UNIVERSITY OF GAZİANTEP
GRADUATE SCHOOL OF
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PRODUCTION OF LOW CALORIC AND SPREADABLE LIPID BY ENZYMATIC INTERESTERIFICATION OF TEREBINTH FRUIT (*Pistacia terebinthus L.*) OIL

Ph.D THESIS
IN
FOOD ENGINEERING

BY
DERYA KOÇAK YANIK
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Production of Low Caloric and Spreadable Lipid by Enzymatic Interesterification of Terebinth Fruit (*Pistacia terebinthus* L.) Oil

Ph.D Thesis
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Supervisor
Prof. Dr. Fahrettin GÖĞÜŞ

by
Derya KOÇAK YANIK
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Approval of the Graduate School of Natural and Applied Sciences

Assoc. Prof. Dr. Metin BEDİR
Director

I certify that this thesis satisfies all the requirements as a thesis for the degree of Doctor of Philosophy.

Prof. Dr. Ali Rıza TEKİN
Head of Department

This is to certify that we have read this thesis and that in our consensus opinion it is fully adequate, in scope and quality, as a thesis for the degree of Doctor of Philosophy.

Prof. Dr. Fahrettin GÖĞÜŞ
Supervisor

Examining Committee Members

Prof. Dr. Aziz TEKİN (Chairman) .....

Prof. Dr. Ali Rıza TEKİN .....

Prof. Dr. Sami EREN .....

Prof. Dr. Sibel FADILOĞLU .....

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Derya KOÇAK YANIK

ABSTRACT

PRODUCTION OF LOW CALORIC AND SPREADABLE LIPID BY ENZYMATIC INTERESTERIFICATION OF TEREBINTH FRUIT (*Pistacia terebinthus L.*) OIL

KOÇAK YANIK, DERYA
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The overall objective of the study is to produce a low-calorie and spreadable structured lipid (SL) from terebinth fruit oil. For this purpose, the *sn*-1,3 specific immobilized lipase (from *Rhizomucor miehei)* catalyzed acidolysis reaction of terebinth fruit oil with caprylic and palmitic acids was performed in a batch stirred tank reactor and recirculating packed bed reactor (PBR) systems.

The effect of reaction conditions and relationship among them were analyzed and optimized by the response surface methodology with a four-factor five-level central composite rotatable experimental design. The major factors chosen were enzyme load, reaction time, temperature, substrate mole ratio and flow rate. The substrate mole ratio, enzyme load, reaction time and temperature were found as key factors for modulating the distribution of reaction products. The PBR system was found more effective to produce target SL. In this system, optimum reaction conditions at 45 ^{o}C for reaction time, enzyme load, flow rate and substrate mole ratio were 5.9 h, 10 %, 4 mL/min and 1:3.10:2.07, respectively. The caloric value of the produced SL was found as 38.96 kJ/g.

The produced SLs were compared to fat phases of some commercial margarine in terms of solid fat content, melting profile and microstructure. The results of the study showed that the produced SL had similar melting characteristics and microstructure with those of commercial margarine fat extracts. So, it could be used in spreadable fats.

Key Words: *Pistacia terebinthus* L., Caprylic acid, Palmitic acid, Structured lipid, Response surface methodology

ÖZ

ENZİMATİK İNTERESTERİFİKASYON İLE MENENGİÇ MEYVE YAĞINDAN DÜŞÜK KALORİLİ- SÜRÜLEBİLİR YAĞ ÜRETİLMESİ

KOÇAK YANIK, DERYA
Doktora Tezi, Gıda Mühendisliği Bölümü
Tez Yöneticisi: Prof. Dr. Fahrettin GÖĞÜŞ
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Bu çalışmanın genel amacı menengiç meyve yağından düşük kalorili ve sürülebilir yapılandırılmış bir yağ üretmektir. Bu amaçla, menengiç meyve yağının *sn*-1,3 spesifik immobilize lipaz katalizörlüğünde kaprilik ve palmitik asit ile asidoliz reaksiyonları kesikli karıştırmalı tank reaktör ve dolgulu yatak reaktör sistemlerinde gerçekleştirildi.

Reaksiyon koşullarının etkisi ve bunlar arasındaki etkileşim dört faktörlü beş seviyeli merkezi bileşik dairesel deney tasarımı kullanılarak yüzey tepki metodu ile araştırıldı ve optimize edildi. Seçilen ana faktörler; enzim miktarı, reaksiyon süresi, sıcaklık, substrat mol oranı ve akış hızıdır. Substrat mol oranı, enzim miktarı, reaksiyon süresi ve sıcaklık reaksiyon ürünlerinin dağılımını düzenleyen anahtar faktörler olarak tespit edildi. Dolgulu yatak reaktör sistemi, istenilen yapılandırılmış yağın üretilmesinde daha etkili bulundu. Bu sistemde, optimum reaksiyon koşulları 45 ^{o}C de reaksiyon süresi, enzim miktarı, akış hızı ve substrat mol oranı için sırasıyla 5.9 sa, % 10, 4 mL/dk ve 1:3.10:2.07'dir. Üretilen yapılandırılmış yağın kalorik değeri 38.96 kJ/g olarak bulunmuştur.

Üretilen yapılandırılmış yağlar; erime profili, katı yağ oranı ve mikroyapı açısından bazı ticari margarinlerin yağ fazları ile karşılaştırıldı. Çalışmanın sonuçları; üretilen yağın, ticari yağlarla benzer erime özelliklerine ve mikroyapıya sahip olduğunu göstermiştir. Bu özellikleriyle sürülebilir yağlarda kullanılabilir niteliktedir.

Anahtar Kelimeler: *Pistacia terebinthus* L., Kaprilik asit, palmitik asit, yapılandırılmış yağ, Yüzey tepki metodu

To my endless love Riza

&

My little angel Deniz, welcome to our life, we are looking forward to holding you.....

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LIST OF SYMBOLS/ABREVIATIONS

ANOVA	Analysis of variance
CA	Caprylic acid
CCRD	Central composite rotatable design
DSC	Differential Scanning Calorimeter
FA	Fatty acid
FAME	Fatty acid methyl ester
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
LCFA	Long-chain fatty acids
MCFA	Medium-chain fatty acids
PA	Palmitic acid
PBR	Packed bed reactor
PLM	Polarized Light Microscope
RSM	Response Surface Methodology
SCFA	Short-chain fatty acids
SFC	Solid fat content
SL	Structured lipid
STR	Stirred tank reactor
TAG	Triacylglycerol
TO	Terebinth oil

CHAPTER 1

INTRODUCTION

Being the most important components, fats and oils have been used as food and for variety of other applications since prehistoric times because of their unique properties (O'Brien, 2000). They are mainly used for their nutritional and physical properties. Natural fats and oils are not always ideal for their ultimate use and sometimes appropriate modification has to be carried out. Modified lipids that have been restructured in terms of their native composition and/or distribution of fatty acids (FAs) in the glycerol backbone are known as structured lipids (SLs) (Foresti and Ferreira, 2010). Today, structurally defined lipids have been developed for special functionality or nutritional use. These modifications can be done chemically or enzymatically. However, enzymatic interesterification is more preferable than chemical synthesis due to its milder reaction condition requirements. Moreover enzymatic modification is providing incorporation of specific fatty acid at specific positions of triacylglycerols to produce novel products (Akoh, 1995).

A variety of fatty acids are used in the synthesis of SL, taking advantage of the functions and properties of each to obtain maximum benefits from a given structured lipid (Akoh and Kim, 2008). Many unique functional, nutritional and metabolic characteristics of these fatty acids make them useful in pharmaceutical and nutritional applications. For instance long chain saturated fatty acids such as stearic and palmitic acid can be used to increase the melting point of oils. Medium chain fatty acids (MCFAs) such as caprylic, capric and caproic are often used to synthesize structured lipids (SLs) which are low caloric and better absorbed.

In the last years, most attention has focused on triacylglycerols (TAGs) with medium-chain fatty acids (MCFA) located in positions 1 and 3 of the glycerol backbone and a functional long-chain polyunsaturated fatty acids in position 2 (Hita et al., 2009).

It is well known that pancreatic lipase is *sn*-1,3 specific and hydrolyzes the ester bonds at positions 1 and 3 of triacylglycerols (TAG). Moreover, this lipase shows higher activity toward medium-chain than toward long-chain fatty acids (Camacho et al., 2007). The liberated medium-chain free fatty acids are directly absorbed because they can be solubilized in the aqueous phase of the intestinal contents absorbed bound to albumin and transported to the liver by the portal vein (Decker, 1996). However LCFA are incorporated into chylomicrons and transported through lymph. Triglycerides, containing MCFA in the sn-1,3 positions and essential fatty acids or long chain polyunsaturated fatty acids in the sn-2 position of the same TAG molecule can be used in malabsorption and cystic fibrosis syndromes providing energy as well as essential fatty acids in a more absorpable manner (Hamam and Shahidi, 2008).

