lipase activity and regioselectivity. However, in the case of our designed MSM

structured TAG, specifically 1,3-dicapryloyl-2-acetylglycerol, this methodology is not applicable because the acetic acid generated is likely to devastate lipase structure and stultify its action, seeing that lipases are more stable over alkaline pH range according to the lipase supplier report.[32]

The second methodology consists of two successive esterification reactions, where the intermediate product is monoacid 1,3-diacylglycerol (1,3-DAG). In terms of the first step, chemical synthesis of monoacid 1,3-DAG can be realized by refluxing fatty acid anhydride and glycidol in petroleum fraction with 56% of theory yield based on the method published by C.M. Lok. *et al.*[33] However, in recent years, more and more researchers use 1,3-specific lipase to catalyze the esterification of fatty acid, fatty acid methyl/ethyl ester or vinyl ester with glycerol for the 1,3-DAG production. Ning Liu *et al.* reported a direct esterification of oleic acid with glycerol to produce 1,3-dioleoylglycerol with a yield of 54.8 wt% by Lecitase® Ultra (phospholipase from Novozymes, Copenhagen, Denmark).[34] In Matthias Berger's paper, 1,3-dilaurin was synthesis by esterifying glycerol with lauric acid (alternatively methyl laurate) (yield 80%), and with vinyl laurate (yield 84%) in the presence of Lipozyme RM IM.[35] Similar reaction was performed by R. John Craven and Robert W. Lencki, who obtained 1,3-dipalmitin in a yield of 35% with Novozym 435.[36] Since the 1,3-specific lipase catalyzed esterification of glycerol and fatty acid can provide fair regioselectivity and higher yield of 1,3-DAG

with much simpler operation requirements, it was selected in the current study for the synthesis of monoacid 1,3-DAG, specifically 1,3-dicapryloylglycerol.

When it comes to the second step, both chemical and lipase were investigated to catalyze the esterification reaction at the sn-2 position. Adam W. Mazur *et al.* prepared 1,3-didecanoyl-2-docosanoylglycerol and 1,3-dioctanoyl-2-docosanoylglycerol in yield of 83% and 25%, respectively, through reaction of 1,3-didecanoylglycerol with corresponding fatty acid anhydrides in the presence of 4-dimethyaminopyridine (DMAP).[37] 1,3-Distearoyl-2-acetylglycerol was synthesized by 12 hours reflux of acetic anhydride and 1,3-distearin in the study of Taek Ho Yang *et al.*[38] In Tsuneo Yamane's research group, a method involving *N*,*N'*-dicyclohexylcarbodiimide (DCC) as condensation agent and DMAP as catalyst was preferred, and 42% yield of 1,3-dicapryloyl-2-eicosapentaenoylglycerol was attained.[39] Gudmundur G. Haraldsson *et al.* followed this method with a minor modification by replacing DCC with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), and successfully achieved 90-95% yield in the synthesis of symmetrically structured TAG molecules comprising one pure EPA or DHA at the sn-2 position and one pure SFA at the sn-1 and sn-3 positions.[40-43] Sirirung Wongsakul and her colleagues utilized lipase from *Burkholderia cepacia* (immobilized, Amano PS-D, Amano Pharmaceuticals Co.) to facilitate the esterification reaction between 1,3-DAG and oleic acid or oleic acid vinyl ester, whereas

the specificity was not good and a mixture of positional isomers was obtained.[44] In another case, an unacceptably low yield of 1,3-oleoyl-2-docosahexaenoylglycerol (9.4%) was obtained after reaction of 1,3-diolein with DHA ethyl ester catalyzed by Novozym 435.[45] Considering the less agreeable performance of enzymatic methods and the fact that chemical catalysts (such as DMAP, EDC and DCC) are not suitable for food ingredient production, Taek Ho Yang's method involving esterification between acetic anhydride and 1,3-DAG under reflux was adopted in our current research.

2.2.2 Synthesis of 1,3-dicapryloylglycerol

In the current study, four lipases, namely Lipozyme RM IM, Lipozyme TL IM, Novozym 435 and Lipase 150 were screened for their catalytic efficiency on the 1,3-regioselective esterification between caprylic acid and glycerol backbone. In the light of previous published literatures, each lipase was subjected to the esterification under their own optimum reaction conditions, and the results were summarized in Table 2.2. Since regioselectivity and esterification catalysis activity are of same importance in lipase evaluation,[35] Lipozyme RM IM clearly outperformed the other lipases in both aspects by providing high 1,3-regioselectivity and high yield of 1,3-dicapryloylglycerol. Hence, it was chosen for further synthesis.

Table 2.2. Evaluation of different lipases on their efficiency in catalyzing the synthesis of 1,3-dicapryloylglycerol via esterification of glycerol and caprylic acid.

Lipase	Reaction conditions	Best result	Esterification activity	1,3-regio-selectivity
Lipozyme RM IM	25 ℃, 8h, vacuum[39]	1,3-DAG yield 75%, purity confirmed by NMR	High	High
Lipozyme TL IM	40 ℃, 8h, vacuum[46-47]	Poor yield of 1,3-DAG	Poor	High
Novozym 435	40 ℃, 6.5h, vacuum[40, 47]	TAG was the major product	Very high	Poor
Lipase 150	-	No obvious reaction under any condition	Scarce	-

There are several factors that should be considered in this enzymatic esterification reaction. Since esterification reaction is reversible, removal of water generated during the reaction from the system plays a crucial role in shifting the esterification equilibrium towards the formation of 1,3-DAG in higher yield as well as in suppressing acyl migration.[48] So far, multiple water-removing methods, such as incubation with molecular sieves, vacuum-driven nitrogen bubbling and magnetic stirring under vacuum, have been evaluated by many researchers.[48-49] Incubation with molecular sieves was found to be less efficient due to limited mass transfer and inefficient water removal caused by the high viscosity of oily reaction mixture.[48-49] In Roxana Rosu's report, the water was efficiently removed by nitrogen stream or by vacuum, and a conversion of 98% was obtained.[39] A solvent-free system under vacuum was the most recommended in recent literatures,[50-51] and therefore it was applied into the current experiments.

Reaction temperature has a great impact on the reaction rate and lipase activity, and therefore influences the distribution of product.[51] Although higher temperature may elevate reaction rate, it also affects the regioselectivity and stability of lipase, leading to an increased extent of esterification and the formation of TAG molecules.[51] Zhang H. *et al.* observed the highest conversion of glycerol and oleic acid to 1,3-DAG under the catalysis of Lipozyme RM IM at 25 ℃,[45] which is also supported by Roxana Rosu's observation.[39] Thus, this temperature was firstly tried in the current study, and provided a fair yield of 1,3-dicapryloylglycerol (75%). Further investigation could be conducted in the future in an attempt to improve the yield of 1,3-DAG.

Substrate molar ratio is believed to have double functions on the enzymatic esterification reaction.[52] Higher molar ratio of glycerol to fatty acid can promote the reaction equilibrium toward synthesis and increase the extent of esterification and product yield. On the other hand, it may also amplify the inhibition effect of FFA and lead to a longer period of reaction time required to reach equilibrium [53]. Roxana Rosu *et al.* studied the effect of different molar ratio of glycerol to caprylic acid on the yield of 1,3-dicapryloylglycerol, and found 1:4 was the optimum where the content of 1,3-DAG was maximum (98%) and that of 1-MAG and TAG was minimum (1% each).[39]

According to Takaaki Watanabe *et al.*, lipase load had little influence on the yield of 1,3-DAG.[51, 54] Higher lipase load could raise the esterification rate, but also would

increase the TAG content in later stage of reactions, resulting in a decrease in the purity of 1,3-DAG. For this reason, good control of reaction time length is of great importance in the current study and should be further optimized. Additionally, the immobilized lipases should be immediately removed from the reaction system once the maximum yield of 1,3-DAG is achieved.

Agitation speed is another factor that have an impact on the esterification efficiency. Since oily reaction system is always with high viscosity, stirring should be applied to facilitate the macromixing.[55] In spite of the reported observation that higher 1,3-DAG yield was attained with higher agitation speed,[47, 51] lipase reusability will be compromised. In this light, to maintain the balance between reusability and efficiency, a mild agitation speed of 250 rpm was selected for the current synthesis. Meanwhile, the used lipases could be recovered and then reused with steady activity.[35]

The intermediate compound 1,3-dicapryloylglycerol was firstly characterized by ^{1}H and ^{13}C NMR spectroscopy (Figure 2.5 and 2.6, respectively). As shown in Figure 2.5, the triplet at 0.85 ppm with a coupling constant of 7.2 Hz corresponds to the six hydrogen atoms in the terminal methyl groups of caprylic acids. The multiplets at 1.25 and 1.60 ppm should be assigned to the 20 hydrogen atoms in the methylene groups of caprylic hydrocarbon chain, while the triplet at 2.31 ppm with a coupling constant of 7.6 Hz could be conserved as manifestation of the four hydrogen atoms at the α position of carboxylic

esters. The hydrogen atom at the sn-2 position and four hydrogen atoms in the methylene groups of the glycerol backbone should both appear around 4.10 ppm, in other words, five hydrogen atoms should be observed together within this chemical shift range in theory. Unfortunately, only four hydrogen atoms were found around 4.10 ppm as a multiplet in the ^{1}H NMR spectrum of 1,3-dicapryloylglycerol.

To clarify this puzzling situation, ^{13}C distortionless enhancement by polarization transfer (DEPT) 135 NMR and hetero-nuclear multiple quantum correlation (HMQC) NMR were conducted. In Figure 2.7, the DEPT 135 spectrum clearly indicates that the positive peak at 68.29 ppm and the negative peak at 65.00 ppm can be assigned to the tertiary carbon and secondary carbons of the glycerol backbone, respectively, which coexist in the intermediate compound. Furthermore, our HMQC analysis shown in Figure 2.8 also demonstrates that the tertiary carbon of glycerol is indeed correlated with one proton and the secondary carbons with a few other protons. Both of these observations prove the multiplet around 4.10 ppm in Figure 2.5 indeed corresponds to two different types of hydrogen atoms. Besides, ^{13}C NMR, FT-IR, and LC-ESI-MS results all precisely accord with our suggestion that the intermediate compound is 1,3-dicapryloylglycerol. Possible explanation to the unclear observation of the hydrogen atom at sn-2 position of glycerol in the ^{1}H NMR spectrum, could be that this specific proton is heavily shielded by the adjacent hydroxyl group and the two bulky aliphatic chains surrounding it.

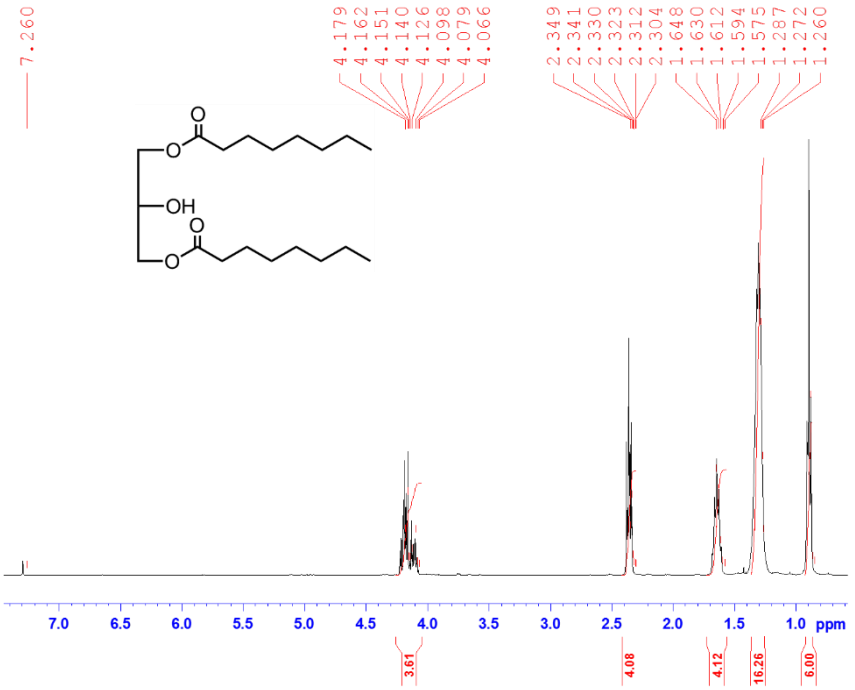

Figure 2.5. The ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of 1,3-dicapryloylglycerol.

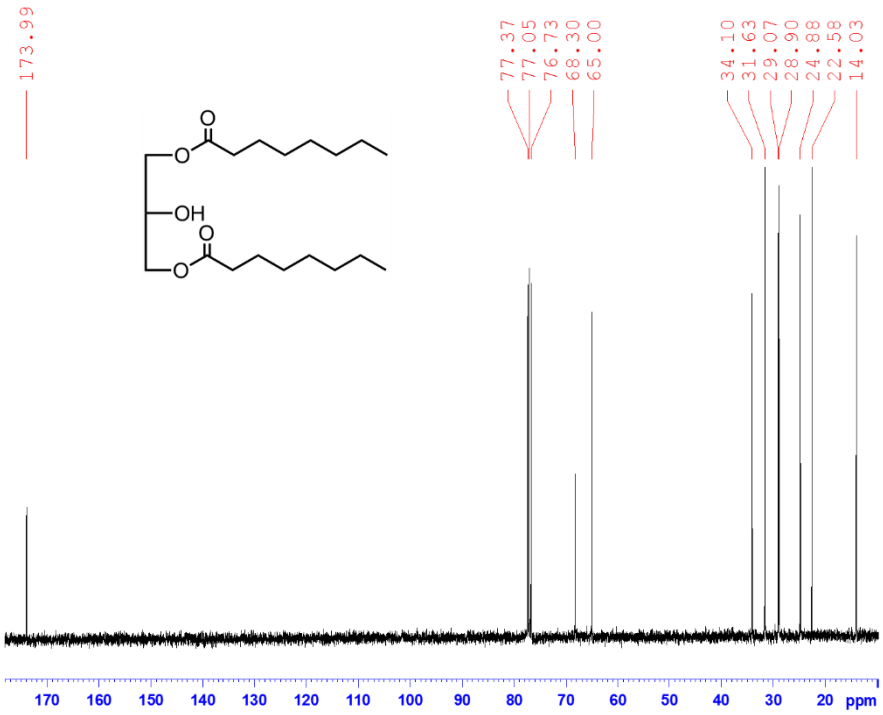

Figure 2.6. The ^{13}C NMR spectrum (400 MHz, $CDCl_3$) of 1,3-dicapryloylglycerol.

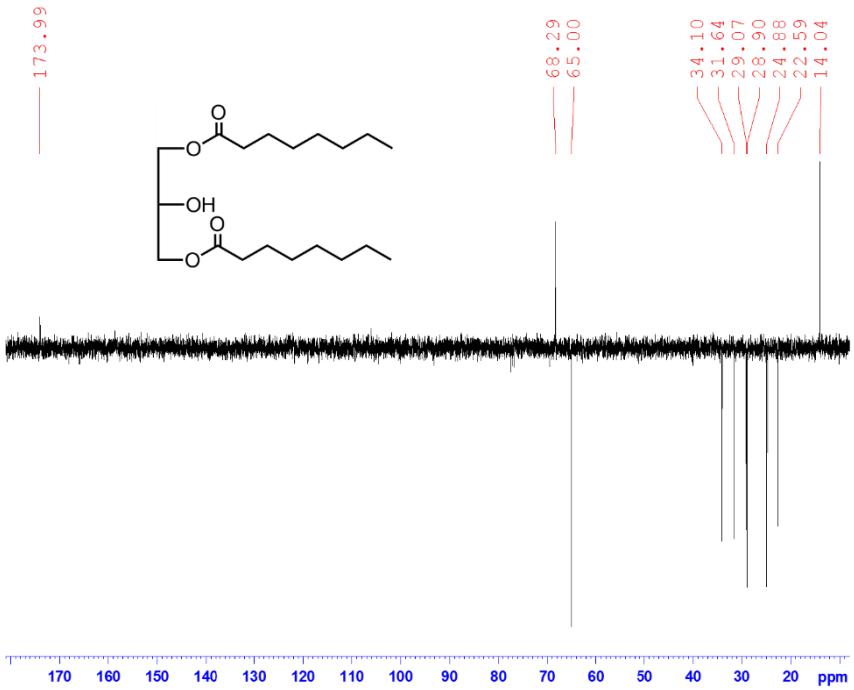

Figure 2.7. The ^{13}C DEPT 135 NMR spectrum (400 MHz, $CDCl_3$) of 1,3-dicapryloylglycerol.