Lipase-catalyzed interesterification reactions are widely used for lipid modification. They can be performed in a stirred tank reactor (STR) or in a packed bed reactor (PBR) (Çiftçi et al., 2009). A major advantage of a PBR in lipase-catalyzed reaction is the relatively short reaction time necessary to approach the equilibrium conditions for the effluent from the reactor. Other major advantage of using a PBR is that the enzyme loss can be reduced by the absence of collisions between enzyme particles and an impeller, and the reduction of liquid shearing (Zhao et al., 2012).

Pistacia terebinthus L. (Anacardiaceae) is one of the 20 *Pistacia* species widely distributed in the Mediterranean region and Asia possessing many biological activities (Topçu et al., 2006). Especially the fruit of this tree is more interesting from the point of view of the oil content. Pistacia terebinthus fruit contains around 40 % oil and this oil is rich in oleic acid (52.3%) followed by palmitic (21.3%) and linoleic (19.7%) acids (Özcan, 2004). However, this valuable oil is used in soap production in some parts of Turkey (Baytop, 1984; Tanker & Tanker, 1998). Moreover the major constituents of sn-2 fatty acids were oleic (67%) and linoleic (23.6) acids (Koçak et al., 2011). Therefore it can be considered as a desirable substrate for synthesis of structured lipids because a diet, rich in oleic acid, has been shown to reduce plasma low-density lipoprotein cholesterol, while leaving high-density lipoprotein cholesterol unchanged (Mensink and Katan, 1987).

In this study, response surface methodology (RSM) was used to evaluate the effect of several variables (reaction time, temperature, enzyme load, substrate mole ratio, substrate flow rate) on the enzymatic process and to optimize the reaction parameters. RSM is an effective statistical technique for the optimization of complicated systems, which enables the evaluation of effects of multiple parameters, alone or in combination, on the response variables (Shieh et al., 1995; Xu et al., 1999). The advantages of using RSM are reported to be the reduction in the number of experimental runs needed to evaluate multiple variables. Therefore, it is less laborious and time-consuming compared to one-variable at a-time. RSM has been widely applied for optimizing conditions and processes in various food studies (Junqueira et al., 2007; Liyana-Pathirana and Shahidi, 2005)

The objective of this study is to characterize the fatty acid and triacylglycerol composition of terebinth fruit oil and then develope biotechnological processes (stirred tank reactor system and recirculating packed bed reactor system) to produce a low-caloric and spreadable structured lipid from terebinth fruit oil. In detail, it has been aimed to synthesize an SL containing caprylic acid (CA) and palmitic acid (PA) at the *sn*-1,3 positions while retaining oleic and linoleic acids of terebinth fruit oil at the *sn*-2 position by using the *sn*-1,3 specific immobilized lipase from *Mucor miehei*. Following the production of SL at defined optimum conditions, it was aimed to evaluate and compare some properties such as solid fat content, microstructure, caloric value, and oxidative stability with orginal terebinth fruit oil and margarines.

CHAPTER 2

LITERATURE REVIEW

2.1. Terebinth (*Pistacia terebinthus L.*) Fruits

Pistacia terebinthus L. (Anacardiaceae) is among the 20 species of Pistacia. (Gülaçtı et al., 2007) *Pistacia terebinthus* L. (terebinth or turpentine tree) is a small tree showing shiny leaves with a strong resinous smell. (Özcan, 2004) Its fruits are small globular nutlets which are dark greenish when riped (Figure 2.1.) (Davis, 1967). It is native to Asia and the Mediterranean region of the world (Figure 2.2.) and widely distributed in Turkey (Figure 2.3). *P terebinthus* is used in the propagation of *P. vera* (pistachio) because of its vigour and resistance to nematodes and soil borne fungi (Özcan, 2004). Turkey is an important gene center for *P. terebinthus* and 66 millions of *P. terebinthus* tree present in here (Kuru and Özsabuncuoğlu, 1990; Özuslu et al., 2009).

Figure 2.1. Picture of terebinth (*Pistacia terebithus L.*) fruits

The fruit of *Pistacia terebinthus*, L. are the oil rich seeds collected between August-October from the trees. The seeds are then available year round being sold in spice and condiments shops and town bazaars. These oily seeds have a typical taste and they are very aromatic. Terebinth fruits are not grown by agronomic culturing and not collected from nature to processes on an industrial scale.

However, this oil and protein rich product is home processed and consumed both raw and roasted and can be served in various forms.

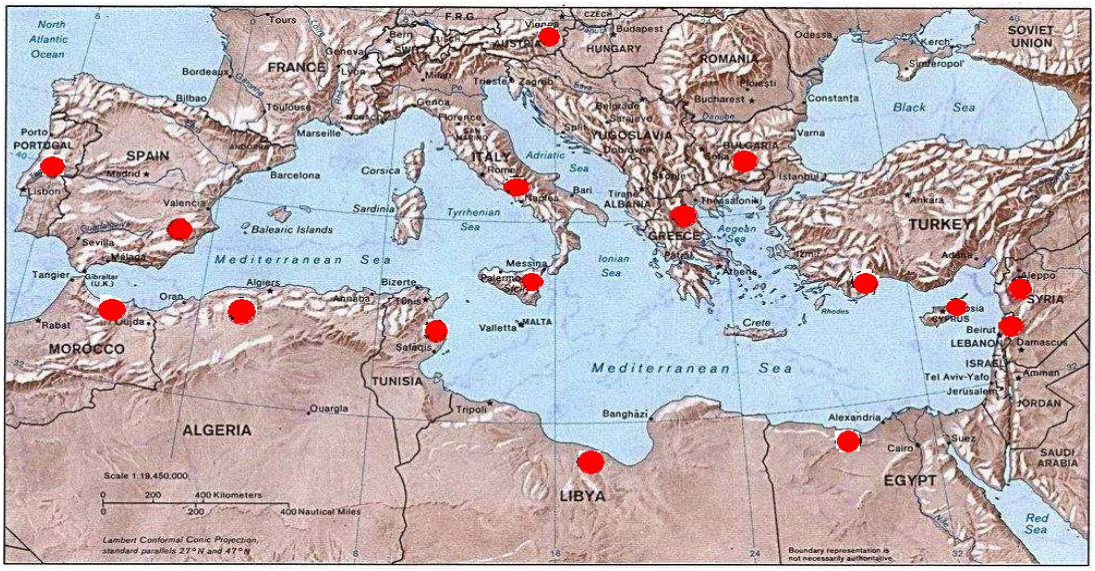

Figure 2.2. Distribution of *P. terebinthus* in the world

Archaeological evidence in Turkey indicates that the nuts were being used for food as early as 7000 BC. Its fresh shoots and fruits are used for human nutrition. The fruits have been regarded as an appetizer in Southern Turkey for several thousand years. (Özcan, 2004). The fruits are used in the baking of village bread and the oil from their seeds is used as cooking oil as well as in soap production in some parts of Turkey (Baytop, 1984; Tanker and Tanker, 1998). The fruits of *P. terebinthus* are also roasted and ground, and used subsequently as coffee in Turkey.

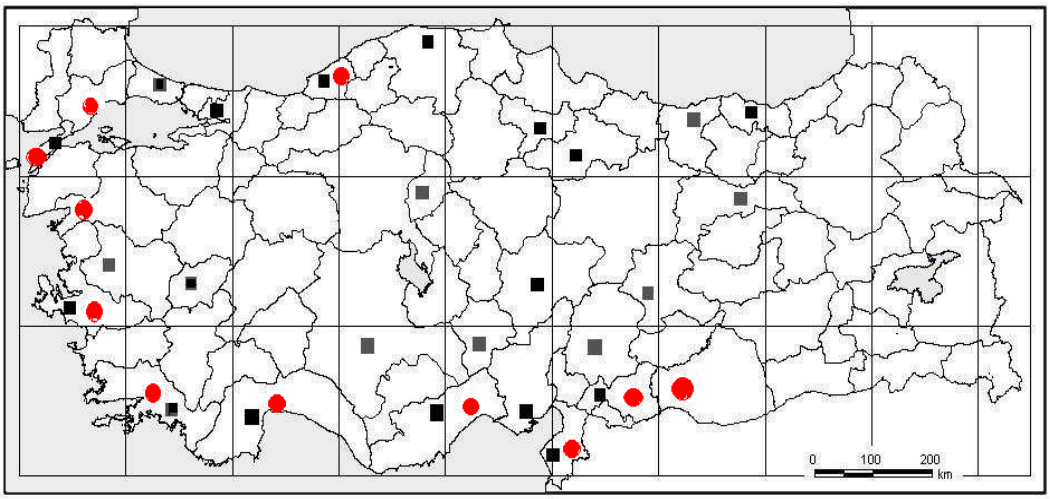

Figure 2.3. Circles represent distribution of *P. terebinthus* in Turkey

Especially the fruits of this tree are more interesting from the point of view of the oil content. Some earlier reports recorded that the crude oil content of *Pistacia terebinthus* was around 40-60 % (Özcan, 2004; Agar et al., 1995). Also, the fruits of terebinthus are used in folk medicine for gastralgia (internally), rheumatism and cough (externally) and as stimulant, diuretic and antitussive (Baytop, 1984; Matthaus and Özcan, 2006; Özcan, 2004; Walheim, 1981, Özcan et al., 2009). Özcan (2004) evaluated various physical and nutritional properties of terebinth fruits and fruit oil. Table 2.1 summarizes the physical and chemical properties of terebinth fruit and fruit oil.

Table 2.1. Physical and chemical properties of terebinth fruit and fruit oil (Özcan, 2004)

Property	**Value**
Fruit[a]	
Moisture (%)	6.17 ± 0.21
Crude protein (Nx6.25) (%)	9.67 ± 0.47
Crude oil (%)	38.74 ± 2.68
Crude fiber (%)	10.9 ± 1.70
Ash (%)	3.1 ± 0.71
HCl-insoluble ash (%)	0.0047 ± 0.0018
Weight of 1000 fruits (g)	59.73 ± 0.97
Essential oil yield (%)	0.084 ± 0.01
Crude energy (cal g^{-1})	6189 ± 13.44
Dimethyl sulphite (µg kg^{-1})	4.1 ± 0.14
Width/length ratio	0.93 ± 0.07
Oil	
Relative density (d_{20}^{20})	0.9742 ± 0.0041
Refractive Index (n_D^{20})	1.477 ± 0.007
Acidity (oleic %)	0.86 ± 0.16
Peroxide value (meq kg−1)	0.47 ± 0.09
Saponification number	156.7 ± 14.64
Unsaponifiable matter (g kg−1)	15.7 ± 3.3
Carotenoid content (mg kg−1)	322 ± 35.4
Iodine value	89.06 ± 0.65

Values are mean ± standart deviation (n=3), [a] values for fruits are dry matter basis

2.2. Structured Lipids

The fatty acid composition, fatty acid distribution, and ratio of saturated to unsaturated, melting point, crystallization behavior, storage stability, nutritional value, caloric value and health-promoting effects of naturally present fats and oils are very different from each other. Sometimes in their original form they are not ideal for specific purposes; in that case it needs to modify their structure to meet desired requirements. Therefore, it is useful in this section to consider the chemistry of fats and oils, and then to review briefly the structured lipids, their synthesis, their application areas and safety and regulatory status.

2.2.1 Chemistry and importance of fats and oils

Domestic consumption of major vegetable oils has grown steadily. Statistics indicate that the world consumption in March 2013 increased to a level of 155.95 million tons per year (USDA, 2013). Fats and oils have a major role in human nutrition. They are essential dietary requirement, providing energy, essential nutrients (essential fatty acids and fat-soluble vitamins), and adding palatability and flavor to cooked food. Fats and oils give the highest calorie among the important nutrients (carbohydrates, proteins, and fats). In human body fats are used for basic energy requirements to produce heat and for physical activity. They are also required for growth. Some fatty acids are classified as essential; they are not synthesized in human body and must be taken externally by food intake. The essential fatty acids (EFA) are important components of membranes in human tissues. So consumption of fats and oils are also important for this reason. Fats and oils are also serving as carrier for some important vitamins, such as vitamin A, D, E and K. All these vitamins have important functions on human mechanism (Gunstone, 2001).

There is a growing awareness about the close relationship between the health and nutritional habit. It is a well known fact that diet and lipids are important in the maintenance of good health and in development and treatment of some diseases. For example, the excessive and low quality fat consumption has been associated with several diseases such as obesity, heart disease, cancer, blood pressure, diabetes, skin diseases, disease of immune system, osteoporosis, etc (Gunstone, 2001).

In addition, fats and oils are the raw materials for liquid oils, shortenings, margarines, and other specialty or tailored products that are functional ingredients in food products prepared by food processors, restaurants, and in the home. These ingredients were used to add flavor, lubricity, texture, and satiety to foods (O'Brien, 2000).

All edible fats and oils are water insoluble substances that consist predominantly of triglycerides, with some nonglyceridic materials present in small or trace quantities. The fat and oil terms are used interchangeably according to physical state of the material at ambient temperature. Generally, when they are present in semi-solid or solid form at ambient temperature are called as fat, in the other case, in liquid form; they are called as oil (O'Brien, 2000).

Chemically, all fats and oils are the same. They are esters of glycerol and fatty acids and they are commonly called as triacylglycerol. Triacylglycerol molecule contains three fatty acids attached to glycerol (Figure 2.4). All triacylglycerols have the same glycerol unit, so the chemical and physical properties of fats and oils are largely determined by the fatty acids that they contain and their position within the glycerol backbone.

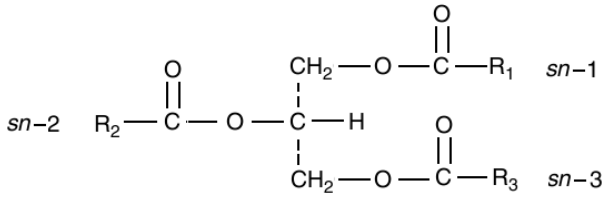

Figure 2.4. A triacylglycerol structure and stereospecific numbering (*sn*) of triacylglycerols (R_1,R_2 and R_3: fatty acyl groups)

Fatty acids can be present in *sn*-1, *sn*-2, and *sn*-3 position of a glycerol. In the numbering that describes the hydroxyl groups on the glycerol molecule in Fisher projection, *sn*-1, *sn*-2, and *sn*-3 designations are used for the top, middle, and bottom OH groups (Figure 2.4). The *sn* term indicates stereospecific numbering (Christie, 1982; O'Keefe, 2008).

2.2.2 What are structured lipids?

Most of the naturally present fats and oils are not necessarily ideal for some physical, chemical and nutritional purposes and have to be modified to meet such requirements. The term modified means any alteration in the structure of the naturally occurring lipids. Structured lipids (SLs) are lipids that have been chemically or enzymatically modified from their natural form. In this definition, lipids include triacylglycerols (TAGs), diacylglycerols, monoacylglycerols, and phospholipids. But in this text and in many cases, SLs are specifically defined as TAGs that have been modified. This modification can be achieved by incorporation of new fatty acids, restructured to change the fatty acids positions, or the fatty acid profile, from the natural state, or synthesized to yield novel TAGs.

Generally, SLs refer to TAGs containing mixtures of both short- (SCFAs) or medium-chain fatty acids (MCFAs) and long-chain fatty acids (LCFAs) in the same

glycerol molecule. Figure 2.5 shows the general structure of SLs. (Akoh and Kim, 2008).

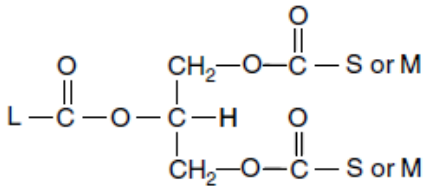

Figure 2.5. General structure of structured lipids: S, L, and M: short-, medium-, and long chain fatty acid, respectively; the positions of S, L, and M are interchangeable

Any fat modified by either chemical or enzymatic methods is included in this group. Cocoa butter equivalents, human milk fat substitutes, some low-calorie fats, oils enriched in essential fatty acids, margarines or other plastic fats are some of them.

2.2.3 Importance of structured lipids

The component fatty acids and their position in the TAG molecule determine the functional and physical properties, the metabolic fate, and the health benefits of the SL (Akoh and Kim, 2008). SLs have been developed to optimize the benefit of fat (Schimdl, 1996). A variety of fatty acids are used in the synthesis of SLs, taking advantage of the functions and properties of each to obtain maximum benefits from produced SLs.

Fats and oils can be manipulated to improve their physical, chemical and nutritional properties. The thermal properties, crystallization and melting profile, are most important physical properties for fats and oils in the food industry. These properties are important in spreads, cooking and baking fats, frying oils and creams. For example, salad oils should be liquid during storage in a refrigerator so it does not contain lipids that will crystallize in this condition. Most frying oils and oils used as food coating should also be free of solid components. On the other hand the successful production of spreads depends on having appropriate levels of solid fat at refrigerator temperature, at ambient temperature and at mouth temperature (Gunstone, 2006). The nutritional properties of lipids can also be manipulated. Nutritional modifications are important for maintenance of good health and treatment of diseases. As an example, the total level of fat in a food with its calorie value is important for consumers and generally they are associated with some serious

diseases. The American Heart Association's Nutrition Committee strongly advises to limit the total fat intake to less than 25–35 percent of total daily calories (AHA, 1996). In that case, foods with lower fat content or fat substitutes with reduced calorie value are preferred. Additionally, an appropriate balance between saturated, monounsaturated and polyunsaturated acids is desirable for a good health. Saturated acids represent a group of acids that raise serum cholesterol levels and can be described as cholesterol-raising.