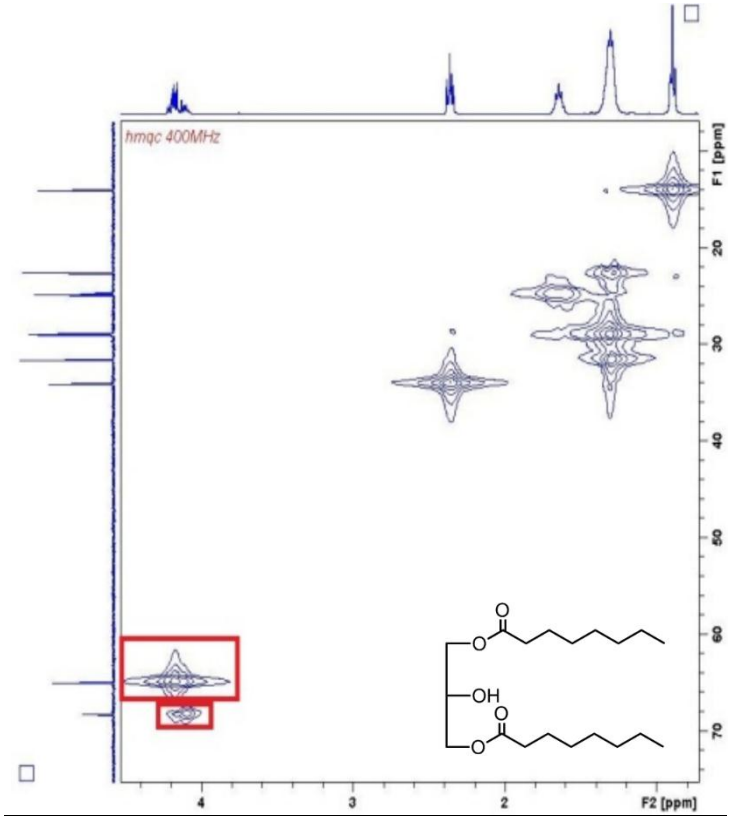

Figure 2.8. The HMQC NMR spectrum (400 MHz, $CDCl_3$) of 1,3-dicapryloylglycerol.

2.2.3 Synthesis of 1,3-dicapryloyl-2-acetylglycerol

As stated in Section 2.2.1, the esterification between fatty acid and 1,3-DAG to generate symmetrically structured TAG could be realized in several methods. Although enzymatic esterification could be used again for the acetylation of the secondary hydroxyl group in glycerol, it is practically difficult to maintain lipase activity in such an acid environment. To esterify the 1,3-DAG with acetic acid under the catalysis of concentrated sulfuric acid might be the simplest, but the existence of abundant H^+ increases the chance for the hydrolyzation of ester bonds formed in the first step. Therefore, in our study, refluxing 1,3-dicapryloylglycerol with acetic anhydride to yield 1,3-dicapryloyl-2-acetylglycerol was considered to be a prime choice, considering its simple operation required and relatively high yield. This method provided a relatively high yield of 60%, and product structure was confirmed by ^{1}H and ^{13}C NMR (Figure 2.9 and 2.10, respectively), FT-IR, and LC-ESI-MS analysis.

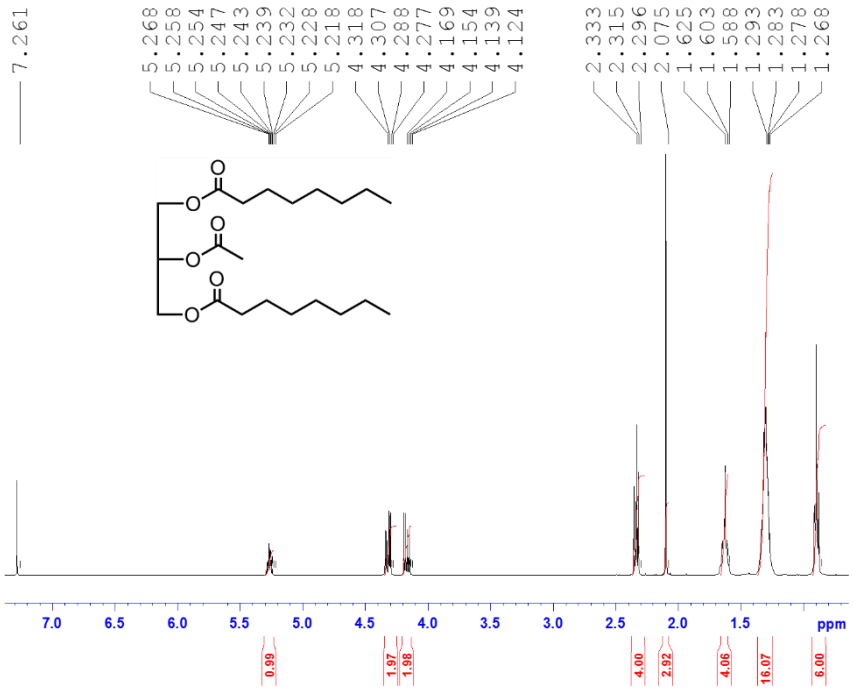

Figure 2.9. The ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of 1,3-dicapryloyl-2-acetylglycerol.

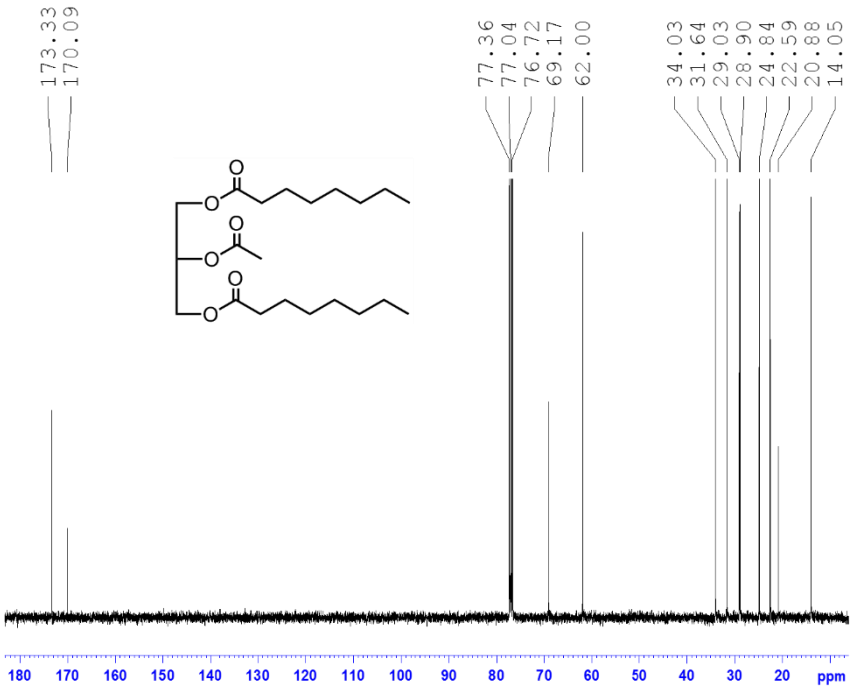

Figure 2.10. The ^{13}C NMR spectrum (400 MHz, $CDCl_3$) of 1,3-dicapryloyl-2-acetylglycerol.

2.2.4 Main side reaction – acyl migration

The acyl migration from 1,3-DAG to 1,2-DAG was the main obstacle in the preparation of 1,3-dicapryloyl-2-acetylglycerol. As shown in Figure 2.11, the mechanism of acyl migration is suggested to involve a five member ring intermediate.[56] The process is initiated under the nucleophilic attack of one lone pair of electrons in the free hydroxyl oxygen of the ester carbonyl carbon, resulting in orthoester as the five member ring intermediate. Cleavage of the original ester bond will open the ring and result in the formation of 1,2-DAG. It can be predicted that efficient nucleophilic attack and stable orthoester formation will both enhance acyl migration. Considering that primary hydroxyl oxygen is a better nucleophile than secondary hydroxyl oxygen, the acyl shift from a secondary position to a primary position is favored. Meanwhile, the process is also governed by substituent steric effects during the formation as well as the subsequent cleavage of orthoester intermediate. Additionally, both acidic and basic environments can facilitate acyl migration rapidly at room temperature.

In the first step esterification, although Lipozyme RM IM provided excellent 1,3-regioslectivity and a good yield of 1,3-DAG, 1,2-DAG could be easily generated from the conversion of 1,3-DAG, since various factors (such as temperature, lipase load, water content and reaction time length) can positively influence the acyl migration.[57] The extent of such influence has been investigated by Takaaki Watanabe and his colleagues,

and they found that higher lipase load and higher reaction temperature would promote acyl migration instead of increase reaction rate.[54] In this light, a smaller enzyme load of 4 wt% and a mild reaction temperature of 25 ℃ were applied, which successfully helped Lipozyme RM IM exert a relatively high 1,3-regioselectivity and the formation of ester bond on the secondary hydroxyl group in glycerol was efficiently restrained during the enzymatic esterification.

However, isomerization of 1,3-dicapryloylglycerol to 1,2-dicapryloylglycerol was observed during purification. The major method used to detect acyl migration was TLC analysis. Our TLC plates were developed in a solution of chloroform/acetone (95:5 v/v), and visualized by potassium permanganate staining. Distillation under reduced pressure led to significant acyl migration which lowered the purity of 1,3-DAG greatly. At the same time, increasing amount of 1,2-DAG was observed in the conventional column chromatography, possibly caused by interaction of 1,3-DAG with the hydroxyl groups on silica gel. To shorten the time 1,3-DAG spent on silica gel and therefore suppress their interaction, a flash column chromatography was utilized as the purification method, with n-hexane/EA (3:1 v/v) as eluent, providing 75% yield of 1,3-dicapryloylglycerol.

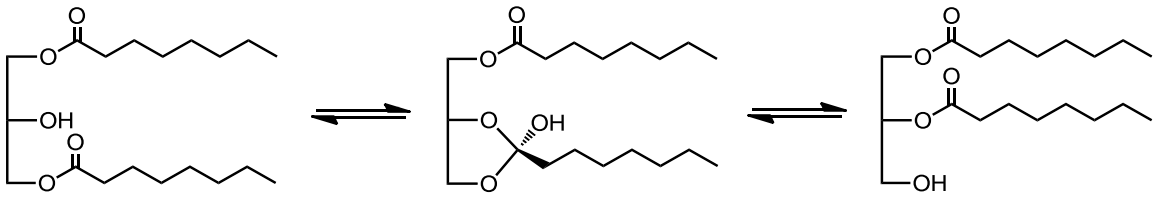

Figure 2.11. Acyl migration between 1,3-DAG and 1,2-DAG.

Further isomerization was also observed when purified 1,3-DAG product was stored at 4˚C after 48 h sealed with Parafilm. As it has been reported that the reverse acyl migration from 1,2-DAG to 1,3-DAG is promoted in solid state,[58] we believe that the isomerization of 1,3-DAG could be significantly minimized via low temperature (< -30˚C) storage in a nitrogen environment. In addition, it is strongly recommended to proceed the following reaction immediately after obtaining purified 1,3-dicapryloylglycerol, minimizing the chance for this side reaction. Acyl migration was also apparent during the second step esterification as well, leading to the formation of a small amount of 1,2-dicapryloyl-3-acetylglycerol. The high temperature involved in reflux was believed to be the factor that enhanced acyl migration, and in response, reaction time was shortened to minimize such influence.

2.3 Conclusions

A typical MSM structured TAG, 1,3-dicapryloyl-2-acetylglycerol, was successfully synthesized in a two-step strategy with a net yield of 45%. Firstly, 1,3-dicapryloylglycerol was prepared via 1,3-specific esterification of glycerol and caprylic acid catalyzed by Lipozyme RM IM, with a molar ratio of 1:4 and an agitation speed of 250 rpm at 25 ℃ for 8 hours under vacuum. Flash column chromatography was preferred as the purification method. 1,3-Dicapryloyl-2-acetylglycerol was then produced by refluxing 1,3-DAG with acetic anhydride for 6 hours. Structural characterization was

conducted by ^{1}H, ^{13}C NMR, FT-IR, and LC-ESI-MS.

The obtained product can be used as molecular probe for further metabolic and nutritional study on MSM structured TAG. It is prospected to be hydrolyzed by pancreatic lipase within human body, and generates 2-MAG with stronger polarity, which would hardly be absorbed into intestinal cells. As a consequence, the recombination of TAG afterwards would be restrained, leading to reduced fat intake into human digestive track. Once the effectiveness is confirmed, our MSM structured TAG could be a competitive reduced-calorie fat substitute candidate and contribute to the control of obesity and associated health problems without side effects.

2.4 Experimental Section

2.4.1 Materials

Commercially available Lipozyme TL IM (lipase from *Thermomyces lanuginosa*) and Lipozyme RM IM (lipase *Rhizomucor miehei*, immobilized on anion exchange resin) were generous gifts from Novo Nordisk A/S, Bagsvaerd, Denmark. Novozym 435 (lipase from *Candida Antarctica*, immobilized on acrylic resin) and Lipase, immobilized on Immobead 150 from *Rhizomucor miehei* (shorten as Lipase 150) and was purchased from Sigma Chemical Co. (Singapore). Chemicals were purchased as follows: caprylic acid (min. 98.0%) and acetic anhydride (min. 98.0%) from Sigma Chemical Co. (Singapore), potassium carbonate (min. 99.0%) from Sinopharm Chemical Reagents (Shanghai,

China), glycerol (min. 99.5%) from USB Corporation (Cleveland, Ohio, USA), and sodium sulfate anhydride from Schedel (Singapore).

2.4.2 Enzymatic synthesis of 1,3-dicapryloylglycerol

Glycerol (0.8 g, 8.7 mmol) and caprylic acid (5.5 mL, 34.8 mmol) in a molar ratio of 1:4 were mixed in a round bottom flask. Different types of lipase (4 wt%) were added into the reaction mixture with an agitation speed of 250 rpm. For Lipozyme RM IM, the reaction temperature and time length were set at 25 ℃ and 8 hours, respectively.[39] For Lipozyme TL IM, 40 ℃ and 8 hours, respectively.[46-47] For Novozym 435, 40 ℃ and 6.5 hours, respectively.[40, 47] For Lipase 150, different temperature and time length were tried. Reaction progress was monitored by TLC which was visualized by potassium permanganate staining. The reaction was terminated by removal of lipase by filtration, and the filtrate was concentrated by rotary evaporation at 35 ℃. 1,3-Dicapryloylglycerol was purified by flash column chromatography with isocratic eluent consisted of n-hexane/EA (3:1 v/v), and obtained as viscous colorless oil in a yield of 75%.

FT-IR (cm^{-1}, air): 3464, 2954, 2926, 2856, 1739, 1716, 1456, 1379, 1165, 1163, 1107.

^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 4.10 (m, 4H, C**H**$_2$C**H**C**H**$_2$), 2.31 (m, *J* = 7.6 Hz, 4H, C**H**$_2$COO), 1.60 (m, 4H, C**H**$_2$CH$_2$COO), 1.27 (m, 16H, (C**H**$_2$)$_4$CH$_3$), 0.88 (m, *J* = 7.2 Hz, 6H, (CH$_2$)$_4$C**H**$_3$).

^{13}C NMR (400 MHz, $CDCl_3$): δ (ppm) = 14.03, 22.58, 24.88, 28.90, 29.07, 31.63,

34.10, 65.00, 68.30, 173.99.

MS (LC-ESI-MS): m/z (%) = 367.18 $[M + Na]^+$; $C_{19}H_{36}O_5$ $[M + Na]^+$ requires 367.25.

2.4.3 Synthesis of 1,3-dicapryloyl-2-acetylglycerol

1,3-Dicapryloylglycerol (1.0 g, 2.92 mmol) and acetic anhydride (0.30 g, 2.92 mmol) were refluxed for 6 hours. Reaction progress was monitored by TLC which was visualized by potassium permanganate staining. Product mixture was cooled to room temperature and dissolved in chloroform. Unreacted acetic anhydride was quenched by adding aqueous potassium carbonate (6.5 wt%), followed by extraction with chloroform (20 mL × 3). The organic layers were combined, treated with anhydrous sodium sulfate and concentrated by rotary evaporation at 35 ℃. 1,3-Dicapryloyl-2-acetylglycerol was purified by flash column chromatography with isocratic eluent consisted of n-hexane and EA, and obtained as viscous colorless oil in a yield of 60%.

FT-IR (cm^{-1}, air): 2954, 2927, 2856, 1745, 1458, 1371, 1230, 1163, 1103.