Designing SL with selected fatty acids at specific locations has also attracted much attention for medicinal applications. For example, the position of FA in the TAG molecules will have a significant impact on their metabolism in the body. In general, FAs at the sn-1 and sn-3 positions of TAGs are hydrolyzed by pancreatic lipase but they are absorbed while those at sn-2 position, remain unchanged and are used in the synthesis of new TAG. Therefore synthesis of a SL containing polyunsaturated fatty acids (PUFA) at the sn-2 position with medium-chain fatty acids (MCFA) at the sn-1,3 positions may be desirable for patients with maldigestion and cystic fibrosis (Hamam and Shahidi, 2008).

The chemical properties of food lipids are also very important. For this reason fats and oils used in food systems can be restructured to obtain more desirable chemical properties. For example, oxidative stability is a parameter affecting the shelf life of foods. So food lipids with increasing oxidatively stability would be desirable to produce foods with a longer shelf life.

Alteration in some properties of fats and oils are also possible by the preparation of physical mixtures of TAGs, but not enough efficient benefits can be obtained from such a mixture. Figure 2.6 shows an example for structure of a physical mixture of medium-chain and long-chain triacylglycerol, and structured lipid molecular species. Although both physical mixtures of TAGs and SL have been used in the diet of patients, an SL emulsion is more attractive due to the modified absorption rates of the SL molecule (Akoh and Kim, 2008).

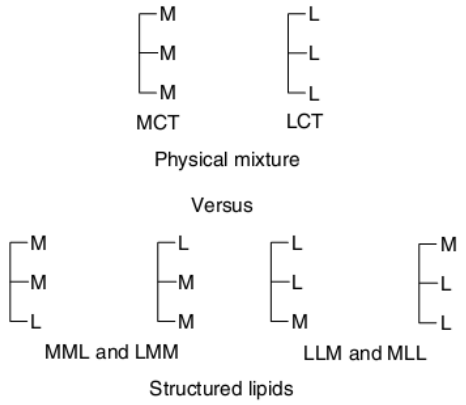

Figure 2.6. Structure of a physical mixture of medium-chain triacylglycerol and long-chain triacylglycerol, and structured lipid molecular species: M, medium-chain fatty acid; L, long-chain fatty acid.

Consequently SLs combine the unique characteristics of component fatty acids such as melting behavior, digestion, absorption, and metabolism to enhance their use in foods, nutrition, and therapeutics.

2.2.4 Synthesis of Structured Lipids

The production of SLs can be accomplished by chemical or enzymatic interesterification and method of synthesis depending on what products are needed. Structured lipids can be manufactured to achieve regiospecific location of fatty acids or to get random location in a glycerol backbone. Regiospecific location of fatty acids can be done by lipase catalyzed reactions by regiospecific lipases, and randomized distribution of fatty acids can be achieved by nonspecific lipases or by a chemical interesterification. As a consequence interesterification allows the rearranging of existing acyl groups or incorporation of new fatty acids to create novel properties (Xu et al., 2006)

2.2.4.1 Sources of acyl donors for structured lipid synthesis

The component fatty acids, their chain length, number and position of the double bonds and their position in the TAG molecule are responsible for the functional and physical properties, the metabolic fate, and the health benefits of the SL. Therefore, function and metabolism of the component fatty acids will be outlined in this section. These fatty acids include short chain fatty acids (SCFAs), medium

chain fatty acids (MCFAs), polyunsaturated fatty acids (PUFAs), saturated LCFAs, and monounsaturated fatty acids (MUFAs).

Short chain fatty acids, also known as volatile fatty acids, and their chain length lower than 8. They are more rapidly absorbed in the stomach because of their higher water solubility, smaller molecular size, and shorter chain length. Due to the positional and chain length specificity of the pancreatic lipase, SCFAs attached to the *sn*-1, 3 positions of TAGs are likely to be completely hydrolyzed in the lumen of the stomach and small intestine. SCFAs are useful ingredients in the synthesis of low-calorie SLs because SCFAs provide fewer calories per unit weight than MCFA or LCFA. In earlier studies, the syntheses of SLs containing SCFAs have successfully achieved. An example, Lee et al. (2008) produced SL as a low-calorie lipid by interesterification of palm oil with tributyrin. Fomuso and Akoh (1997) prepared a reduced-calorie SL, with the potential of improving nutrition, from triolein, butyric acid, and caproic acid by using lipase as the biocatalyst.

Medium chain fatty acids range from C8:0 to C12:0. Advantages of MCFA consumption include the following: (1) MCFAs are more readily oxidized than LCFAs; (2) MCFAs do not require chylomicron formation; and (3) MCFAs are transported back to the liver directly by the portal system. In the SL, MCFAs provide not only a source of dense calories but also potentially fulfill a therapeutic purpose. Most reports show successful lipase-catalyzed synthesis of MLM-type SLs. MLM-type SLs is used for clinical purposes as rapid energy and essential FAs sources for patients (Brenda, 1999; Yugo and Tsuneo, 2000). In previous studies some MCFAs have also be incorporated into different oil samples. Nunes et al. (2011) produced MLM-type structured lipids by acidolysis of virgin olive oil with caprylic or capric acid. In another study, Shuang et al. (2009) incorporated caprylic acid into soybean oil. Foresti and Ferreira (2010) produced MLM-type SLs by the acidolysis reaction of tripalmitin with capric acid. The fatty acid composition of lard was modified to contain medium-chain fatty acid (MCFA), capric acid (C10:0), by using lipases as the biocatalysts by Zhao et al. (2007).

Long-chain saturated fatty acids are another important fatty acid sources for synthesis of SLs. Generally, saturated fatty acids are believed to increase plasma cholesterol levels, but stearic acid (18:0) has been reported not to raise plasma

cholesterol levels (Bonanome and Grundy, 1988). They are generally used to synthesize plastic fats. As an example, Carrín and Crapiste (2008) modified the sunflower oil with palmitic-stearic acid mixture to obtain an alternative of hydrogenation product. Lipase-catalyzed interesterification of fully hydrogenated soybean oil, rapeseed oil and palm stearin was performed to produce solid fat by Lee et al. (2008).

Omega-3 and Omega-6 fatty acids are known as EFAs because humans like all mammals, cannot synthesize them and therefore must get them from their diets. The ω-3 fatty acids are represented by linolenic acid, which is commonly found in soybean and linseed oils and in the chloroplast of green leafy plants. A common ω-6 fatty acid is linoleic acid. Linoleic acid is mainly found in most vegetable oils and in the seeds of most plants except coconut, cocoa, and palm nuts. Linoleic acids have a reducing effect on plasma cholesterol and an inhibitory effect on arterial thrombus formation (Gottenbos, 1988). Yeşilçubuk and Karaali (2008) studied the production of Gamma-linolenic acid included SL by enzymatic acidolysis reaction. In another study, Sharma et al. (2009) synthesized structured lipid with balanced omega-3: Omega-6 ratio by lipase-catalyzed acidolysis reaction.

Other polyunsaturated ω-3 fatty acids (*n*-3 PUFAs) of interest in SL synthesis are eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), which are commonly found in fish oils, particularly fatty fish. Children without enough ω-3 PUFAs in their diet may suffer from neurological and visual disturbances, dermatitis, and growth retardation (Holman et al., 1982). Therefore ω-3 PUFAs such as DHA must be included in their diet and in SL design. Senanayake and Shahidi (2002), studied the lipase-catalyzed incorporation of docosahexaenoic acid (DHA) into borage oil. In another study, the fatty acid composition of fish oil (tuna oil) was chemically modified to enrich ω-3 PUFAs, primarily EPA and DHA, by using sodium methoxide as catalyst (Klinkesorn et al., 2004). Paez et al. (2002) studied the synthesis of SLs by acidolysis between a commercial heterogeneous triglyceride (cod liver oil) as source of ω-3 PUFAs and caprylic acid.

Oleic acid, ω-9 is one of the most important fatty acids found in vegetable oils such as canola, olive and peanut. It plays a moderate role in reducing plasma cholesterol

in the body (Gottenbos, 1988). Oleic acid is useful in SLs for fulfilling the long chain acid requirements of SLs.

2.2.4.2 Chemical interesterification

Chemical interesterification has long been used in modification of fats and oils in industry. The commonly used chemical synthesis of SLs is transesterification; it involves hydrolysis of a mixture of medium chain triacylglycerols and long chain triacylglycerol and then re-esterification of released FAs. Chemical interesterification can be performed under relatively mild conditions with chemical catalysts. Chemical randomization can be done at 60-90 ^{o}C, even down to 30 ^{o}C, depending on the oils used. However, the reaction time is increased at low temperatures (Hoy and Xu, 2001). Alkali metals or alkali metal alkylates are used as catalyst and this process requires high temperature and anhydrous conditions. Chemical interesterification results in desired randomized TAG molecular species. The choice of catalyst and the load, reaction temperature and time and the substrate molar ratios are important parameters and those can be set up for a product design.