^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 5.26–5.22 (m, 1H, $CH_2C\mathbf{H}CH_2$), 4.28 (dd, J_1 = 12.0 Hz, J_2 = 4.4 Hz, 2H, $C\mathbf{H}_2CHC\mathbf{H}_2$), 4.15 (dd, J_1 = 12.0 Hz, J_2 = 6.0 Hz, 2H, $C\mathbf{H}_2CHC\mathbf{H}_2$), 2.32 (t, J = 7.2 Hz, 2H, $C\mathbf{H}_2COO$), 2.08 (s, 3H, $C\mathbf{H}_3COO$), 1.60 (m, 4H, $C\mathbf{H}_2CH_2COO$), 1.28 (m, 16H, $(C\mathbf{H}_2)_4CH_3$), 0.87 (t, J = 7.2 Hz, 6H, $(CH_2)_4C\mathbf{H}_3$).

^{13}C NMR (400 MHz, $CDCl_3$): δ (ppm) = 14.05, 20.87, 22.58, 24.84, 28.89, 29.03, 31.64, 34.03, 62.00, 69.17, 170.08, 173.33.

MS (LC-ESI-MS): m/z (%) = 409.29 $[M + Na]^+$; $C_{21}H_{38}O_6$ $[M + Na]^+$ requires 409.26.

2.4.4 Analytical techniques

All NMR measurements were proceeded with Bruker Avance III 400 MHz NMR, BBFO probe, with $CDCl_3$ as the solvent and tetramethylsilane (TMS) as the reference compound. LC-ESI-MS analysis were conducted using a Thermo UPLC with Hypersil C18 3 μm, 15 × 2.1 mm^2 (ACE) column. The spectrometer was a Thermo LCQ FLEET equipped with ESI source. The mass spectrum was recorded in positive mode between 50 and 2000 Da. Capillary tension was 3000 V and cone tension 20 V. Flow rate: 0.5 mL/min, Gradient: from A/B 90/10 at t = 0 min to A/B 0/100 at t = 15 min (A: H_2O + 0.1% Formic acid, B: Acetonitrile + 0.1% Formic acid). The injection volume was 10 μL.

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Chapter 3

Enzymatic Interesterification of Jojoba Oil for Producing Functional Monoesters and Their Structure Characterization

3.1 Introduction

Fatty acid ethyl ester (FAEE) and fatty alcohol acetate (FAA) are major compounds used as emulsifiers, flavor and fragrance agents in food industry.[1-3] Along with fatty acids, glycol stearate, and glycerol monostearate, FAEE is a common emulsifier used on films and coatings to guarantee good wetting and spreading of the surface, and adhesion to food surface.[4] Monoesters formed by ethanol and fatty acids with aliphatic tails of 6-24 carbons could readily emulsify the oil phase which containing Coenzyme Q10 and other lipophilic dietary supplements of low water solubility in particular aqueous medium (e.g., simulated gastric fluid), providing high oral bioavailability of the lipophilic dietary ingredients.[5] It could also be formulated into mixed phase co-crystals to impart desirable physical properties and stability of active agent (e.g., agro-chemicals, human and animal medicines, and dietary supplements) otherwise not achievable for its pure form or in simple homogenous combination with other materials incorporated, and deliver the active agent.[6] FAA can provide ready emulsions of coating reagents when mixed with water and produce stable coatings for nursery stock, nuts, fruits, and the like to reduce decay and desiccation and improve product appearance and conditions.[7] Short- and medium-chain FAEE and FAA always have a pleasant fruity aroma, and are regular artificial flavors in food (e.g., ice cream, baked good, candy, and cheese) and beverages.[3, 8-11]

Particularly, long chain monounsaturated fatty acid ethyl ester (LCMFAEE) and long

chain monounsaturated fatty alcohol acetate (LCMFAA), are not only active compounds in aroma and fresh fruit coatings,[12-16] but also of special functions in human body metabolism. For instance, ethyl oleate has been patented as body weight managing element that can induce a sensation of satiety and control appetite in both animal and human subject.[17] In addition, they are also extensively applied in various high-end products, including cosmetics, fragrances and pharmaceutical formulations.[18-28] LCMFAA with 18 to 22 carbons are identified as insect pheromone of exceptional value for social behavior study and insecta mate investigation.[29-30] Given the growing application scope and quantity demanded, interests has been provoked to develop novel and convenient sources of LCMFAEE and LCMFAA.

Simmondsia chinensis Link Schneider, commonly known as jojoba, is the unique species in the Simmondsiaceae family, which is native to the Mohave and Sonoran deserts of Mexico, Arizona and southern California, and later spread to Australia, Israel, Argentina and Middle East.[31-33] This economically important shrub is evergreen, wind pollinated and perennial dioecious and has a long life span (about 100–200 years).[34] The jojoba seeds contain 40-65 wt% wax ester (also known as jojoba oil or jojoba wax) of light gold color, of which the structure is fundamentally different from other plant oils due to the absence of typical TAG as the oil molecule.[31] As shown in Figure 3.1, it is actually a mixture of linear monoesters composed of LCMFAC and LCMFAL, while the

LCMFAC composition is dominated by *cis*-11-eicosenoic acid (71.3%) and *cis*-11-eicosenol and *cis*-13-docosenol (43.8% and 44.9%, respectively) predominate in the LCMFAL part.[33, 35-37] This unique structure imparts jojoba oil a 'liquid wax' nature, including high boiling, smoke, flash and fire point, low chemical reactivity, excellent lubricity, low and constant viscosity over different temperatures, etc.[38-39] Till now, jojoba oil has almost replaced sperm whale oil to be the major natural source of liquid wax esters for lubricants and other applications in industry.[32]

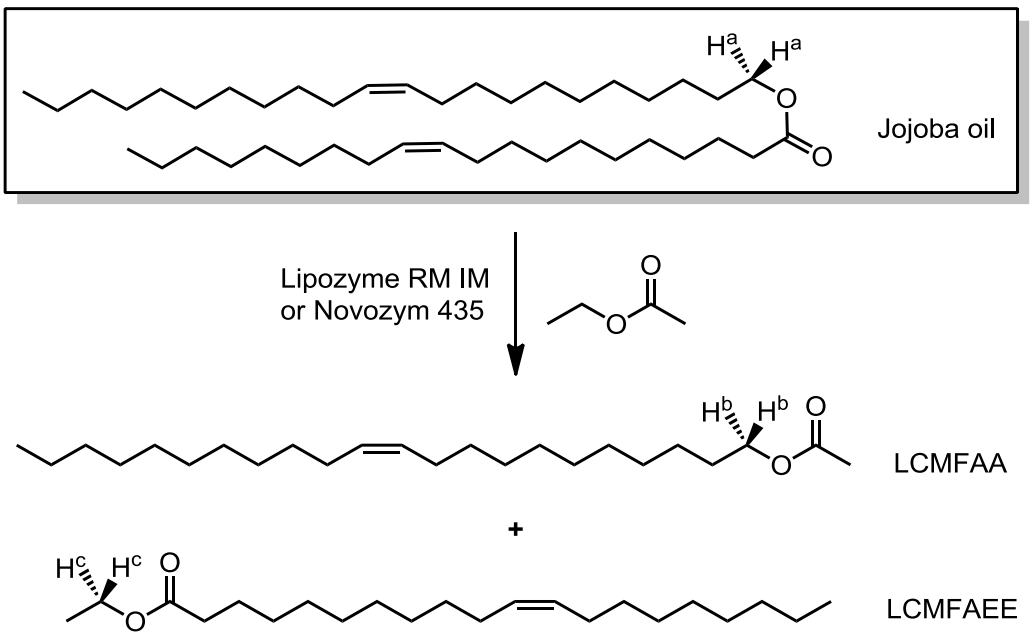

Figure 3.1. Typical structure of jojoba oil ester molecule and its interesterification reaction with EA catalyzed by Novozym 435 and Lipozyme RM IM, respectively.

In the recent two decades, various modification of jojoba oil has been developed as an effort to explore its new applications.[32] It can be easily hydrogenated to form lustrous pearly-white solid wax, which can be utilized in penicillin drugs, candle coating, polish waxes and fruit waxing.[32] By reacting with potassium hydroxide, jojoba oil can also be

hydrolyzed to be an ester emollient that has been commercialized to help human skin enhance formulation barrier function, remain constant moisture and sensory silky-feel in personal care and cosmetic products.[40] However, to the best of our knowledge, little research has been done to convert such rarely enriched source of LCMFAC and LCMFAL to their LCMFAEE and LCMFAA derivatives for food applications.

In the current study, the LCMFAC and LCMFAL within jojoba oil has been fully utilized for a combined production of jojoba LCMFAEE and LCMFAA on the basis of one simple enzymatic interesterification reaction with EA (Figure 3.1). Two commercialized lipases, namely Novozym 435 and Lipozyme RM IM, were studied on the catalytic efficiency under different conditions where lipase load, EA content, reaction temperature and time were varied. ^{1}H NMR analysis as a fast and convenient technique that recently involved in many studies to quantify lipid production,[41-44] was used to monitor reaction progress and determine interesterification yield (IY, %). The produced jojoba LCMFAEE and LCMFAA were purified by preparative TLC and characterized by ^{1}H NMR, FT-IR and GC-MS. Operational stability of Novozym 435 was also investigated. The jojoba LCMFAEE and LCMFAA obtained provide great versatility and potential in a wide range of applications in food industry, such as bio-functional ingredients, edible coatings, emulsifiers, flavor, and fragrance agents in both purified form or as mixture.

3.2 Results and Discussion

3.2.1 IY quantification via ^{1}H NMR analysis

As shown in Figure 3.1, the α methylene hydrogen atoms of alcohol to ester bond in the three monoester molecules (H^{a-c}) are not the same, and their difference can be reflected clearly as differentiable signals in 4.00-4.15 ppm region of the ^{1}H NMR spectra of the treated interesterification reaction mixture. Specifically, the quartet generated by H^{c} was around 4.11 ppm, while the triplets generated by H^{a} and H^{b} perfectly overlapped at about 4.05 ppm. No overlap of the triplet and the quartet has been observed and both of them are perfect as its own (Figure 3.2). Since IY indicates the conversion efficiency of the current interesterification reaction, namely the yield of jojoba LCMFAEE and LCMFAA, it can be calculated as the percentage of reacted jojoba oil, in other words, the content of jojoba LCMFAEE divided by the sum content of jojoba LCMFAA and unreacted jojoba oil. Considering that the methylene hydrogen atoms in EA molecule can also generate a quartet in the same region as the H^{c} in jojoba LCMFAEE, the remaining EA in reaction mixture would affect the quantification of IY, and therefore must be completely removed before ^{1}H NMR analysis. Then, direct IY quantification in the EA-removed reaction mixture was obtained from the area ratio of selected signals as shown in the following equation:

$$\text{IY}\ (\%) = \frac{\text{Area(quartet)}}{\text{Area(triplet)}} \times 100$$

Where Area(quartet) and Area(triplet) are the areas of signals of the quartet and the triplet, respectively, in 4.00-4.15 ppm region of the ^{1}H NMR spectra of the treated interesterification reaction mixture.

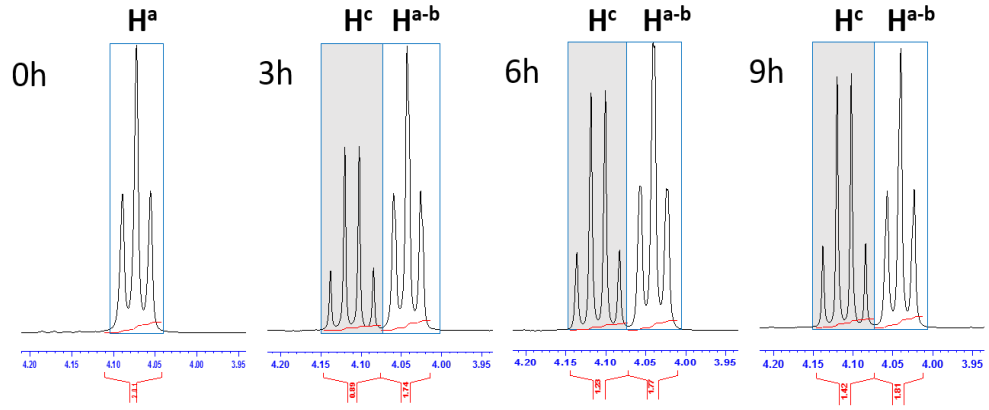

Figure 3.2. Expansion of δ 4.00-4.15 region and signal assignments in the ^{1}H NMR spectra (400 MHz, $CDCl_3$) of the treated reaction mixture at different time points of the interesterification of jojoba oil with EA.

Agreement between values obtained via ^{1}H NMR analysis and those calculated on the basis of weights was good (within 2% of error). To confirm the results, each reaction assay was triplicated and the data shown are expressed as the mean values (within 5% experimental uncertainties).

3.2.2 Interesterification with Lipozyme RM IM

Due to environmental issues and health concerns, there is an increasingly strong demand to optimize industrial processes and meet new quality criteria.[45] For instance, reduce effluent and waste, minimize usage of chemicals and solvents, avoid neutral oil loss, etc.[45] For this reason, enzymatic interesterification has attracted more attention of

both industry and researchers over the traditional chemical interesterification. As a biocatalyst, lipase can provide not only environmentally friendly connotation (e.g., less severe process conditions, less side reactions and by-products, limited post-treatment) but also remarkably high conversion efficiency.[45] Therefore, it was chosen as the catalyst for the interesterification of jojoba oil and EA in the current study.

In the beginning, the reaction was catalyzed by Lipozyme RM IM, which has been utilized extensively by many researchers on interesterification reactions.[45-48] Its catalytic efficiency was evaluated under different reaction conditions where lipase load, EA content, reaction temperature and time were varied. A stirring speed of 300 rpm was kept as constant in all experiments, seeing that it is high enough for Lipozyme RM IM to maximize mass transfer within the reaction system as well as avoid significant mechanical destruction to the lipase carrier during reactions at the same time.[49-50]

Firstly, the effect of reaction temperature on the enzymatic interesterification reaction was studied for it can directly influence the rate of reaction, and stability and catalytic activity of the lipase.[51] Figure 3.3a shows the change of IY obtained when reaction temperature was varied and other conditions were constant (75 wt% EA content, 5.00 wt% lipase load, 9 hours), and obviously 30 ℃ assay provided the best result (IY 76%). Lower temperature (25 ℃) was not sufficient to accelerate reaction rate and to enhance the mass transfer within reaction system.[48-49] Meanwhile, temperatures higher than 30 ℃

were believed to be more important in reducing the operational stability and catalytic activity of Lipozyme RM IM than in accelerating mass transfer in terms of the current interesterification reaction.[48-49, 52-53] This was also observed by Roxana Irimescu and her coworkers in the research on the synthesis of 1,3-dicapryloyl-2-eicosapentaenoylglycerol by interesterification of ethyl caprylate with trieicosapentaenoylglycerol in the presence of Lipozyme RM IM, where the best yield was provided by 30 ℃ assay.[46] Based on these results, 30 ℃ was kept for Lipozyme RM IM to perform interesterification in this reaction system.

Figure 3.3b shows the effect of EA content on the efficiency of Lipozyme RM IM on the current interesterification reaction. Stoichiometrically, one mole EA (12 wt%) is required to complete the conversion of one mole jojoba oil to jojoba LCMFAEE and LCMFAA. However, in our experiments, the content of EA was raised to 60, 65, 70, 75, 80, and 85 wt% as an effort to promote the equilibrium of interesterification reaction to obtain higher yield. Additionally, the extra EA played an important role as solvent to dilute the oil and decrease the viscosity of reaction system, which would facilitate the mass transfer greatly. Mukesh Kumar Modi *et al.* used the same strategy to boost the yield of biodiesel to 90%, 91.3% and 92.7% with crude karanj, jatropha and sunflower oils, respectively.[54] As illustrated in Figure 3.3b, the highest conversion (76%) was gained in 75 wt% assay while the rest of reaction conditions were fixed (5.00 wt% lipase

load, 9 hours, 30 ℃). EA content exceeding this value gave similar low IY, which is believed to be a result of over-dilution of jojoba oil and thereby inefficient interactions between substrates and the biocatalyst.[55] An EA content of 75 wt% was therefore selected for Lipozyme RM IM in the following experiments.

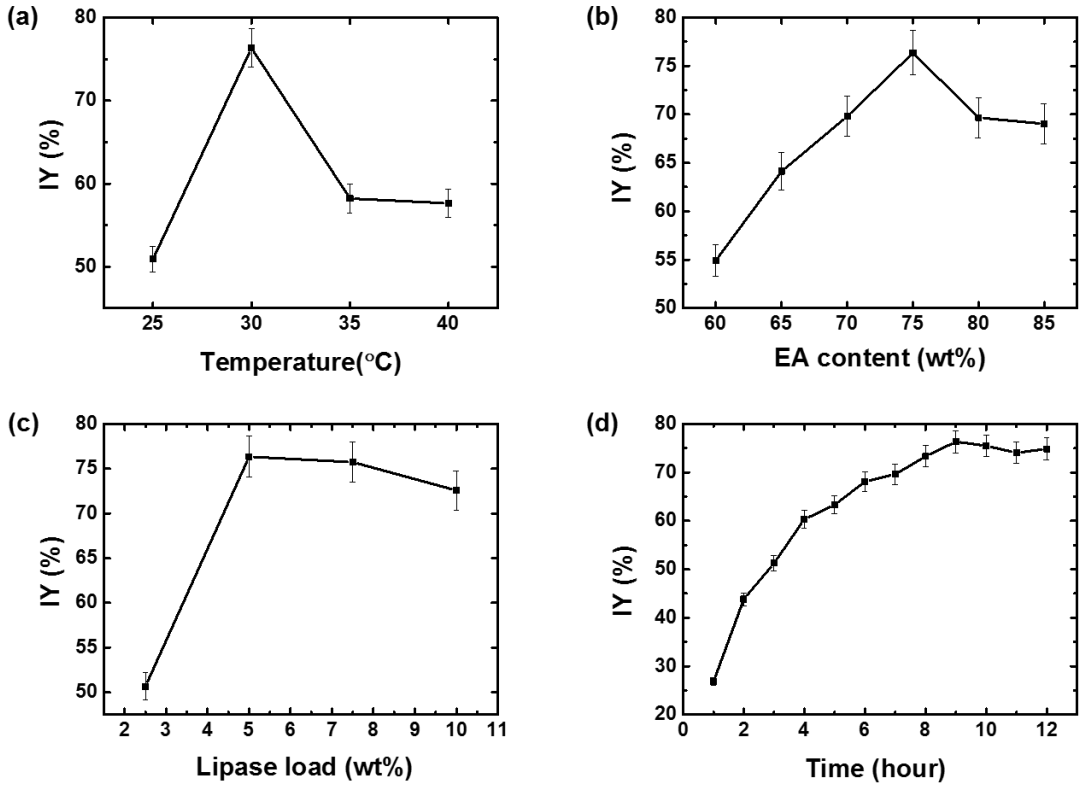

Figure 3.3. Effects of (a) reaction temperature (℃), (b) EA content (wt%), (c) lipase load (wt%) and reaction time (hour) on the interesterification of jojoba oil with EA catalyzed by Lipozyme RM IM. Reaction conditions: (a) 75 wt% EA, 5.00 wt% lipase load, 9 hours; (b) 5.00 wt% lipase load, 9 hours, 30 ℃; (c) 75 wt% EA, 9 hours, 30 ℃; (d) 75 wt% EA, 5.00 wt% lipase load, 30 ℃.

Figure 3.3c shows the influence of immobilized lipase load on the yield of current interesterification reaction. In our study, the dosage of lipase was varied at 2.50, 5.00, 7.50 and 10.00 wt% on the total weight of substrates, while the rest of reaction conditions

were kept constant (75 wt% EA, 9 hours, 30 ℃). For enzymatic reactions, generally, the reaction conversion would increase with an increasing amount of lipase, until it comes to a saturation point where substrate concentration cannot satisfy the lipase.[48] And in this case, 5.00 wt% seemed to be the saturate point of Lipozyme RM IM and provided the highest IY 76%. A decrease in IY was observed when higher dosage of lipase was charged, which is in accordance with other reports showing that increased lipase load might hinder the reaction as a result of poor mixing and limited mass transfer.[56-57] As suggested by Hanen Ghamgui *et al.*, the decreased IY may also relate to the protein aggregations caused by lipase overdosage, preventing the exposure of lipase active site to the substrates.[58] Hence, the load of Lipozyme RM IM was fixed at 5.00 wt% in the following study.

Last but not least, the time course of the current interesterification reaction was recorded in an attempt to identify the optimum reaction time length under the previous determined conditions (75 wt% EA, 5.00 wt% lipase load, 30 ℃). During the course of reaction, samples were withdrawn hourly from the reaction vessel, and prepared to be analyzed by ^{1}H NMR. As shown in Figure 3.3d, the IY curve rose very fast from the beginning to the fourth hour, and climbed slowly in the following five hours to reach the highest IY, after which the IY kept relatively stable with a slight drop. In this light, a period of 9 hours was chosen for Lipozyme RM IM to catalyze the current

interesterification reaction. The highest yield of jojoba LCMFAEE and LCMFAA obtained in our study was 76% in this interesterification system. Considering the overall moderate yield provided by Lipozyme RM IM, which was not as high as our expectations and other published results,[46] further optimization on the reaction conditions was believed to be pointless.

3.2.3 Interesterification with Novozym 435

Since the IY obtained in Lipozyme RM IM assays were not as high as expected, another commercially available lipase, Novozym 435, was then evaluated for its catalytic efficiency on the current interesterification under different reaction conditions. Surprisingly, we found a remarkable improvement in the yield of jojoba LCMFAEE and LCMFAA and therefore a detailed optimization was conducted for the reaction conditions, including lipase load, EA content, reaction temperature and time length. Since 300 rpm is also enough to maximize the mass transfer and minimize mechanical destruction to Novozym 435, such agitation speed was still maintained in the following experiments.[49-50, 52]

Figure 3.4a shows the effect of reaction temperature on the IY obtained in the current interesterification, while other reaction conditions were kept constant (75 wt% EA, 5.00 wt% lipase load, 9 hours). Firstly, a temperature range of 25 ℃-50 ℃ in 5 ℃ interval was tested, and the highest IY appeared in the assay at 35 ℃. As an effort to get more favored

temperature, the interval was squeezed and 27 ℃, 33 ℃, and 37 ℃ were also evaluated. Clearly, Novozym 435 performed best in the 37 ℃ assay and higher temperature led to a moderate decrease of IY. It is obvious that this lipase provided high yield (90-95%) of jojoba LCMFAEE and LCMFAA over the whole range of temperature tested, indicating an overall stronger catalytic activity than Lipozyme RM IM in the current interesterification reaction, which gave us more confidence in the following optimization. Similarly high yield (92%) was also achieved in the biodiesel production from soybean oil interesterified with methyl acetate in the presence of Novozym 435 at 40 ℃ by Wei Du *et al.*[59] As well as in Mukesh Kumar Modi's report on FAEE synthesis via interesterification of crude vegetable oils and EA at 50 ℃.[54] The reason higher temperatures were preferred in their works might be that a warmer condition was needed to reduce system viscosity and enhance mass transfer, seeing that the viscosity of TAG molecules in normal oils are much higher than that of monoesters in jojoba oil.[48-49] Grounded on these results, 37 ℃ was determined to be the optimum temperature for Novozym 435 in this interesterification reaction system and fixed for subsequent experiments.

Figure 3.4b shows how EA content influence the yield of the interesterification reaction under the catalysis of Novozym 435. A wider range 50-90 wt% was evaluated in this case, and the maximal IY was attained when the amount of EA was set at 75 wt%,

which coincided with the result from Lipozyme RM IM assay. This observation might suggest that the viscosity of 75 wt% EA content system was suitable for both lipases, and such a substrate ratio shifted the interesterification equilibrium most efficiently. In other words, the effect of EA content on the yield of jojoba LCMFAEE and LCMFAA was regardless of lipase type in the current studied system. Therefore, the content of EA was maintained at 75 wt% in the rest of our study.

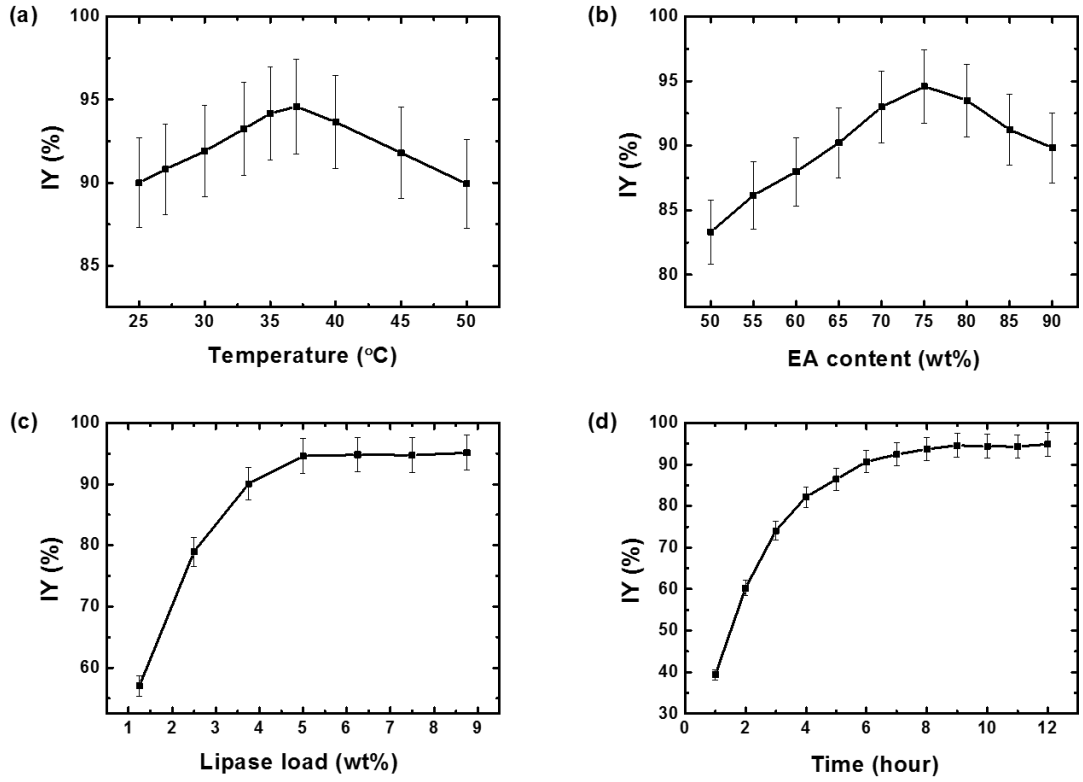

Figure 3.4. Effects of (a) reaction temperature (℃), (b) EA content (wt%), (c) lipase load (wt%) and reaction time (hour) on the interesterification of jojoba oil with EA catalyzed by Novozym 435. Reaction conditions: (a) 75 wt% EA, 5.00 wt% lipase load, 9 hours; (b) 5.00 wt% lipase load, 9 hours, 37 ℃; (c) 75 wt% EA, 9 hours, 37 ℃; (d) 75 wt% EA, 5.00 wt% lipase load, 37 ℃.

In a further effort to improve IY, the effect of Novozym 435 dosage was studied by ranging the lipase load from 1.25 to 8.75 wt% in 1.25 wt% interval while keeping other reaction conditions constant (75 wt% EA, 9 hours, 37 ℃). As shown in Figure 3.4c, the IY value increased rapidly when raising the lipase load from 1.25 to 5.00 wt%, where the peak yield of jojoba LCMFAEE and LCMFAA (95%) was achieved. Similar with the case of Lipozyme RM IM, a lipase load of 5.00 wt% seemed to be the saturated point of Novozym 435 as well. However, further addition of Novozym 435 (5.00 to 8.75 wt%) led to a stable IY curve instead of a decreasing one as shown in Figure 3.3c, indicating that the mass transfer hindrance caused by high dosage of Novozym 435 was not significant as that of Lipozyme RM IM. And this might relate to the differences in lipase structure as well as the immobilization carrier, seeing that Lipozyme RM IM was immobilized on anion exchange resin while Novozym 435 was immobilized on the much more hydrophobic acrylic resin.[60] Taking both efficiency and economy into consideration, the optimum amount of Novozym 435 for the current reaction was determined to be 5.00 wt%.

Time course of the interesterification reaction in the presence of Novozym 435 was recorded under the previously optimized reaction conditions (75 wt% EA, 5.00 wt% lipase load, 37 ℃). Similar with the time course of Lipozyme RM IM catalyzed interesterification (Figure 3.3d), the Novozym 435 involved one as shown in Figure 3.4d

also began with a fast reaction rate in the first four hours, and reached the highest IY slowly in the following period (5^{th} – 9^{th} hour), after which a stable yield of jojoba LCMFAEE and LCMFAA was observed. However, the yields of Novozym 435 assay were much higher than those of Lipozyme RM IM assay at each time point, and the maximal IY in the presence of Novozym 435 was 95%, whereas it was only 76% for Lipozyme RM IM. Hence, it can be concluded that Novozym 435 outperformed Lipozyme RM IM in this specific reaction system and it was recommended for further studies.

3.2.4 Operational stability of Novozym 435

In terms of enzymatic process economics, lipase recycle has significant implications in decreasing the process cost of enzyme use as well as waste production.[59] The immobilized lipases were therefore utilized in our research, since a solid carrier can improve the enzyme stability in organic solvents and facilitate the recovery of both products and the biocatalyst.[60] For interesterification reaction system, properties of the acyl acceptors play an important role in the recycle of lipase.[54, 59] In Wei Du's study, both methanol and methyl acetate were evaluated for their influence on enzyme activity, and the former was found to inactivate the lipase seriously while the latter showed no negative impacts and continually gave high conversion of biodiesel without observable loss in lipase activity.[59] Mukesh Kumar Modi and his colleagues have proved that EA has

no negative effect on the catalytic activity of Novozym 435 by comparing the IY provided by normal lipase and lipase that treated with EA for 72 hours, where no significant difference has been observed.[54] Meanwhile, they also investigated the reusability of Novozym 435 over 12 repeated cycles, and found that the yields of their interesterification reactions were constantly high, indicating an outstanding operational stability of such lipase.[54]

In our study, the operational stability of Novozym 435 was examined when the previously optimized reaction conditions (75 wt% EA, 9 hours, 37 ℃, 5.00 wt% lipase load) were applied. After each interesterification reaction cycle, the same lipase was regenerated through hexane wash and stored at 4 ℃ until reuse. As shown in Figure 3.5, the immobilized lipase exhibited excellent operational stability as a biocatalyst for the current interesterification reaction for at least 17 cycles (153 hours). The obtained average IY was 88%, slightly lower than both Wei Du's and Mukesh Kumar Modi's results (> 90%). It can be well explained because less vigorous agitation can help reduce the mechanical destruction to the enzyme and its carrier, and the stirring speed was set at 300 rpm in our experiments while theirs were only 150 rpm. Nevertheless, the operational stability of Novozym 435 was good enough for the current study and its cost can be reduced significantly, making the method more economically attractive for industrialized production of functional jojoba LCMFAEE and LCMFAA.

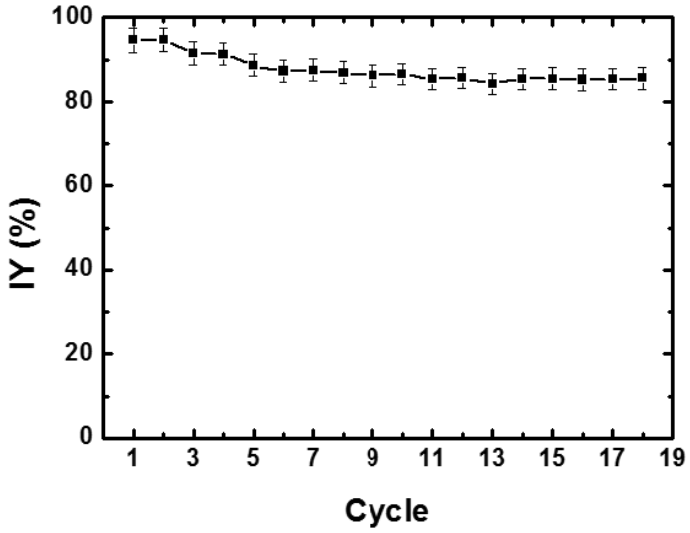

Figure 3.5. Operational stability of Novozym 435 in the interesterification of jojoba oil with EA. Reaction conditions: 75 wt% EA, 9 hours, 37 ℃, 5.00 wt% lipase load, 300 rpm.