The process and procedure of chemical interesterification are well established in laboratory and industry. Normally the reaction is conducted in batch stirrer tank reactor. The process starts with oil drying after which sodium methoxide (catalyst) is added either as dry powder or in alcohol solution while stirring. After the reaction, a water solution of phosphoric acid is added to neutralize the sodium methoxide and a water solution of citric acid is used to wash the product until the separated solution is not basic. The product is further dried and subjected to bleaching and physical refining if necessary. In addition to randomized SL, this procedure generally results in a number of unwanted products, which can be difficult to remove (Gunstone, 1994).

Chemical interesterification is an important technology for producing trans-free plastic fats to replace hydrogenation technology. Chemical interesterification can also be used to produce some commercial products for nutritional purposes. An example, Caprenin, Benefat, Captex, and Neobee are commercially available chemically synthesized SLs. Caprenin is a SL containing C8:0, C10:0, and C22:0 fatty acids esterified to glycerol moiety. It is manufactured from coconut, palm kernel, and rapeseed oils by chemical transesterification process. Benefat is another

example, contains C2:0–C4:0, and C18:0 esterified to glycerol moiety. Benefat is produced by base-catalyzed interesterification of highly hydrogenated vegetable oils with TAGs of acetic, propionic, and butyric acids (Smith et al., 1994; Akoh and Kim, 2008). SLs, both Caprenin and Benefat are low calorie fats.

2.2.4.3 Enzymatic interesterification

Enzymatic interesterification is a general term for the reactions between an ester and a fatty acid, an alcohol, or another ester, which includes acidolysis, alcoholysis, and ester-ester exchange by lipase catalyst. Therefore, the triacylglycerol lipases, interesterification in micro-aqueous organic system, factors that affect enzymatic process and product yield, reaction types and interesterification bioreactors will be covered in this section.

2.2.4.3.1 Triacylglycerol lipases

TAG lipases, also known as TAG acylhydrolases belong to the super class of hydrolases that act on the carboxylic ester bonds (Rajendran, 2009). Depending on the medium employed, these enzymes are able to catalyze both the hydrolysis of esters and acyl-transfer reactions, such as esterifications (acid and alcohol), transesterifications (alcohol and ester), interesterifications (ester and acid), and transfer of acyl groups from esters to other nucleophiles such as amines, thiols or hydroperoxides (Alcántara, 1998). Lipases are serine hydrolases which do not require any cofactor. Due to this unique feature, they remain dissolved in oil water interface and under the natural conditions they hydrolyse the triacylglycerols which have low solubility in the water. In the presence of traces of water, they reverse the reaction leads to esterification and formation of glycerides from the fatty acids and glycerols (Ghosh et al., 1996; Sharma et al., 2001; Rajendran, 2009).

Extensive studies have reported the applications of the animal, plant and microbial lipases to various industrial purposes. Current applications mostly involve the microbial lipases at greater length (Macrae and Hammond, 1985). Microbial lipases which are regiospecific and fatty acid specific could be exploited for the esterification and transesterification reactions (Gupta et al., 2003). Lipases are being developed to carry out the transformations without the extreme temperature

and pressure conditions which are essential for the traditional industrial processes (Rajendran, 2009).

Certain lipases show positional specificity, that is, specificity toward ester bonds in positions sn-1,3 of the triacylglycerol. This results from an inability of lipases to act on sn-2 position of triacylglycerol due to steric hindrance. Steric hindrance prevents the fatty acid in position sn-2 from entering the active site (Macrae, 1983; Macrae and How, 1988). During the interesterification reaction by a 1,3-specific lipase, initially a mixture of triacylglycerols, 1,2- and 2,3-diacylglycerols, and free fatty acids are produced. Then, acyl migration can occur due to prolong reaction periods, it cause to formation of 1,3-diacylglycerols, which allows some randomization of the fatty acids existing at the *sn*-2 position of the triacylglycerols. A variety of specific lipases available and they could be used for the production of specific SLs due to their regiospecificity or stereospecificity. A list of regiospecific lipases is given in Table 2.2. Beside sn-1,3 specific lipases, there are a few lipases available possessing sn-2 specificity or sn-1 and sn-3 specificity. The specificity of individual lipases can change due to microenvironmental effects on the reactivity of functional groups or substrate molecules (Pabai et al., 1995).

Table 2.2. Specific lipases for the production of specific structured triacylglycerols (Xu, 2000)

Lipase source	Fatty acid specificity	Regio specificity
Aspergillus niger	S, M, L	1, 3 >> 2
Candida lipolytica	S, M, L	1, 3 > 2
Humicola lanuginosa	S, M, L	1, 3 >> 2
Mucor javanicus	M, L	>> S 1, 3 > 2
Rhizomucor miehei	S > M, L	1 > 3 >> 2
Pancreatic	S > M, L	1, 3
Pre-gastric	S, M	>> L 1, 3
Penicillium camembertii	MAG, DAG > TAG	1, 3
Penicillium roquefortii	S, M	>> L 1, 3
Rhizopus delemar	M, L >> S	1, 3 >> 2
Rhizopus javanicus	M, L > S	1, 3 > 2
Rhizopus japonicus	S, M, L	1, 3 > 2
Rhizopus niveus	M, L > S	1, 3 > 2
Rhizopus oryzae	M, L > S	1, 3 >>> 2
Pseudomonas fluofescens	M, L > S	1, 3 > 2
Pseudomonas sp.	S, M, L	1, 3 > 2
Rhizopus arrhizus	S, M > L	1, 3

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

Certain lipases show no positional or fatty acid specificity during interesterification. Interesterification with these lipases after extended reaction times gives complete

randomization of all fatty acids in all positions and gives the same products as chemical interesterification (Figure 2.6). Examples of nonspecific lipases include lipases derived from *Candida cylindraceae, Corynebacterium acnes*, and *Staphylococcus aureus* (Macrae, 1983; Gunstone, 1994).

Biocatalysts are more expensive then chemical catalysis. In order to make them economically useful in industry they should be immobilized. Immobilization is necessary to enable enzyme reuse and to facilitate continuous processes. Immobilization of enzymes can simply be accomplished by mixing an aqueous solution of the enzyme with a suitable support material and removing the water at reduced pressure, after which small amounts of water are added to activate the enzyme. Suitable support materials for enzyme immobilization include glass beads, Duolite, acrylic resin, and Celite. The introduction of cheap and thermostable enzymes changes the economic balance in favor of lipase use for the commercial production of SL and lipid modifications (Akoh and Kim, 2008). During the last years there has been an increasing interest shown by the industry towards enzymes and especially lipases, due to their extensive applications (Masse et al., 2001; Takamoto, 2001; Bornscheuer, 1994).

Some properties of lipase from Rhizomucor miehei

The lipase from *Rhizomucor miehei*, formerly *Mucor miehei*, is a commercially available enzyme. *Rhizomucor miehei* (soluble and immobilized forms) has a very high activity and good stability under diverse experimental conditions. It is mainly produced for oils and fats modifications. Its high stability under anhydrous systems is main advantages when compared to other lipases and this is the main reson of their use in industry. Its high esterification activity in anhydrous media makes the *Rhizomucor miehei* the choice lipase mainly in esterifications or in any reaction that proceed via esterification in some of the initial steps (e.g., acidolysis and interesterification) (Rodriguesa and Fernandez-Lafuente, 2010).

Regarding the selectivity of the reaction, *Rhizomucor miehei* is described as *sn*-1,3-specific. Between these two positions, the enzyme exhibits a *sn*-1 or *sn*-3 preference depending on the reaction. In hydrolysis, the enzyme prefers the *sn*-1 position (Rodriguez, 2008), that means that in acidolysis and interesterification (where the first step is the hydrolysis of the triglyceride) the enzyme is mainly sn-1

specific. However, in esterification, the enzyme exhibits a slight sn-3 preference (Deng, 2008).

Use of Rhizomucor miehei in oil modifications

Lipase from *Rhizomucor miehei* has long been used as a biocatalyst for the hydrolysis of glycerides, transesterification, esterification, acidolysis and interesterification. It is one of the most studied ones of lipases. The first report of the use of *Rhizomucor miehei* as catalyst was in the hydrolysis of animal fat and vegetable oil to improve the characteristic flavor of Italian cheese when the enzyme was added to the milk (Huge-Jensen et al., 1987). Extensive studies have reported the applications of the *Rhizomucor miehei* on the modification of different oils. In many cases, the objective is to improve the physical properties of the fats and oils like its melting point, etc. In others, objectives is to prepare healthier products, low-calorie structured lipids, conversion of cheap commercial oils to high value added products such as cocoa butter equivalents, human milk fat substitutes etc. The list of studies in which *Rhizomucor miehei* used as catalyst is given in Table 2.3.