3.2.5 Composition analysis of jojoba LCMFAEE and LCMFAA

Composition analysis on the jojoba LCMFAEE and LCMFAA was conducted by GC-MS as shown in Table 3.1 and 3.2, respectively. FT-IR was also utilized to analyze the configuration of the carbon-carbon double bonds within the monoesters. Given the absence of absorption around 970 cm^{-1} and the presence of absorption around 730 cm^{-1}, it was concluded that all ethylenic bonds were in *cis* configuration. The jojoba LCMFAEE was dominated by ethyl *cis*-11-eicosenoate with a content of 88.37%. On the other hand, the jojoba LCMFAA was mainly composed of *cis*-11-eicosenyl acetate (49.66%) and *cis*-13-docosenyl acetate (44.26%). In other words, the compositions of the entire interesterified product were mainly ethyl *cis*-11-eicosenoate (44.19%), *cis*-11-eicosenyl acetate (24.83%) and *cis*-13-docosenyl acetate (22.13%). The interesterified product can

be used as bio-functional ingredients and flavor and fragrance agents in the purified form, or as emulsifiers and edible coatings in mixture for a wide range of food products.

Table 3.1. Composition of jojoba LCMFAEE profiled by GC-MS analysis.

Compound	Fatty acid component	R_t (min)	Content (wt%)
Ethyl tetradecanoate	C(14:0)	7.83	0.01
Ethyl *cis*-9-hexadecenoate	C(16:1)	10.34	0.02
Ethyl hexadecanoate	C(16:0)	10.62	0.49
Ethyl *cis*-9-octadecenoate	C(18:1)	13.19	3.25
Ethyl *cis*-11-eicosenoate	C(20:1)	16.28	88.37
Ethyl eicosanoate	C(20:0)	16.75	0.11
Ethyl *cis*-13-docosenoate	C(22:1)	19.30	7.27
Ethyl docosanoate	C(22:0)	19.67	0.18
Ethyl *cis*-15-tetracosenoate	C(24:1)	22.21	0.27
Ethyl tetracosanoate	C(24:0)	22.56	0.02

Table 3.2. Composition of jojoba LCMFAA profiled by GC-MS analysis.

Compound	Fatty alcohol component	R_t (min)	Content (wt%)
Cis-9-tetradecenyl acetate	C(14:1)	7.91	0.18
1-Hexadecanyl acetate	C(16:0)	10.60	0.03
Cis-9-octadecenyl acetate	C(18:1)	13.56	0.59
1-Octadecanyl acetate	C(18:0)	13.84	0.12
Cis-11-eicosenyl acetate	C(20:1)	16.51	49.66
1-Eicosanyl acetate	C(20:0)	16.92	0.18
Cis-13-docosenyl acetate	C(22:1)	19.54	44.26
1-Docosanyl acetate	C(22:0)	19.87	0.59
Cis-15-tetracosenyl acetate	C(24:1)	22.41	4.30
1-Tetracosanyl acetate	C(24:0)	22.72	0.09

3.3 Conclusions

The jojoba LCMFAEE and LCMFAA have been successfully synthesis through the enzymatic interesterification of jojoba oil with EA with a yield of 95%. Novozym 435 overall outperformed Lipozyme RM IM in the current reaction, for it gave the highest IY whereas the IY was only 76% in the case of Lipozyme RM IM. The optimum reaction conditions were determined to be 9 hours reaction at 37 ℃ with an EA content of 75 wt% in the presence of 5.00 wt% Novozym 435. The preferred lipase also exhibited excellent operational stability over 17 repeated interesterification cycles with an average IY of 88%. Profiled by GC-MS, the compositions of the entire interesterified product were mainly ethyl *cis*-11-eicosenoate (44.19%), *cis*-11-eicosenyl acetate (24.83%) and *cis*-13-docosenyl acetate (22.13%).

The proposed method produces the functional jojoba LCMFAEE and LCMFAA in one simple enzymatic reaction, which not only avoided costly separation and purification but also maximized the utilization of the LCMFAC and LCMFAL from jojoba oil. The resulted jojoba LCMFAEE and LCMFAA, in both mixture and purified form, have great versatility and potential for a wide range of applications in food industry, such as bio-functional ingredients, flavor and fragrance agents, emulsifiers and edible coatings.

3.4 Experimental Section

3.4.1 Oils and Reagents

Jojoba oil was purchased from NOW® Solution (IL, USA) and characterized by ^{1}H NMR (Table 3.3). Lipozyme RM IM (lipase *Rhizomucor miehei*, immobilized on anion exchange resin) and Novozym 435 (lipase from *Candida Antarctica*, immobilized on acrylic resin) were generous gifts from Novo Nordisk A/S, Bagsvaerd, Denmark. EA and hexane were purchased from RCI Labscan Limited (Bangkok, Thailand). $CDCl_3$ with TMS (v/v 0.03%) was purchased from Cambridge Isotope Laboratories Inc. (MA, USA).

Table 3.3. Assignment of ^{1}H NMR signals (400 MHz, $CDCl_3$) of commercial jojoba oil.

Proton(s)	Functional group	Chemical shift, δ(ppm)	Integration
C**H**$_3$-C	terminal methyl protons	0.88(t) *J* = 6.8 Hz	6
-(C**H**$_2$)$_n$-	backbone CH_2	1.27(m)	54.5
-C**H**$_2$CH$_2$COOR and -C**H**$_2$CH$_2$OCOR	β-methylene protons	1.61(m)	4
=CH-C**H**$_2$-	α-methylene protons to one double bond	2.00(m)	8
-C**H**$_2$COOR	α-methylene protons of fatty acid to ester	2.28(t) *J* = 7.6 Hz	2
-C**H**$_2$OCOR	α-methylene protons of fatty alcohol to ester	4.05(t) *J* = 6.8 Hz	2
-C**H**=C**H**-	olefinic protons	5.34(m)	4

3.4.2 Enzymatic Interesterification

In a typical reaction, about 3.0 grams of jojoba oil and a certain amount of EA were mixed at 300 rpm for 5 min at given temperature, a certain amount of lipase was added to initiate reaction. Optimization on reaction conditions were conducted via different assay of experiments where EA content, lipase load, reaction temperature and time length were varied independently. To monitor reaction progress, aliquots of 700 μL were collected hourly from the reaction system, treated by rotary evaporation at 35℃ to remove extra EA and submitted for ^{1}H NMR analysis. Reaction was terminated by removal of lipase by filtration, and the resulted reaction mixture was purified through preparative TLC with an eluent of n-hexane/EA (25:1 v/v). The obtained jojoba LCMFAEE and LCMFAA were in the form of light yellow liquid and characterized by ^{1}H NMR (Table 3.4 and 3.5, respectively) and FT-IR.

Jojoba LCMFAEE: FT-IR (cm^{-1}, air): 3154, 2957, 2928, 2857, 2253, 1730, 1466, 1375, 1250, 1217, 1096, 1045, 912, 905, 745, 727, 650.

Jojoba LCMFAA: FT-IR (cm^{-1}, air): 3154, 2926, 2855, 2253, 1726, 1466, 1379, 1252, 1215, 1096, 1036, 908, 735, 650.

3.4.3 IY quantification via ^{1}H NMR analysis

Table 3.4. Assignment of ^{1}H NMR signals (400 MHz, $CDCl_3$) of jojoba LCMFAEE.

Proton(s)	Functional group	Chemical shift, δ(ppm)	Integration
C**H**$_3$-C	terminal methyl protons of fatty acid	0.88(t) J = 6.8 Hz	3
-(C**H**$_2$)$_n$- and C**H**$_3$CH$_2$OCOR	backbone CH_2 and terminal methyl protons of ethyl group	1.27(m)	27.5
-C**H**$_2$CH$_2$COOR	β-methylene protons of fatty acid to ester	1.61(m)	2.6 (contain H_2O)
=CH-C**H**$_2$-	α-methylene protons to one double bond	2.00(m)	4
-C**H**$_2$COOR	α-methylene protons of fatty acid to ester	2.28(t) J = 7.6 Hz	2
-C**H**$_2$OCOR	methylene protons of ethyl group	4.11(q) J_1 = 7.2 Hz, J_2 = 14.4 Hz	2
-C**H**=C**H**-	olefinic protons	5.34(m)	2

Table 3.5. Assignment of ^{1}H NMR signals (400 MHz, $CDCl_3$) of jojoba LCMFAA.

Proton(s)	Functional group	Chemical shift, δ(ppm)	Integration
C**H**$_3$-C	terminal methyl protons of fatty alcohol	0.88(t) J = 6.8 Hz	3
-(C**H**$_2$)$_n$-	backbone CH_2	1.27(m)	30.9
-C**H**$_2$CH$_2$OCOR	β-methylene protons of fatty alcohol to ester	1.61(m)	2.9 (contain H_2O)
=CH-C**H**$_2$- and C**H**$_3$COOR	α-methylene protons to one double bond, and methyl protons of acetyl	2.00(m)	7
-C**H**$_2$OCOR	α-methylene protons of fatty alcohol to ester	4.05(t) J = 6.8 Hz	2
-C**H**=C**H**-	olefinic protons	5.34(m)	2

The samples were dissolved in $CDCl_3$ in a ratio of 1:5 (v/v) and analyzed in 5 mm NMR tubes. Chemical shifts were referred to TMS signal (δ = 0.0 ppm) indirectly by calibrating the residual signal from CHD_2Cl_3 to 7.26 ppm. ^1H NMR spectra were recorded on a Bruker Avance 400 spectrometer operating at 400 MHz with QNP probe (5 mm) and the figures were processed with Bruker's TopSpin™ software.

3.4.4 Jojoba LCMFAEE and LCMFAA composition analysis via GC-MS

The samples were processed on a Thermo TR-5MS column (30 m × 0.25 mm I.D. × 0.25 μm film) in a ThermoFinnigan PolarisQ MS system which was equipped with Thermo Trace GC. Both injector and detector temperature were set at 300 ℃. Column temperature was programmed from 80 ℃ to 180 ℃ at 25 ℃/min, from 180 ℃ to 280 ℃ at 5 ℃/min, from 280 ℃ to 300 ℃ at 15 ℃/min, and then kept at 300 ℃ for 8 min. Injection volume was 0.5 μL and sample concentration was 1 ppm.

3.5 References

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Chapter 4

Enrichment of Omega-3 PUFA in Cod Liver Oil via Alternate Solvent Winterization and Enzymatic Interesterification

4.1 Introduction

Omega-3 fatty acids are PUFA of biofunctional significance, which contain carbon-carbon double bonds starting at the third carbon atoms from the terminal methyl group of the fatty acids.[1] EPA and DHA are the most common omega-3 PUFA, of which the nutritional importance and curative implications to human body have been demonstrated, including reduction in risk of cardiovascular disease, cognitive decline, Alzheimer's disease and even cancers, as well as regulations of blood pressure and thickness, hormone production, inflammation, and function of immune and central nervous systems.[2-11] Generally, they exist in the form of TAG in food, which is more readily absorbed and metabolized by human body than their methyl or ethyl esters.[1, 12-15] Due to human's limited ability to produce EPA and DHA, direct intake of omega-3 fats is clinically advised to maintain the normal metabolism of human body.[16-18]

Nowadays the main source of dietary omega-3 fatty acids, mainly EPA and DHA, come from the oils of cold water fishes, such as salmon and cod fish.[19] For instance, natural CLO contains 18.7 wt% omega-3 fatty acids on average while the rest are mainly monounsaturated fatty acids (MUFA) (53.0 wt%) and SFA (23.2 wt%).[20] It is well recognized that MUFA is beneficial for human body, whereas SFA is the so-called 'bad fatty acid'.[21-22] Hence, in order to intake the recommended omega-3 fatty acids and avoid unhealthy SFA in the meantime, enrichment of omega-3 PUFA content in dietary oils has

gained comprehensive attention and been studied widely.[2, 19, 23-27]

So far, two major methodologies have been developed to enrich the content omega-3 PUFA in edible oils in the form of TAG. The first methodology mainly relies on physical property differences between omega-3 PUFA and non-omega-3 fatty acids, such as boiling point and melting point. Distillation of Menhaden oil was studied and the content of EPA and DHA in TAG form has been increased to 37 wt% on the basis of 24 wt%.[28] Homayooni B. reported the concentration of omega-3 PUFA in rainbow sardine fish oil from 34.52 wt% to 47.53 wt% via low-temperature fractional crystallization at -5℃ for 24 hours in the TAG form.[19] The corresponding values were 25.53 wt% and 31.50 wt%, respectively, when refined hoki oil was subjected to solvent winterization at -80℃ for 6 hours in Tengku Mohamad Tengku-Rozaina's study.[2] Despite the advantages in easy operation and less chemical usage, the increment of omega-3 fatty acids content was not remarkable.

The second methodology involves the change of fatty acid compositions within oils by introducing lipases as biocatalyst in hydrolysis, interesterification, transesterification, and re-esterification reactions. In Tsao-Jen Lin's research, transesterification of Menhaden oil with pre-accumulated PUFA catalyzed by Lipozyme RM IM was used to raise the content of omega-3 PUFA from 21 wt% to 58 wt%.[27] Gerald McNeill and his colleagues enriched EPA and DHA from 30 wt% to 45 wt% in fish oil by selective enzymatic hydrolysis in

the presence of *Candida rugosa* lipase and therewith nonselective re-esterification of FFA and partial glycerides that mainly contained omega-3 PUFA.[26] According to Michel Linder *et al.*, enzymatic hydrolysis, followed by membrane filtration and enzymatic re-esterification, can concentrate omega-3 PUFA in salmon oil from 39.2 mol% to 43.3 mol%.[23] However, the oil products produced by such methods always contain a great amount of FFA, MAG and DAG fractions, since the multiple enzymatic reactions broke the TAG structure and generated various by-products. Further purification process, such as molecular distillation, membrane filtration and urea complexation, was therefore required, resulting a higher production cost.[29]

The objective of the current study is to propose a novel solution for the enrichment of omega-3 fatty acids in the form of TAG in CLO, namely the alternate conduction of solvent winterization and enzymatic interesterification (Figure 4.1). Different solvents, oil/solvent concentrations, temperatures and time lengths of the solvent winterization of CLO was investigated to achieve better removal of TAG molecules with high melting point fatty acids.[30-34] Also, enzymatic interesterification of the winterized CLO was studied under different conditions (system air condition, reaction temperature and time length) with different lipases, including Lipozyme RM IM, Lipozyme TL IM and Novozym 435. The aim of this procedure is to re-distribute the fatty acids to form TAG molecules accumulated with high melting point fatty acids, facilitating their removal in

the following solvent winterization. The content of omega-3 PUFA in oil sample was quantified by ^{1}H NMR,[35] and the fatty acid profile was analyzed by GC-MS. Our proposed method could enrich the content of omega-3 PUFA in CLO with two simple food techniques, providing excellent industrial feasibility and also reducing the production cost drastically.

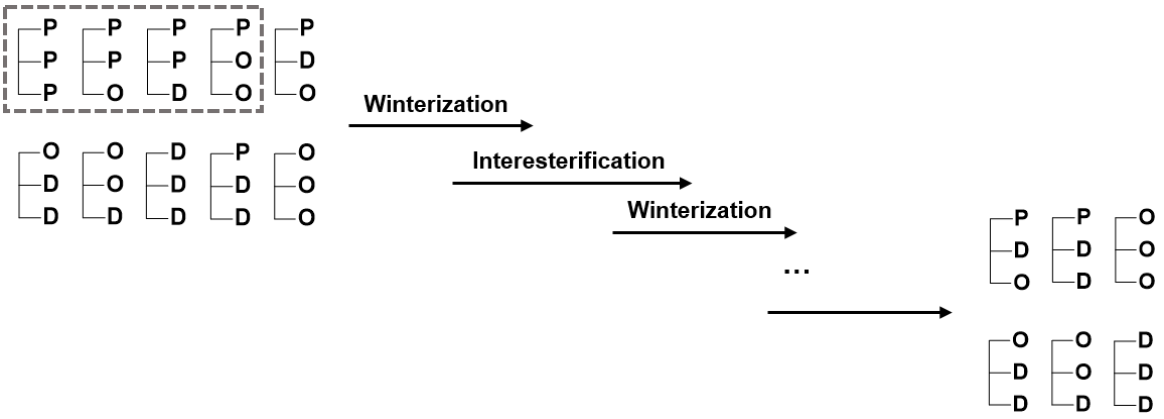

Figure 4.1. Illustration of the proposed method for the enrichment of omega-3 PUFA in CLO in TAG form through alternate solvent winterization and enzymatic interesterification. (D, docosahexaenoic; O, oleic; P, palmitic. The designation PDO, etc., does not indicate the TAG PDO alone, but all the positional isomers — PDO, ODP and POD, etc.)

4.2 Results and Discussion

4.2.1 ^{1}H NMR quantification of omega-3 PUFA content

Direct determination of the content of omega-3 PUFA in the winterized oil samples was realized by ^{1}H NMR analysis following Raffaele Sacchi's method, which was firstly proposed in 1993 and later fortified by many researchers.[35-38] As shown in Figure 4.2, one of the major differences between omega-3 PUFA fractions and non-omega-3 fatty acid

fractions is the chemical environments of their terminal methyl protons. Influenced by the omega-3 carbon-carbon double bond, the methyl protons in omega-3 PUFA (H^a) should exhibit a triplet around 0.96 ppm in the ^{1}H NMR spectrum, while the manifestation of methyl protons in non-omega-3 fatty acids (H^b) is around 0.87 ppm as another triplet. These two different signals are in theory differentiable in ^{1}H NMR spectra and have been verified with substantial studies.[35, 37] Figure 4.2 shows the ^{1}H NMR spectrum of the original CLO, and clearly there is no overlap of those two triplet signals. Hence, the content of omega-3 PUFA in the CLO sample can be determined using the following equation[35]:

$$\text{Content (mol\%)} = \frac{\text{mole}(\omega - 3)}{\text{mole}(\omega - 3) + \text{mole}(\text{non} - \omega - 3)} \times 100$$

$$= \frac{\text{Area}(0.96)}{\text{Area}(0.96) + \text{Area}(0.87)} \times 100$$

Where mole(ω-3) and mole(non-ω-3) indicate the relative amounts of omega-3 PUFA fractions and non-omega-3 fatty acid fractions in mole, respectively; Area(0.96) and Area(0.87) indicate the areas of the triplet signals at 0.96 ppm and 0.87 ppm, respectively. The content of omega-3 PUFA in original CLO was determined to be 21.30 mol%, consistent with our GC-MS result 19.92 mol% (Table 4.1). Therefore, this ^{1}H NMR quantification method was applied in our entire research to determine the content of omega-3 PUFA.

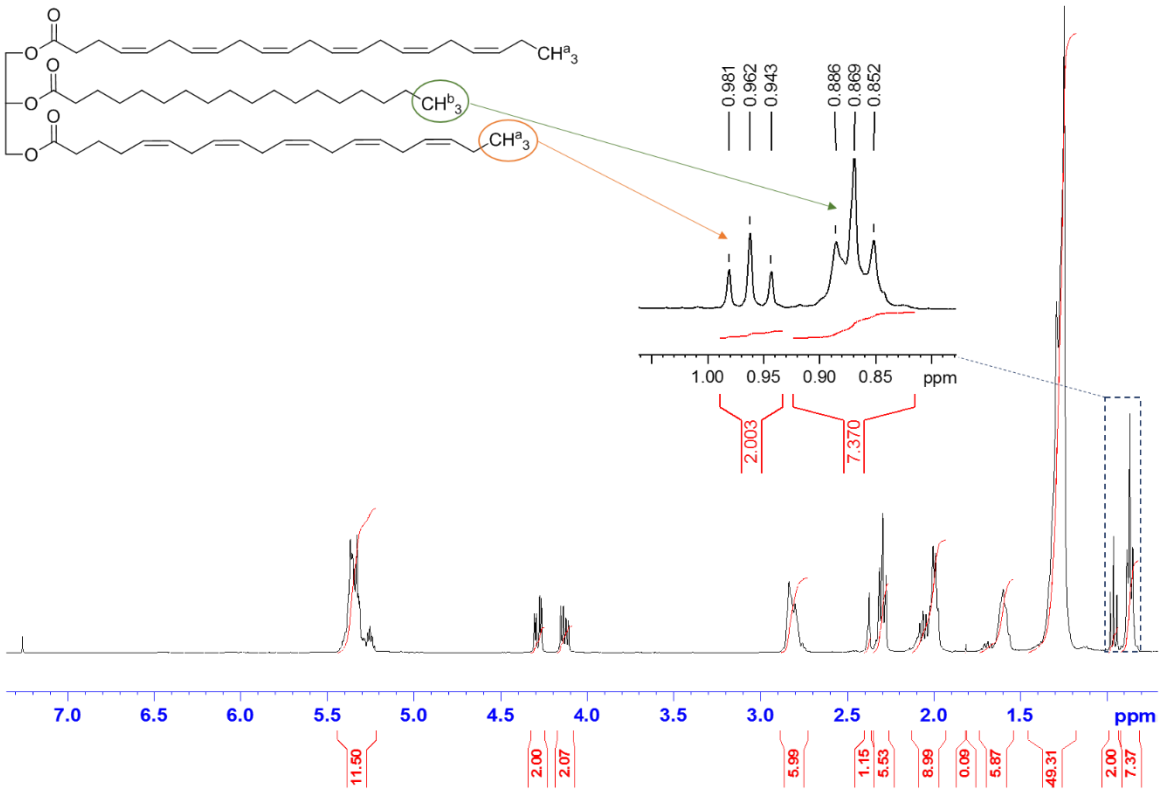

Figure 4.2. ^{1}H NMR spectrum of the original CLO (400 MHz, $CDCl_3$) and the difference in the chemical shift between the methyl protons of omega-3 PUFA fractions and that of non-omega-3 fatty acid fractions.

No internal standard was needed in the current study, since the quantification is on the basis of structure-specific spectroscopic measurement and relative amounts of omega-3 PUFA and non-omega-3 fatty acids which can be obtained directly by measuring the areas of the characteristic signals in the ^{1}H NMR spectra.[35] Good control on NMR sample concentration as well as nice shimming on the NMR instrument are of great importance to avoid signal overlap and attain more precise quantification.[39] Each experiment assay was triplicated and the data shown here are expressed as the mean value (within 3% relative standard deviation). Results calculated from ^{1}H NMR spectra data were in agreement with those obtained from GC-MS analysis (within 2% of error) as shown in

Table 4.1. It can be observed that ^{1}H NMR quantification got slightly higher values, which was opposite to the phenomena reported by other researchers.[36-37] Sample oxidation during the pretreatment of GC-MS might contribute to these deviations.

4.2.2 Preliminary study

4.2.2.1 Solvent winterization

Firstly, the effect of solvent type on the winterization of CLO was investigated at -20 ℃ and -80 ℃ while other conditions were constant (oil/solvent concentration 0.1 g/mL, 24 hours). Since acetone, hexane and ethanol are approved organic solvent for food application and have been adopted in other winterization research, they were all evaluated for the current study.[2, 19, 24] As shown in Figure 4.3, increments in the omega-3 PUFA content were not obvious in all assays, except the one with acetone at -20 ℃ (4.33 mol%, recovery 68%). The outperformance of acetone was believed to be a result of its proper polarity, which can provide good selectivity for TAG molecules accumulated with PUFA into the liquid phase (LP) and maintain the efficiency of the separation process.[1, 24] The less polar one, hexane, reserved the majority of oil in the LP and little was left in the solid phase (SP), leading to inefficient separation.[24] On the contrary, the strong polarity of ethanol decreased oil solubility and therefore diminished the selectivity, in consideration of the insignificant increment of omega-3 fatty acid content together with a very low recovery. In addition, it was found that all assays at -80 ℃ had similarly low increments

of omega-3 PUFA content and high recoveries. Possible explanation could be referred to Fourier's law, that heat transfer rate is proportional to the temperature difference within the same system. On this occasion, the big temperature gap from -80 ℃ to room temperature accelerated the decalescence of winterized oil during the two-minute centrifugation, leading to fast thawing and thereby futile winterization. Hence, winterization with acetone at -20 ℃ was chosen for the rest of our preliminary study.

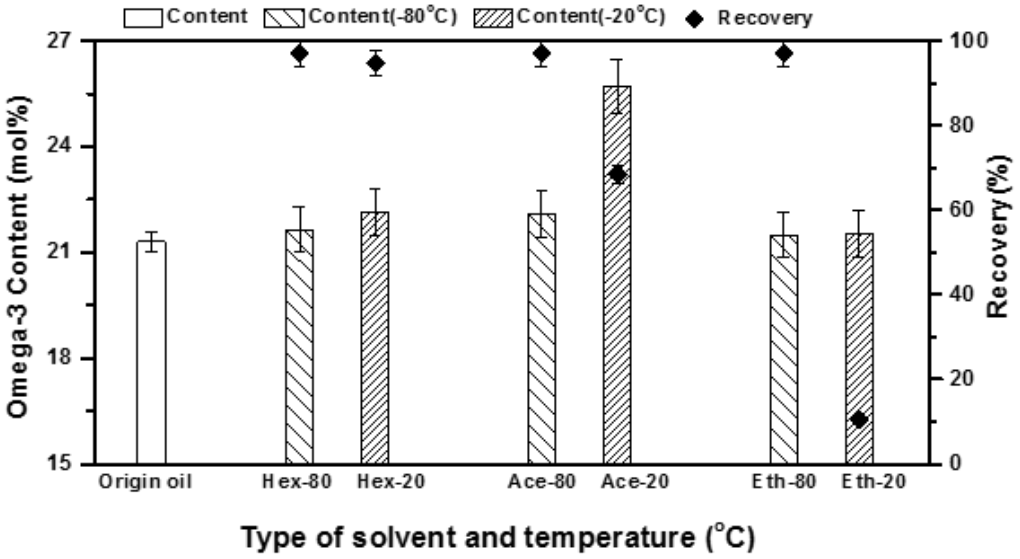

Figure 4.3. Preliminary study on the effects of solvent type on the solvent winterization efficiency at -20 ℃ and -80 ℃. Conditions: (Ace-20/80) acetone at -20/-80 ℃; (Hex-20/80) hexane at -20/-80 ℃; (Eth-20/80) ethanol at -20/-80 ℃.

The effect of different oil/acetone concentrations on winterization efficiency was then studied at -20 ℃ for 24 hours (Figure 4.4). The highest increment in omega-3 PUFA content in LP was 4.4 mol% at a concentration of 0.1 g/mL. Further increase of the oil concentrations elevated the content of omega-3 PUFA in SP instead of LP. Equivalent observation has been reported by López-Martínez, J. C. and his coworkers, who

suggested that low oil concentration would promote the formation of a meta-stable solution which facilitates spontaneous crystal growth.[40] On the other hand, high oil concentration would help generate supersaturated solution where nucleation is favored against crystal growth, resulting in loss of selectivity on different TAG molecules.[40] Furthermore, samples with higher oil/acetone concentration unfroze faster during centrifugation, leading to a further decrease of separation efficiency. Despite the fact that 0.05 g/mL assay provided lowest contents of omega-3 PUFA in SP, suggesting an efficient removal of non-omega-3 fatty acids, its increment in LP was not efficient (2.96 mol%). Therefore, an oil/acetone concentration of 0.1 g/mL was determined to be the optimum for the current winterization.

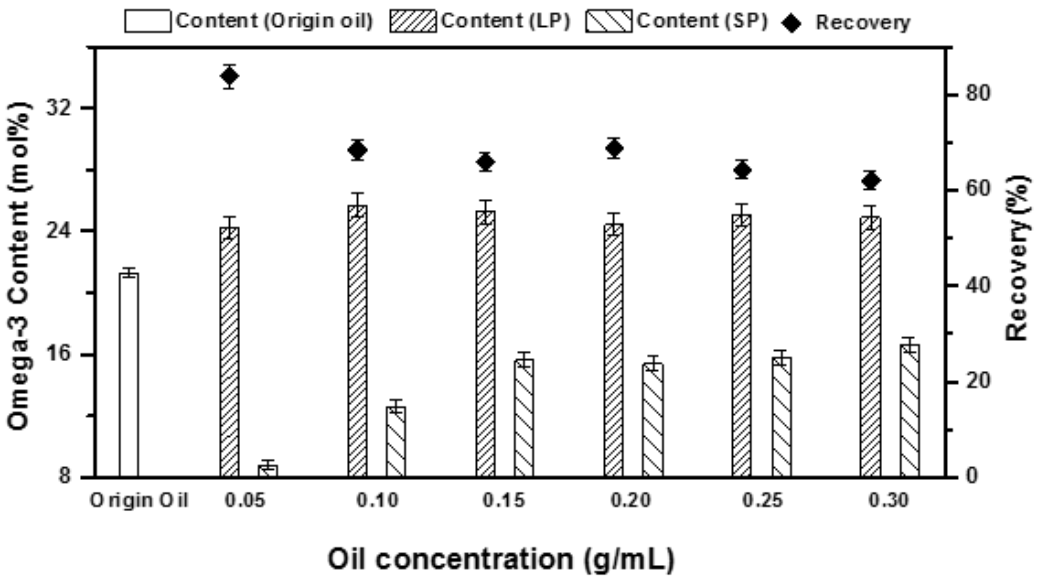

Figure 4.4. Preliminary study on the effect of oil/acetone concentration on the solvent winterization efficiency. (SP, solid phase; LP, liquid phase.)

The effect of time on the solvent winterization efficiency was also examined when other conditions were fixed as 0.1 g/mL and 24 hours. As shown in Figure 4.5, no significant difference has been observed in their results which ranged from 25.01 mol% to 25.70 mol%, indicating that time length might not be a major factor for the current solvent winterization. Similar conclusion was also drawn by Luis Vazquez and Casimir C. Akoh in their research on stearidonic acid enrichment in modified soybean oil via solvent winterization.[24] Since the 24 hours assay gave the largest omega-3 PUFA content difference between LP and SP, suggesting a slightly better separation, it was chosen as the winterization time length in our following experiments.

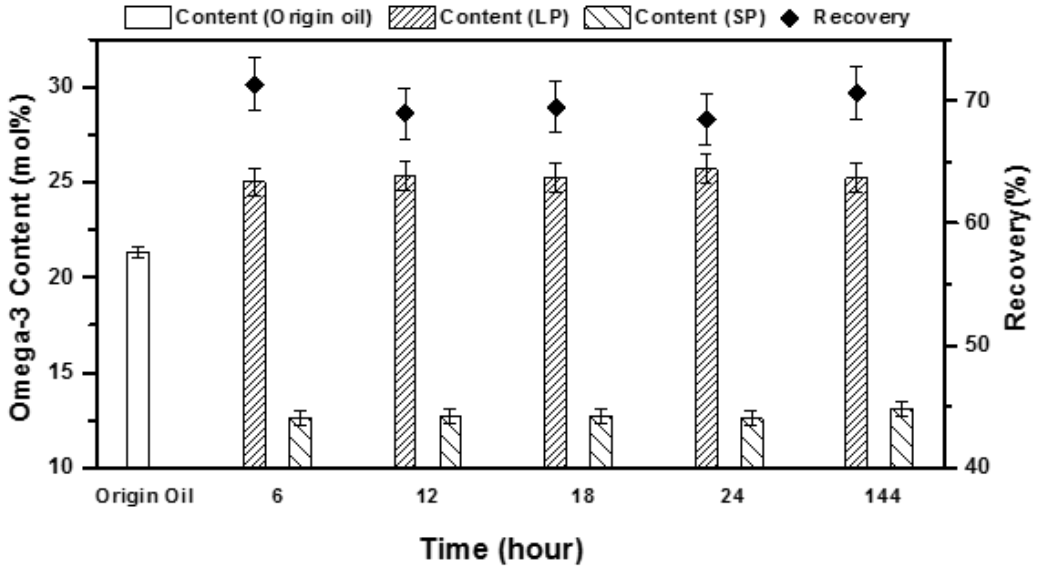

Figure 4.5. Preliminary study on the effect of time on the solvent winterization efficiency. (SP, solid phase; LP, liquid phase.)

4.2.2.2 Enzymatic interesterification

The objective of the current interesterification reaction is to re-arrange the fatty acid

distributions within the winterized CLO. For example, POD and PDD TAG molecules that have lower melting point, might be reserved during the first round of solvent winterization (D, docosahexaenoic; O, oleic; P, palmitic. The designation PDO, etc., does not indicate the TAG PDO alone, but all the positional isomers — PDO, ODP and POD, etc.). Upon the enzymatic interesterification, the palmitic acids reserved in such molecules could be re-arranged into new PPP, PPD and PPO TAG molecules that have higher melting points, and would be removed by the following winterization. As a result, content of such SFA with high melting points would decrease gradually, whereas the low melting point PUFA, especially EPA and DHA in the case of CLO, would be accumulated.