Table 2.3. Summary of studies reported in the literature with *Rhizomucor miehei*

Substrates	Reaction Type	Aim	References
Fully hydrogenated soybean oil and rapeseed oil and palm stearin	Interesterification	To produce solid fat for trans-Free Bakery Shortening	Lee et al. (2008)
Olive oil and caprylic acid	Acidolysis	To produce SL with the benefits of the medium chain fatty acid while retaining the benefits of oleic acid	Fomuso and Akoh (2002)
Triolein and Stearic acid	Acidolysis	To produce margarine-type fats.	Seriburi and Akoh (1998)
Trilinolein and tricaproin	Transesterification	To produce SL that contains linoleic acid and caproic acid	Fomuso and Akoh (1998)
Triolein with caproic and butyric	Acidolysis	To produce reduced calorie SL	Fomuso and Akoh (1997)
Menhaden fish oil and capric acid	Acidolysis	To produce a SL containing both, fish oil EPA and DHA and MCFA	Jennings and Akoh (2001)
Palm olein with caprylic acid	Acidolysis	To produce SL that can confer metabolic benefits when consumed	Lai et al. (2005)
Tributyrin with palm oil	Transesterification	To produce mixtures of structured triacylglycerol species as a low-calorie lipid.	Lee et al. (2008)
Lard and soybean fatty acids	Acidolysis	To modify lard into human milk fat substitutes	Yang et al. (2003)
Refined olive pomace	Acidolysis	To produce a Cocoa butter	Çiftçi et al. (2010)

oil, Palmitic acid and stearic acid		like fat from refined olive pomace oil.	
Menhaden oil and caprylic acid	Acidolysis	To produce SL that contains medium-chain fatty acids, and EPA and DHA	Xu et al. (2000)
fish oil and capric acid	Acidolysis	To produce SL based on fish oil, known as MEG-3, and capric acid	Hamam and Budge (2010)
Cod liver oil as source of n-3 PUFAs and caprylic acid	Acidolysis	To produce of SL rich in n-3 polyunsaturated fatty acids	Paez et al. (2002)
Roasted sesame oil with caprylic acid	Acidolysis	To produce a SL of roasted sesame oil with caprylic acid	Kim and Akoh (2006)
Lard and soybean oil fatty acids	Acidolysis	To produce a human milk fat substitute on a kg scale	Nielsen et al. (2006)
Phytosterols and oleic acid	Esterification	To produce phytosteryl esters of oleic acid	Kim and Akoh (2007)
DHA and borage oil	Acidolysis	To incorporate DHA into borage oil	Senanayake and Shahidi (2002)
Omega-3 fatty acid extracted from linseed oil into groundnut oil	Acidolysis	To synthesis a SL with balanced omega-3: Omega-6	Sharma et al. (2009)
Sunflower oil with palmitic–stearic acids	Acidolysis	To obtain an alternative of hydrogenation products.	Carrín and Carapiste (2008)
Palm stearin and anhydrous milk fat	Transesterification	To produce the desired melting properties for use, in or as standard table margarine or shortening.	Lai et al. (2000)
Tripalmitin with capric acid	Acidolysis	To produce a model SL of the MLM type	Foresti and Ferreira (2010)
Capric and stearic acids with glycerol	Esterification	To produce medium- and long-chain triacyglycerols fat suitable for margarine and shortening applications	Arifin et al. (2010)
Virgin olive oil with caprylic or capric acids	Acidolysis	To produce MLM type SL with important clinical and nutritional properties.	Nunes et al.(2011)

Factors That Affect Enzymatic Process and Product Yield

The factors that affect the yield of product in enzymatic processes are namely pH, water content, temperature, substrate composition and substrate molar ratio, reaction time, lipase content and type of solvent, etc. It is important to remark that in almost all cases the reactions conditions were different and particular for each case, and this makes difficult a comparison of the results since the enzyme presents different activities and selectivities depending on its concentration, the reaction medium, pH, temperature and other parameters, that may affect enzyme properties and acyl migrations (Rodriguesa and Fernandez-Lafuente, 2010).

Enzymes show different activity at various water content ranges. Although most reactions require water contents of <1% for effective interesterification, the optimal

water content for interesterification by different lipases ranges from 0.04% to 11% (w/v), (Bornaz et al., 1994; Malcata et al., 1992; Li and Ward, 1995). The water content in a reaction system is an important factor which determines the reaction equilibrium will shift toward hydrolysis or ester synthesis. Low water activity is essential for ester synthesis but too low water activity prevents all reactions so lipases need a certain amount of water to remain hydrated for enzymatic activity (Briand et al., 1994; Svesson et al., 1994).

The pH value has an effect on the catalytic activity of lipases. Depending on the origin of enzyme and the ionization state of residues in their active sites, enzymes show different activity at different pH ranges. Lipases show activity in a wide pH range, from 4 to 10 and for most lipases optimum pH lies between 7 and 9. The nature of enzyme, substrate concentration, stability of the enzyme, temperature, and the length of the reaction are affected on this pH range (Malcata et al., 1992; Yamane, 1987).

The choice of appropriate solvent is also critical point in enzymatic reactions because reaction kinetics and catalytic efficiency of an enzyme are also dependent on type of organic solvent used. Lipases differ in their sensitivity with respect to solvent type. Polarity of solvent is most important characteristic which determines the catalytic activity of enzyme. In addition, the solubility of the reactants in the chosen solvent, presence of chemical interference, solvent density, viscosity, surface tension, toxicity, flammability, waste disposal, and cost are other factors that must be taken into account (Dordick, 1989).

Temperature is another parameter affecting the enzymatic processes. Adequate temperature control is important for the reproducible assay of enzyme-catalyzed reactions. The optimal temperature for most immobilized lipases falls within the range of 30 to 62 ^{o}C, whereas it tends to be slightly lower for free lipases (Malcata et al., 1992). In general, increasing the temperature improves the interesterification, but too high temperatures can damage the enzyme structure so cause reduction in reaction rate. Sometimes high temperature can be necessary to provide substrate solubility as in solvent-free system but it is not needed in organic solvents included systems because substrates are readily solubilized in organic solvents (Willis and Marangoni, 2008).

Product accumulation also affects the rate of reaction. The production and accumulation of high amount free fatty acids can reduce the reaction rate due to acidifying of micro-aqueous phase surrounding the lipase besides it can cause desorption of water from the interface (Willis and Marangoni, 2008).

2.2.4.3.2 Enzymatic acidolysis

Acidolysis is the transfer of an acyl group between an acid and an ester. In most applications in the lipid field, esters can be tri-(di-, or mono-) acylglycerols, glycerol-phospholipids, alkyl fatty acid esters, *etc.* and acids can be fatty acids or other acids (Xu, 2003). It is an effective means of incorporating novel free fatty acids into triacylglycerols.

Enzymatic acidolysis reaction is a reversible reaction. The reaction is commonly considered as a two-step reaction: hydrolysis and esterification. For this consideration, diacylglycerols are considered as the reaction intermediates. Through steps of hydrolysis and esterification, the new fatty acids in the system are incorporated into triacylglycerols. The reaction will finally reach equilibrium. The procedure for the reaction between a TAG (LLL) and a fatty acid (M) is depicted in Figure 2.7. (Xu, 2003).

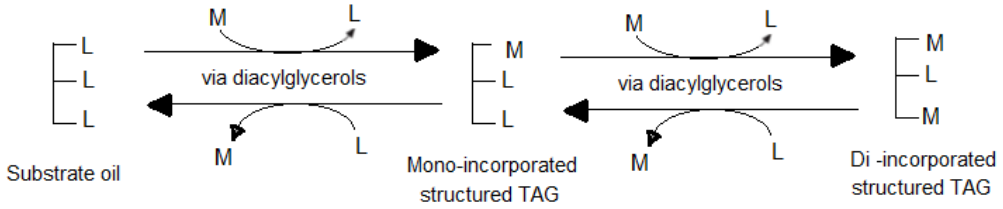

Figure 2.7. Reaction principle of the lipase-catalyzed interesterification between LLL and M and the dynamic balance between LLL, *sn*-MLL/LLM and *sn*-MLM, L = long-chain fatty acid, M = medium-chain fatty acid

A particular interest in this area is that 1,3 specific lipases can selectively catalyze the acyl exchange at the *sn*-1 and *sn*-3 positions whilst leaving the *sn*-2 acyl group unchanged. This provides an opportunity to tailor some functional lipids with special requirements for the fatty acid types located in 1,3- or 2-positions. With this technology, a series of functional lipids, such as cocoa butter equivalents, human milk fat mimics or structured lipids containing medium- or short-chain fatty acids

have been developed (Macrae, 1985; Balcao and Malcata, 1998; Gunstone, 1999; Xu, 2000; Baljit et al., 2002).