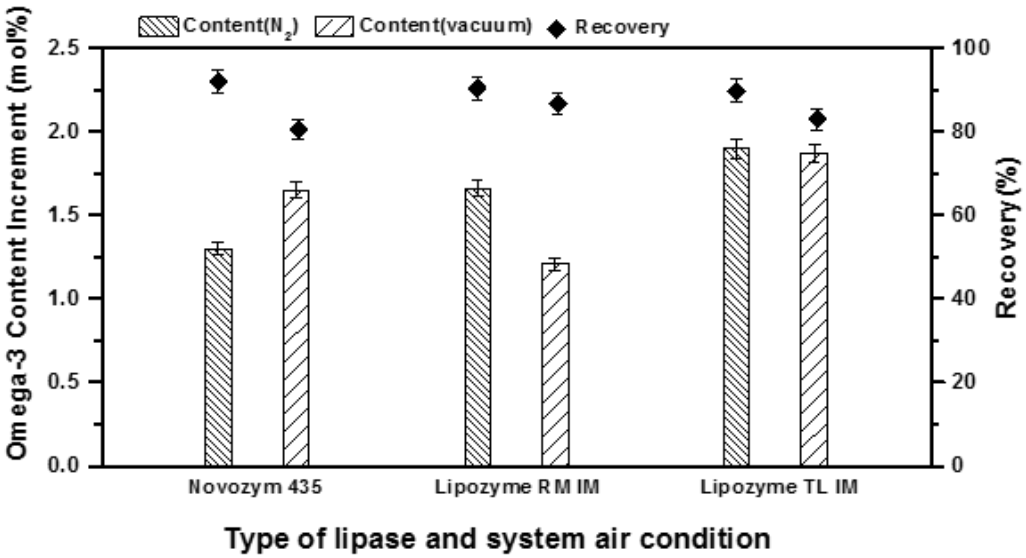

Figure 4.6. Effect of system air condition on the efficiency of different lipases in catalyzing the enzymatic interesterification of CLO. Reaction conditions: 5 wt% lipase load, 60 ℃, 6 hours, 300 rpm.

Three commercialized lipases, namely Lipozyme RM IM, Lipozyme TL IM and Novozym 435, were screened for their efficiency in catalyzing the current interesterification. A stirring speed of 300 rpm was kept as constant in all experiments, seeing that it is high enough for all three lipases involved to maximize mass transfer within the reaction system as well as avoid significant mechanical destruction to the lipase carrier during reactions at the same time.[41-44] The amount of lipase was all fixed at 5 wt%, which is sufficient to maintain lipase activity and efficiency as well as minimize high viscosity induced hindrance in system mass transfer.[44-47] After reaction, the oil product was subjected to a following solvent winterization with 0.1 g/mL oil/acetone concentration at -20 ℃ for 24 hours, and the increment in omega-3 fatty acid content was then used as the standard to evaluate the reaction conditions.

Firstly, catalytic efficiency of the three lipases on the current interesterification reaction were studied at 60 ℃ for 6 hours. Given that fact that omega-3 fatty acids are highly vulnerable to oxidation and thereby generate various genotoxic and cytotoxic compounds,[48] the air condition of the current reaction system was maintained in nitrogen flow or under vacuum in an attempt to prevent the oxidation.[49] As shown in Figure 4.6, both Lipozymes was found to be more efficient in nitrogen flow than under vacuum, whereas Novozym 435 behaved contrarily. Possible explanation might relate to the lipase water content during reaction, since the major difference in these two conditions lies in

that nitrogen flow still remains a certain amount of water in the reaction system while rare is left under vacuum. Despite the recommendations on lower water content to minimize by-product formation (e.g., FFA, MAG and DAG), a little amount of water is essential to preserve the enzymatic activity for all three lipases.[49-59] Comparing the observations reported by Hong Zhang and Malgorzata Kowalska,[49, 59] Novozym 435 gave better performance in drier condition (e.g., 2 wt% water content), while Lipozyme RM IM and Lipozyme TL IM required a little more water (4 wt% and 3 wt%, respectively) for interesterification reactions, in agreement with our results. In this light, all the following experiments with Novozym 435 were fixed under vacuum, and in nitrogen flow for the two Lipozymes.

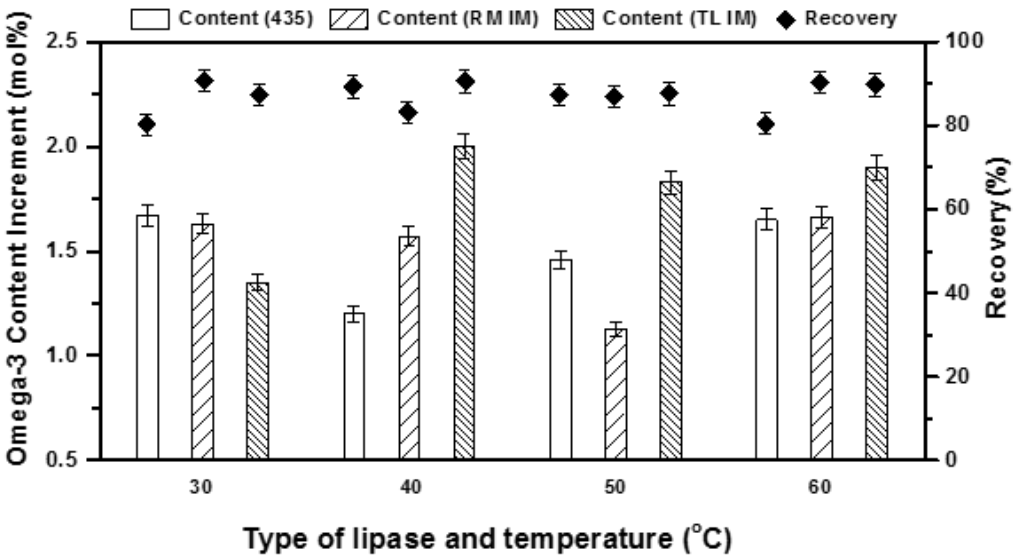

Figure 4.7. Effect of temperature on the efficiency of different lipases in catalyzing the enzymatic interesterification of CLO. Reaction conditions: 5 wt% lipase load, 6 hours, vacuum for Novozym 435, N_2 flow for Lipozyme TL IM and Lipozyme RM IM, 300 rpm.

Effect of reaction temperature on lipase efficiency for the current interesterification reaction was investigated after 6 hours reaction under their preferred system air conditions. As shown in Figure 4.7, both Lipozyme RM IM and Novozym 435 provided moderate increments of omega-3 PUFA content, except for exceptionally low values at 40 and 50 ℃, respectively. The reason might relate to the dual character of temperature, on the one hand, lipase stability and catalytic activity can be ensured in mild temperature for most lipases,[42, 60] on the other hand, warmer conditions can reduce system viscosity and enhance mass transfer between substrates and lipase particles, leading to an accelerated reaction.[45] In terms of Lipozyme TL IM, it was obvious that higher temperatures (40-60 ℃) were preferred, which is in equivalent with the observations reported by Cristina Otero *et al.*[41] Moreover, it outperformed Lipozyme RM IM and Novozym 435 with 2.00 mol% increment in the content of omega-3 PUFA (recovery 91%) at 40 ℃ reaction. Hence, further investigation on the current interesterification reaction was conducted in the presence of Lipozyme TL IM at 40 ℃.

Last but not least, the time course of the current interesterification reaction was recorded in an attempt to identify the optimum reaction time length under the previous determined conditions (5.00 wt% Lipozyme TL IM, 40 ℃, N_2 flow). As illustrated in Figure 4.8, the increments of omega-3 PUFA content ranged widely over time, suggesting the distribution of fatty acids shifted greatly over time. Although the re-distribution of

fatty acids in interesterification reaction in the presence of Lipozyme TL IM was not as random as that of Novozym 435,[59] our results showed the possibility to make it favor the formation of TAG accumulated with higher melting point fatty acids through time control. The maximal increment of omega-3 fatty acid content was 2.42 mol%, which was obtained in the 2.5 hours assay, indicating that more TAG molecules accumulated with higher melting point fatty acids were formed and later removed during the following solvent winterization. Based on these results, 2.5 hours was determined to be the optimum time length for the current interesterification reaction.

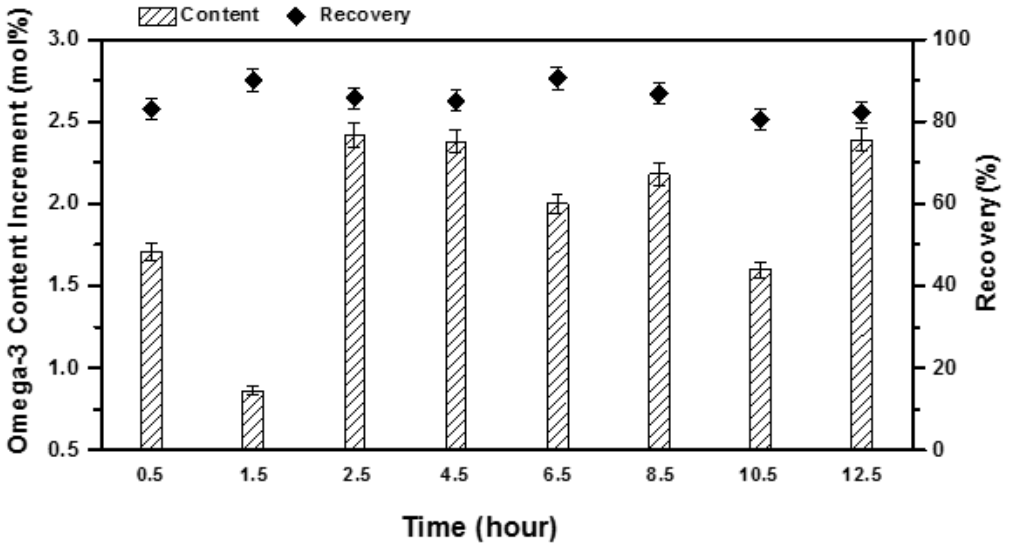

Figure 4.8. Effect of time on the efficiency of Lipozyme TL IM in catalyzing the enzymatic interesterification of CLO. Reaction conditions: 5 wt% lipase load, 40 ℃, N_2 flow, 300 rpm.

4.2.3 Optimized method

After four rounds of interesterification-winterization under the preliminary studied conditions, the total increment in omega-3 PUFA content was only 9.13 mol%, which

was considered to be less than expected (Figure 4.9). The centrifuge separation procedure after winterization was believed to be the major defect, seeing that the oil crystals thawed obviously during the two minutes centrifugation. In order to shorten the separation time and avoid oil crystal exposure to warmer condition, a Büchner vacuum filtration was involved as an improved process, where the Büchner funnel was precooled at the winterization temperature for three hours. Such operation could minimize the temperature difference between winterization and separation, as well as shorten the separation process, both contributed greatly to the prevention of oil crystal unfreezing. Such improved procedure was only applied to the assays gave best results in the preliminary study, namely acetone as the solvent, 0.1 g/mL as the oil/solvent concentration and 24 hours as the winterization time length, while -80 ℃ and -20 ℃ were re-evaluated as the winterization temperature. Considering that all evaluation on the efficiency of the current interesterification reaction was conducted under the same conditions in preliminary study, the efficiency of separation procedure after winterization was believed to be unable to affect the judgement. Hence, the determined optimum conditions for the enzymatic interesterification reaction were all adopted in the following experiments.

Figure 4.9 summarized the changes of omega-3 PUFA content following the improved enrichment methods that involved winterization at -80 ℃ and -20 ℃, respectively. It is obvious that the new separation procedure indeed helped enhance the efficiency of

winterization and raised the increment of omega-3 PUFA content greatly, comparing with that of preliminary studied method. With the winterization temperature of -20 ℃, our proposed enrichment method increased the content of omega-3 PUFA to 28.82 mol% after the first winterization, and on this basis, to 33.34 mol% after two rounds of interesterification-winterization. It is noteworthy that there was moderate decrease in the recoveries of each winterization, suggesting that more TAG crystals were retained during the separation procedure and later removed from the LP. Such observations positively validated that the precooled Büchner vacuum filtration was indeed more efficient in the prevention of oil crystal unfreezing. In the case of winterization at -80 ℃, the content of omega-3 PUFA was boosted up to 35.78 mol% from the original CLO. In spite of the low recovery (20%), it was still considered to be more efficient than the -20 ℃ assay, because the net recovery of three rounds of winterization was 17% while the omega-3 PUFA content was 33.34 mol% in the oil product. Remarkably, the omega-3 PUFA content reached 43.20 mol% after two rounds of interesterification-winterization in the -80 ℃ assay, doubled the origin level (21.30 mol%). Based on these results, winterization at -80 ℃ followed by precooled Büchner vacuum filtration was preferred, and together with the enzymatic interesterification, our proposed method was proved to be efficient in the enrichment of omega-3 PUFA in CLO.

In addition, new experiments revealed that time length did have an impact on the

winterization efficiency, opposite to the observations in the preliminary study. It was found that there was no crystal formed after four hours refrigeration at -80 ℃ during the third winterization, whereas a lot was obtained 20 hours later. And equivalent results was also reported by Luis Vazquez and Casimir C. Akoh.[24] Further prolonged winterization would hardly increase the amount of crystals (data not shown), and therefore the optimal winterization time length was still fixed at 24 hours. Scaled-up experiments was also investigated, where 50 g of CLO was employed as the starting material. No significant differences in the increments of omega-3 PUFA content and oil recovery have been detected. Hence the results from different reaction scales were combined in the study.

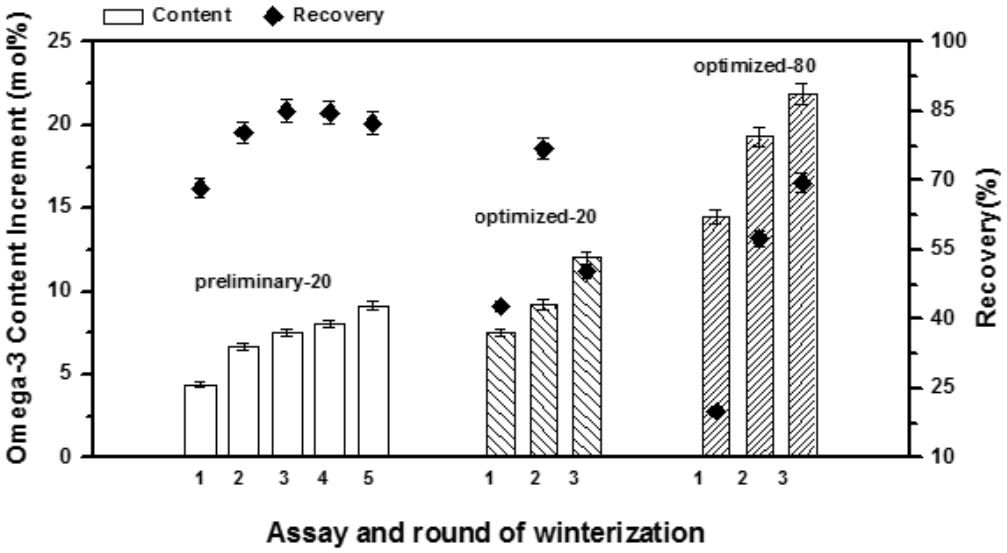

Figure 4.9. Comparison between preliminarily studied method and optimized methods on their efficiency in omega-3 PUFA enrichment in CLO. Conditions: (preliminary-20) -20 ℃, (optimized-20) -20 ℃, (optimized-80) -80 ℃. 24 hours and 0.1 g/mL oil/acetone concentration applied to all winterization. Lipozyme TL IM (5 wt%), 2.5 hours, 40 ℃, N_2 flow and 300rpm were applied to all interesterification.

4.2.4 Fatty acid composition analysis of winterized CLO

Table 4.1. Profiles of original CLO and the winterized oil product obtained after two rounds of interesterification-winterization (-80 ℃) analyzed by GC-MS and the total omega-3 PUFA content analyzed by ^{1}H NMR.

Fatty acids	R_t (min)	Content of original CLO		Content of winterized oil	
		(wt%)	(mol%)	(wt%)	(mol%)
C(14:0)	8.08±0.02	4.03	4.91	0.85	1.06
C(16:1) ω-7	10.62±0.02	12.57	13.81	16.04	18.15
C(16:0)	10.91±0.02	16.58	18.07	0.83	0.93
C(18:4) ω-3	13.49±0.02	3.49	3.54	5.25	5.48
C(18:2) ω-6	13.62±0.02	0.96	0.96	1.67	1.72
C(18:1) ω-9	13.72±0.02	20.65	20.54	23.73	24.30
C(18:1) ω-7	13.82±0.02	5.19	5.16	5.18	5.31
C(18:0)	14.21±0.04	1.83	1.80	nd	nd
C(20:5) ω-3	17.32±0.02	10.53	9.81	21.08	20.23
C(20:1) ω-9+11	18.22±0.02	13.69	12.44	6.89	6.45
C(22:6) ω-3	22.68±0.02	7.63	6.57	17.83	15.81
C(22:1) ω-9+11+13	24.15±0.04	2.85	2.39	0.65	0.56
∑SFA (GC-MS)	-	22.43	24.78	1.68	2.00
∑MUFA (GC-MS)	-	54.96	54.34	52.49	54.76
∑PUFA (GC-MS)	-	22.60	20.88	45.83	43.24
∑ω-3 PUFA (GC-MS)	-	21.65	19.92	44.16	41.52
∑ω-3 PUFA (NMR)	-	-	21.30	-	43.20

nd – not detected.

The fatty acid profile of original CLO and that of the oil product obtained after two rounds of optimized interesterification-winterization process were analyzed by GC-MS. Seeing that the proportions of each fatty acids were on the basis of weight from GC analysis, calculations were conducted to convert the weight proportion (wt%) to molar proportions (mol%). As shown in Table 4.1, the content of omega-3 PUFA was successfully enriched to 41.52 mol%, including 20.23 mol% EPA and 15.81 mol% DHA, which was in good agreement with the results from ^{1}H NMR analysis (43.20 mol%). It should be noted that most of SFA has been removed and only 2.00 mol% was left in the oil product. Whereas, the content of MUFA in the oil product maintained the same as original CLO (54.76 mol% and 54.34 mol%, respectively). This observations suggests that our proposed method is efficient to remove SFA and thereby concentrate PUFA, especially omega-3 PUFA in the case of CLO.

4.3 Conclusion

The omega-3 PUFA content in CLO has been successfully enriched to 43.20 mol% via our proposed method which consists of alternate solvent winterization and enzymatic interesterification. Non-omega-3 fatty acid fractions, specifically SFA, were efficiently removed in the winterization of 0.1 g/mL oil/acetone solution at -80 ℃ for 24 hours. Meanwhile, Lipozyme TL IM outperformed Lipozyme RM IM and Novozym 435, and the interesterification reaction at 40 ℃ for 2.5 hours with N_2 flow provided the best

results in favoring fatty acids re-distribution to form TAG molecules accumulated with higher melting point fatty acids. EPA (20.23 mol%) and DHA (15.81 mol%) were the two major omega-3 PUFA concentrated in the oil product.

The proposed method realized the concentration of omega-3 PUFA with two simple food techniques, and smoothed away the costly separation and purification procedures. Additionally, quantification of omega-3 PUFA content in oil product via ^{1}H NMR analysis simplified the characterization process greatly. Overall, our method not only offers great industrial feasibility but also reduces the production cost drastically.

4.4 Experimental Section

4.4.1 Materials

Arctic CLO was purchased from Nordic Naturals® (CA, USA) and characterized by ^{1}H NMR (Table 4.2). Commercially available Lipozyme TL IM (lipase from *Thermomyces lanuginosa*), Lipozyme RM IM (lipase *Rhizomucor miehei*, immobilized on anion exchange resin) and Novozym 435 (lipase from *Candida Antarctica*, immobilized on acrylic resin) were generous gifts from Novo Nordisk A/S (Bagsvaerd, Denmark). Sodium methoxide, sodium hydroxide, and boron trifluoride-methanol solution (14% in methanol) were purchased from Sigma–Aldrich® (Singapore). All organic solvents were purchased from RCI Labscan Limited (Bangkok, Thailand). $CDCl_3$ containing TMS (v/v 0.03%) was purchased from Cambridge Isotope Laboratories Inc. (MA, USA).

Table 4.2. Assignment of ^{1}H NMR signals (400 MHz, $CDCl_3$) of original CLO.

Proton(s)	Functional group	Chemical shift, δ(ppm)	Integration
C**H**$_3$-C	terminal methyl protons of non-omega-3 fatty acids	0.87(t) J = 6.8 Hz	7.37
C**H**$_3$-C	terminal methyl protons of omega-3 fatty acids	0.96(t) J = 7.6 Hz	2.00
-(C**H**$_2$)$_n$-	backbone CH_2	1.25(m)	49.31
-C**H**$_2$CH$_2$COOR	β-methylene protons of non-DHA fatty acids	1.60(m)	5.87
=CH-C**H**$_2$-	α-methylene protons to one double bond	2.00(m)	8.99
-C**H**$_2$COOR	α-methylene protons of non-DHA fatty acids	2.30(m)	5.53
=CHC**H**$_2$C**H**$_2$COOR	α- and β-methylene protons of DHA	2.38(m)	1.15
=CHC**H**$_2$CH=	methylene protons between two double bonds	2.80 (m)	5.99
-C**H**$_2$CHC**H**$_2$-	protons on the secondary carbon of glycerol backbone	4.15 (dd) J_1 = 12.0 Hz, J_2 = 6.0 Hz	2.07
-C**H**$_2$CHC**H**$_2$-	protons on the secondary carbon of glycerol backbone	4.28 (dd) J_1 = 12.0 Hz, J_2 = 4.4 Hz	2.00
-C**H**=C**H**- and **-CH$_2$C**H**C**H$_2$-**	olefinic protons and the proton on the tertiary carbon of glycerol backbone	5.35(m)	11.50

4.4.2 Preliminary study

In a typical solvent winterization experiment, 1.00 g of CLO was treated with different amounts of organic solvent (acetone, ethanol or hexane) in a 15 mL centrifuge vial and winterized at -80 ℃ or -20 ℃ for different time length. TAG accumulated with higher

melting point fatty acids was crystallized and formed the SP, while the rest mainly contained lower melting point fatty acids was reserved in the LP. Samples were centrifuged in a Hettich EBA 20 centrifuge (Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany) at 6000 rpm for 2 min. The centrifugation time should be short in order to avoid overexposure of the sample to warmer temperature which would surly affect the crystallization. Thereafter, the sample was refrigerated at the winterization temperature for 3 min and later the SP was removed from the LP through decantation. Both fractions were treated with rotary evaporation at 35 ℃ to remove the organic solvent and their content of omega-3 PUFA were determined by ^{1}H NMR analysis. Recovery of the winterized oil was calculated using the following equation:

$$\text{Recovery (\%)} = \frac{\text{weight of dry fraction from LP}}{\text{weight of CLO before winterization}} \times 100$$

In a typical enzymatic interesterification experiment, a certain amount of the winterized CLO were placed into a round bottom flask and maintained in nitrogen flow or under vacuum at tested temperature (30, 40, 50 and 60 ℃) for 5 min. The enzymatic reaction started immediately after the addition of lipase (Lipozyme RM IM, Lipozyme TL IM or Novozym 435, 5 wt%) under 300 rpm agitation, and stopped upon the removal of lipase by filtration. The interesterified oil was ready for the next round of winterization. Time course of the current interesterification reaction was recorded in a series of batches under the optimum reaction conditions determined above.

4.4.3 Optimized method

To further increase the content of omega-3 PUFA in CLO, the separation after solvent winterization was realized by Büchner vacuum filtration where the Büchner funnel was precooled at the winterization temperature for three hours, instead of centrifugation for 2 min at room temperature. Based on our preliminary results, winterization in acetone with 0.1 g/mL oil concentration for 24 hours gave the highest increment in omega-3 fatty acid content and therefore were maintained in the subsequent experiments to re-evaluate -80 ℃ and -20 ℃ as the winterization temperature. For the enzymatic interesterification, conditions optimized in the preliminary study (Lipozyme TL IM, 40 ℃, 2.5 hours) were maintained.

4.4.4 Quantification of omega-3 PUFA content by ^{1}H NMR

Oil aliquots were dissolved in $CDCl_3$ at a ratio of 1:9 (v/v) and analyzed in 5 mm NMR tubes. Chemical shifts were referred indirectly to TMS signal (δ = 0.0 ppm) by calibrating the residual signal from CHD_2Cl_3 to 7.26 ppm. ^{1}H NMR spectra were recorded on Bruker Avance 400 spectrometer operating at 400 MHz with QNP probe (5 mm). Figures were processed with Bruker's TopSpin^{TM} software.

4.4.5 Fatty acids methyl ester analysis by GC-MS

Fatty acid compositions of the original CLO and the winterized oil product were analysed by converting the TAG to the corresponding fatty acid methyl esters (FAME)

with the boron trifluoride-methanol method.[24] About 75 mg oil sample and 2 mL 0.5 M NaOH methanol solution was placed into a sample vial. Sealed with nitrogen, the vial was caped tightly and incubated at 100 ℃ for 5 min to saponify the lipid. After cooled to room temperature, 2 mL of 14% boron trifluoride methanol solution were added and the vial was vortexed for 1 min after sealed with N_2. The following Incubation at 100 ℃ for 5 min allowed the methylation. After the reaction, the produced FAME was extracted by adding 2 mL saturated NaCl solution and 2 mL hexane, followed by 2 min vortex and 3 min centrifugation at 2000 rpm. The upper organic phase was collected and treated with anhydrous sodium sulphate before submitted to GC-MS analysis.

The samples were analyzed on a Thermo TR-5MS column (30 m × 0.25 mm I.D. × 0.25 μm film), in a ThermoFinnigan PolarisQ MS system which was equipped with Thermo Trace GC. Both injector and detector temperature were set at 250 ℃. Column temperature was programmed from 170 ℃ to 215 ℃ at 5 ℃/min (holding for 1 min at 215 ℃), from 215 ℃ to 220 ℃ at 1 ℃/min, from 220 ℃ to 240 ℃ at 2 ℃/min, and from 240 ℃ to 290 ℃ at 20 ℃/min. Injection volume was 0.5 μL and sample concentration was 1 ppm. Data reported were the average of two independent experiments for each assay.

4.5 References

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Chapter 5

Conclusions

In this thesis, the author has developed efficient solutions for the innovation of lipid-based functional food ingredients as an effort to address human health problems. The emphasis is on reducing energy intake while delivering beneficial fatty acids into human diets, as well as on improving the utilization methodology and processing technologies of natural oil resources. All the material required in this work, including chemicals, oils and lipases, are approved for food application by FDA, as a consideration of food safety. In addition, the technologies involved are simple operations in food industry, where the costly separation and purification procedures are avoided in most cases, facilitating the industrialization of these innovations. Various methods has been utilized for the product structural characterization, TLC, NMR, FT-IR, LC-ESI-MS, GC-MS included. The fatty acid composition of oil product is analyzed by GC-MS.

The major accomplishments of the study could be summarized as follows:

1. A typical MSM structured TAG has been designed as potential calorie-reduced fat substitute. It is anticipated to be hydrolyzed by pancreatic lipase within human body, and generates 2-MAG with stronger polarity, which would suppress its combination into micelles and thereby limit its absorption into intestinal cells. Consequently, the recombination of TAG afterwards would be decreased, leading to reduced lipid absorption in human digestive track.
2. A molecular probe, 1,3-dicapryloyl-2-acetylglycerol, has been successfully

synthesized in a two-step strategy with a net yield of 45%. Firstly, 1,3-dicapryloylglycerol was prepared via 1,3-specific esterification of glycerol and caprylic acid catalyzed by Lipozyme RM IM, with a molar ratio of 1:4 and an agitation speed of 250 rpm at 25°C for 8 hours under vacuum. Flash column chromatography was preferred as the purification method. 1,3-Dicapryloyl-2-acetylglycerol was then produced by refluxing 1,3-DAG with acetic anhydride for 6 hours.

3. The jojoba LCMFAEE and LCMFAA have been successfully synthesized through the enzymatic interesterification of jojoba oil with EA with a yield of 95%. Novozym 435 overall outperformed Lipozyme RM IM in the current reaction, for it gave the highest IY, whereas the IY was only 76% in the case of Lipozyme RM IM. The optimum reaction conditions were determined to be 9 hours reaction at 37°C with an EA content of 75 wt% in the presence of 5.00 wt% Novozym 435. The preferred lipase also exhibited excellent operational stability over 17 repeated cycles. The compositions of the entire interesterified product were mainly ethyl *cis*-11-eicosenoate (44.19%), *cis*-11-eicosenyl acetate (24.83%) and *cis*-13-docosenyl acetate (22.13%). The resulted jojoba LCMFAEE and LCMFAA, in both mixture and purified form, have great versatility and potential for a wide range of applications in food industry, such as bio-functional ingredients, flavor and fragrance agents, emulsifiers and edible coatings, etc.

4. The omega-3 PUFA content in CLO has been successfully enriched to 43.20 mol% via our proposed method which consists of alternate solvent winterization and enzymatic interesterification. Non-omega-3 fatty acid fractions, specifically SFA, were efficiently removed in the winterization of 0.1 g/mL oil/acetone solution at -80°C for 24 hours. Meanwhile, Lipozyme TL IM outperformed Lipozyme RM IM and Novozym 435, and the interesterification reaction at 40°C for 2.5 hours with N_2 flow provided the best results in favoring fatty acids re-distribution to form TAG molecules accumulated with higher melting point fatty acids. EPA (20.23 mol%) and DHA (15.81 mol%) were the two major omega-3 PUFA concentrated in the oil product.
5. Quantification methods using ^{1}H NMR analysis are successfully established, with good agreement with traditional quantification methods. In the preparation of jojoba LCMFAEE and LCMFAA, IY is directly obtained from the area ratio between the quartet and the triplet in the 4.00-4.15 ppm region of ^{1}H NMR spectra of the EA-removed interesterification reaction mixture. For the quantification of omega-3 PUFA content in the winterized oil product, it is realized by calculating the area ratio between the triplet signal of methyl protons in omega-3 fatty acids and that of non-omega-3 fatty acids.
6. Various lipase types have been involved in the study and their catalytic activities for esterification and interesterification reactions have been evaluated. Novozym 435

performs excellently in catalyzing interesterification reactions in a wide temperature range, whereas it is not suitable for regioselective reactions due to its poor regioselectivity. Both Lipozyme TL IM and Lipozyme RM IM have good 1,3-regioselectivity in esterification and interesterification reactions. The former lipase provides the maximum increment in the content of omega-3 PUFA in CLO, while the latter is chosen for the synthesis of 1,3-DAG.

Overall, the work succeeds in methodology design, providing both novelty and feasibility for the development of lipid-based functional food ingredients. It shows great potential for basic food techniques to produce multiple functional ingredients if elaborately designed. Meanwhile, there are still more work to be done in the future for the accomplishment of commercializable functional food ingredients. Some of them has been listed below and may be of particular interest in my following work:

1. Fatty acid chain length in the MSM structured TAG will be varied to provide a series of molecular probes to investigate their potential in reducing calorie intake. And synthesis reaction conditions will be optimized accordingly.
2. Biology test (both in vitro and in vivo) and metabolism test shall be conducted with the molecular probe, 1,3-dicapryloyl-2-acetylglycerol, to confirm its biological behavior and the potential as calorie-reduced fat substitute. Specifically, radioactive-labeling in the acetyl group of 1,3-dicapryloyl-2-acetylglycerol with ^{14}C or ^{3}H is a promising

method. If the radioactivity associated with 2-monoacetin is quickly excreted by the subject when the subject is fed with radioactive labeled molecular probe in advance, it would indicate that the MSM type TAG could be easily absorbed by intestinal cells. If this presumptive process could occur in human body, such MSM structured lipid could be a brand new type of calorie-reduced fat substitute candidates, which could contribute to obesity control and other associated health problems.

List of Publications

1. **Qiong Lei**, Sai Ba, Hao Zhang, Yanyan Wei, Yiqin Jasmine Lee, Tianhu Li. Enrichment of Omega-3 Fatty Acids in Cod Liver Oil via Alternate Solvent Winterization and Enzymatic Interesterification. Food Chemistry. *Under review*.

2. **Qiong Lei** and Tianhu Li. Functional Monoesters of Jojoba Oil Can Be Produced by Enzymatic Interesterification: Reaction Analysis and Structural Characterization. European Journal of Lipid Science and Technology 2015. **117**(5): p. 630-636.

3. **Qiong Lei**, Wilson Liangwei Lee and Tianhu Li. Design and Synthesis of 1,3-Dicapryloyl-2-acetylglycerol as Molecular Probe for TAG Metabolism Study. European Journal of Lipid Science and Technology 2013, 115(2), 232–238.

4. Sai Ba, **Qiong Lei**, Chee Wee Ng, Hao Zhang, Dejian Huang and Tianhu Li. Synthesis of New Types of Brominated Diesters as Potential Food Emulsifiers. Journal of Chemical Sciences (JChem) 2014, 1(1).

5. J. Yin, M. Kumar, **Q. Lei**, L, Ma, R. S. S. Kumar, G.G. Gurzadyan, and C. Soci, Small-Size Effects on Electron Transfer in P3HT/InP Quantum Dots. ACS Applied Materials & Interfaces. *Under review.*