2.2.4.3.3 Enzymatic alcholysis

Alcoholysis is the esterification reaction between an alcohol and an ester. Alcoholysis is also a reversible reaction as shown in Figure 2.8. The starting ester can be acylglycerols, TAGs or alkyl esters for example. The starting alcohol can be glycerol, methanol, ethanol, or sterol. The product esters can be partial acylglycerols. Enzymatic alcoholysis has been widely studied for the production of partial acylglycerols, such as MAGs and DAGs, and biodiesels such as fatty acid methyl esters or ethyl esters (Xu, 2003).

Figure 2.8. Lipase-catalyzed alcoholysis reaction between an acylglycerol and an alcohol

The alcoholysis of triacylglycerols with glycerol is called glycerolysis and is widely used for production of mono- and diacylglycerols. The reaction of triacylglycerols with monohydric alcohols generates the simple alcohol esters of fatty acids, which have found applications in the production of biodiesel, as well as alternative acyl donors of fatty acids, e.g. fatty acid ethyl esters. The intermediate product of 1,3-specific lipase-catalyzed alcoholysis, 2-monoglycerides, can also be used as starting materials for the synthesis of ABA-typed structured lipids.

2.2.4.3.4 Enzymatic transesterification

Transesterification is the exchange of acyl groups between two esters, namely, two triacylglycerols (Figure 2.9.). Chemical transesterification often referring to as chemical interesterification is dominantly used for the randomization of oils and fats, which has been widely applied in industry for oil and fat modification. Enzymatic transesterification is an alternative to the chemical method for the modification of oils and fats (Xu, 2003). Transesterification is used predominantly to alter the physical properties of individual fats and oils or fat–oil blends by altering the

positional distribution of fatty acids in the triacylglycerols (Willis and Marangoni, 2008).

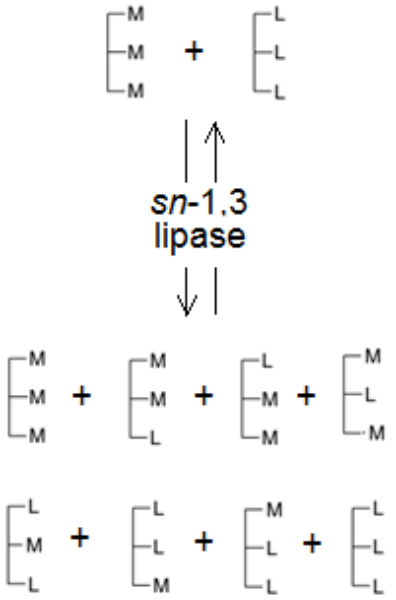

Figure 2.9. Reaction scheme of enzymatic transesterification between two triacylglycerols (MMM and LLL) with *sn*-1,3 specific lipases.

This reaction has been applied to margarine or shortening fat production and the manufacture of other structured lipids with specific functions (Foglia et al., 1993; Chang et al., 1990; Seriburi and Akoh, 1998; Lai et al., 2000).

2.2.4.3.5 Enzymatic interesterification reactors

Bioreactors are important part of enzymatic processes. Reactors designed for immobilized enzyme reactions can be batch or continuous systems. Different reactors have long been studied and used for enzyme reactions, some of these are stirred tank reactors, packed bed reactors, membrane reactors, fluidized bed reactors, gas-lifting reactors, simple circulating reactors, spraying reactors, foam reactors, *etc*. There are no universal criteria for the choice of a suitable reactor for a particular system. It shows differences for each case. But some parameters such as the reaction efficiency, product quality, enzyme reusability, process feasibility and practicality can be accounted as essential requirements for all enzyme reactors (Xu, 2003). Stirred batch reactors, continuous stirred tank reactors, and fixed bed reactors are most investigated reaction systems used for modification of fats and oils.

Stirred batch reactor

A stirred batch reactor consists of a tank with an agitator and integral heating/cooling system which is illustrated in Figure 2.10. There is no addition and removal of substrate and products throughout the reaction. This reactor system is relatively easy to build and its simplicity and low cost makes it useful in lipase catalyzed reactions.

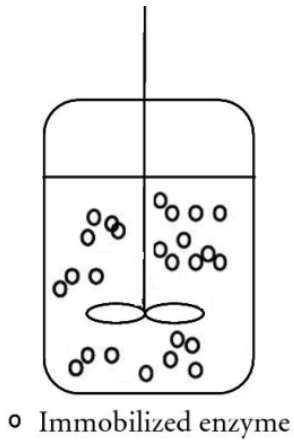

Figure 2.10. Stirred batch reactor for immobilized or free lipase–catalyzed interesterification

In a stirred batch reactor system there is a high level of substrate at the beginning of reaction so the rate of conversion is higher at initial stage and it decreases over time due to reduction of substrate level. Therefore more immobilized enzyme should be added to maintain the same rate of conversion throughout the reaction (Willis and Marangoni, 2008). Acyl migration is a major problem in the synthesis of SLs in batch reactors and this causes a decrease of yield in the targeted SLs. The high substrate/enzyme ratio demands long reaction times to reach equilibrium, and consequently results in acyl migration (Paez et al., 2002).

Continuous Stirred Tank reactor

A continuous stirred tank reactor system is an agitated tank in which substrate and products are added and removed at the same rate throughout the reaction (Figure 2.11). Additionally this system requires a screen or filter at the outlet to prevent losses of the immobilized lipase. The continuous stirred tank reactors require higher power cost due to continuous agitation and it can cause damage on immobilized enzyme structure (Malcata, 1990; Cheetham, 1988).

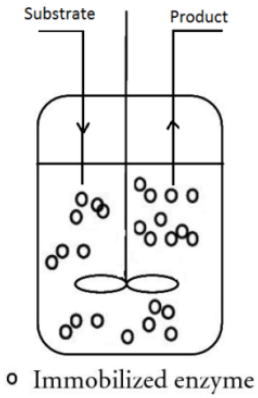

Figure 2.11. Continuous stirred tank reactor for immobilized lipase–catalyzed interesterification

Fixed and Fluidized bed reactors

Fixed bed reactors are always used in a continuous flow mode. In this system immobilized enzyme is packed in a column and the substrate and product streams are pumped in and out of the reactor at the same rate (Figure 2.12a). Fixed bed reactors have been investigated and applied in a wide range of enzymatic applications both in the laboratory and in industry, especially for immobilized enzymes. When compared to a stirred tank reactor, a continuous process employing a packed-bed reactor (PBR) is the most appropriate technology for lipase-catalyzed reactions involving fats and oils for industrial applications (Xu et al., 1998).

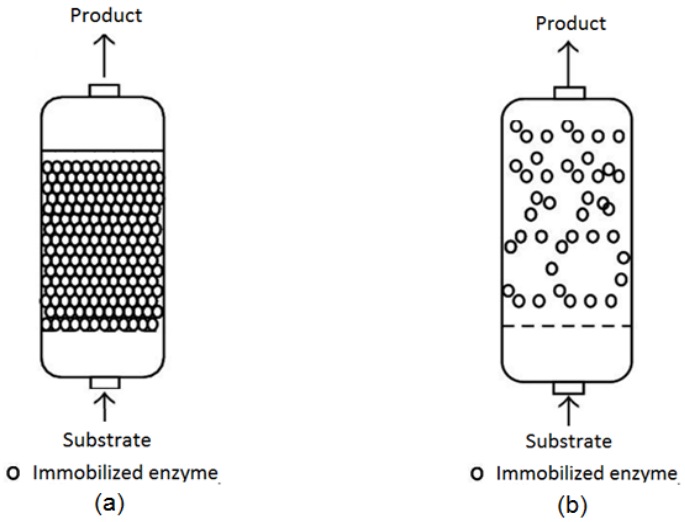

Figure 2.12. (a) Fixed bed reactor, (b) fludized bed reactor for immobilized lipase–catalyzed interesterification

Fluidized bed reactors are reactors in which the immobilized enzymes are kept suspended by the upward flow of substrate or gas at high flow rates (Figure 2.12b) (Cheetham, 1988). In this system small concentrations of enzyme are used since a large void volume is required to keep the enzyme suspended. So, channeling problems, huge pressure changes at high flow rate and coalescence problem of emulsion droplets are eliminated in fluidized bed reactor system (Willis and Marangoni, 2008).

2.2.4.4 Comparison of chemical and enzymatic interesterification

The main differences between chemical and enzymatic interesterification is that chemical interesterification typically results in complete randomization of the fatty acids on the glycerol backbone, while enzymatic interesterification provide positional and/or fatty acid specific manipulation to achieve the production of desired TAGs. Each type of interesterification possesses advantages and disadvantages. Chemical interesterification is a mature technology which has many applications in industry and industrial procedures and equipment are available for this process (Xu, 2000). Chemical catalysts are much cheaper than lipases. Chemical transformation has advantages over enzymatic transformations due to its lower cost and capital investment. But the harsh process conditions and non-specificity of chemical catalyst are main disadvantages of chemical transformations.

Specificity of enzymes allows scientists to design SLs with beneficial end-use properties. From this point of view chemical methods usually are impossible for the production of specific triacylglycerols due to the lack of positional specificity.

The most important and also obvious advantages of biocatalyst for the production of SLs besides the specificity of lipases can be accounted as:

- efficacy of lipases under mild reaction conditions,
- utility in “natural” reaction systems and products,
- reduced environmental pollution,
- availability of lipases from a wide range of sources,
- ability to improve lipases by genetic engineering, and in special situations
- the use of lipases for the production of particular biomolecules.

For these reasons, many nutritional and functional SLs have been produced enzymatically and a lot of these studies have been published (Xu, 2000).

Figure 2.13 shows schemes for the reaction between two triacylglycerols with chemical catalysts and sn-1,3 specific lipases. The reaction will result in similar triacylglycerol species to those produced by randomization, even though their contents may not be totally the same (Mohamed et al., 1993).

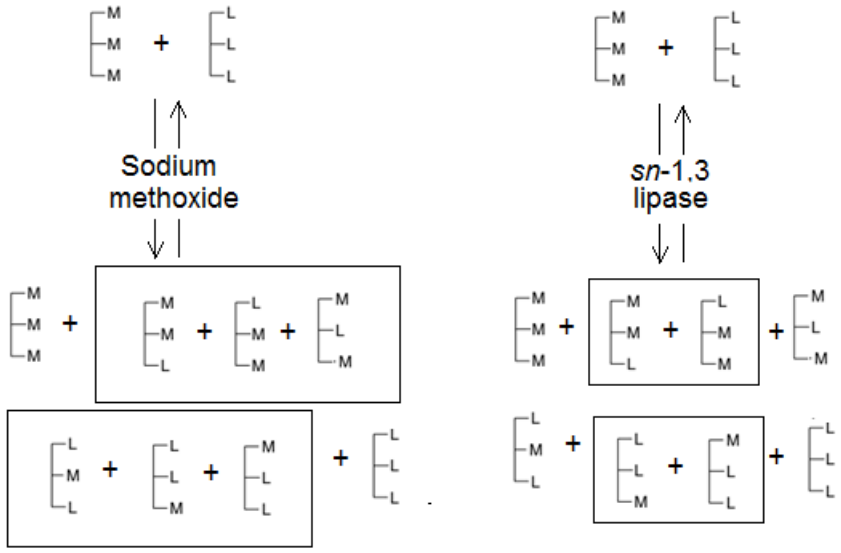

Figure 2.13. Reaction schemes for the lipase-catalyzed interesterification between two triacylglycerols with chemical catalysts and sn-1,3 specific lipases

Chemical interesterification produces randomized products in which fatty acids are randomly distributed in the three positions of the glycerol backbone. Sn-1,3 specific lipase-catalyzed interesterification produces similar triacylglycerol species, but the proportions of each triacylglycerol specie are not the same as those of the randomized products even at the same substrate molar ratios (MMM/LLL). Triacylglycerol species in each rectangle should have the same content for randomized products and also for sn-3 specific lipase-catalyzed products provided that the sn-1,3 specific lipase has the same specificity towards *sn*-1 and *sn*-3 positions and also different fatty acids (Xu, 2000).

Some information is available in literature regarding comparison of chemically and enzymatically produced SLs. For example, fats produced from the enzyme process in general can be used for margarine production without significant change in properties, though there are some unusual aspects compared to either blend or

chemically randomized fats (Zhang, 2005). From the point of view of oxidation during storage, the enzymatically-produced fats have advantages over the chemically randomized product; the latter develops high peroxide values during storage. With regard to physical properties, the former can form the required crystal type and textures along with suitable solid fat profiles which are comparable to those of the chemically randomized fats (Xu, 2006).

2.2.5 Application area of SL: medical, nutritional and functional

SLs can be designed for beneficial end-usage. When designing SLs with particular chemical structure, it is possible to control their behavior, improve the nutritional and pharmaceutical properties. SLs have many industrial applications such as lipid emulsifiers and surfactants in dairy and bakery products, *trans*-free fats for use in food, reduced and zero calorie lipids in food, chocolate and confectionery fats, frying oils etc. Therefore, in this section, some common examples of SL and their possible application area will be overviewed.

Cocoa butter equivalents

Cocoa butter is an important ingredient for chocolate and other confectionery products, having a major influence on the organoleptic and physical properties. The trend of increasing price and the degree of uncertainty in supply of cocoa butter drives manufacturers to use cocoa butter equivalents (Long, 2009). Therefore attempts have been made to prepare cocoa butter-like fat by enzymatic interesterification reaction using a cheap raw starting material (Çiftçi et al., 2009). In the Unilever process triacylglycerols (from e.g. high-oleic sunflower oil) react with fatty acids (e.g. stearic acid) at a moisture content of 0.2% - 1%. In the process created by Fuji Oil, triacylglycerols (a palm fraction) react with fatty acid esters (e.g. ethyl stearate) at a maximum moisture content of 0.18 % (Timms, 2003). Also the Kao Corporations patented a process: a palm mid-fraction is reacted with stearic acid by hexane (Tanaka et al., 1989).

Human milk fat substitutes

Human milk fat substitutes (HMFS) are another important example for the application of SLs in foods. The feeding of human breast milk is the ultimate and ideal method for healthy infants until six months of life (Jensen, 2001). Human milk

fat (HMF) is the richest energy source for infants, supplying about 50–60% of dietary calories, and approximately 98% of the fat is in the form of triacylglycerol (TAG) with a unique fatty acid distribution (López-López et al., 2002; Morea et al., 2003). Palmitic acid is the major saturated fatty acid, representing about 20–25% of the total fatty acids. It is located primarily more than 60% at sn-2 position of TAG whereas monounsaturated fatty acid (i.e. oleic acid) is mainly esterified at sn-1,3 positions. However, cow's milk fat or vegetable oil, commonly used in infant formula contains palmitic acid located predominantly at sn-1,3 positions (Nelsom and Innis, 1999). HMFS, a SL resembling TAG of HMF, can be synthesized by an interesterification using a sn-1,3-specific lipase. BetapolTM, commercialized by Loders Croklaan (Unilever) was manufactured from palm oil fraction rich in tripalmitin and a mixture of canola and sunflower oils high in oleic acid by Rhizomucor miehei (Lee et al., 2010).

Plastic fats

The alteration of physical properties of fats and oils to produce desired plastic fats is one of the important applications of interesterification. Different kinds of food preparation demand a range of physico-chemical characteristics from fats and oils, to achieve a particular functionality (Long, 2009). Physical functionality of fats is strongly related to the presence of saturated and trans fatty acids (Flöter and van Duijn, 2006). Plastic fats account for a big portion of the fat intake in the diet of the people. Normally such products are produced by partial hydrogenation process in which vegetable oils are chemically transformed into a solid state. However, the hydrogenation is accompanied with the formation (up to 50 %) of trans-fatty acids (TFA) which are associated with increased risk of developing cardiovascular disease, thus they play an important role in the health of people (Aro et al., 1998; Tekin et al., 2002; Ascherio et al., 1999). Thus lipase-catalyzed interesterification is one of the alternative technologies for replacing conventional hydrogenation process to reduce or eliminate TFA in plastic fats.

Low-calorie fats

SLs are increasingly being used by the food industry and have recently attracted the attention of food manufacturers for production of low-caloric lipids. Low-caloric lipids are characterized by a mixture of short-chain fatty acids (SCFA) and/or MCFA

and LCFA in the same glycerol moiety. Low calorie concept is coming from the lower caloric content of SCFA or MCFA compared to LCFA. Reduced-calorie specialty lipids are intended for use in baking chips, dips, coatings, bakery and dairy products, or as a cocoa butter replacer (Hamam and Shahidi, 2008). The principle of combining SCFA and LCFA has been used by Nabisco Foods Group (East Hanover, NJ) in the chemical synthesis of Salatrim® which is a reduced-calorie fat (Finley et al., 1994). Caprenin is another commercially available low-calorie fat produced by Procter & Gamble Company. It is a randomized triacylglycerol comprising caprylic, capric and behenic acids.

MLM-type triacylglycerols

Most attention has focused on triacylglycerols (TAGs) with medium-chain fatty acids (MCFA) located in *sn*-1 and *sn*-3 position of the glycerol backbone and a functional long-chain polyunsaturated fatty acid (PUFA) in *sn*-2 (Hita et al., 2009). It is well known that pancreatic lipase is 1,3 specific and hydrolyzes the ester bonds at positions 1 and 3 of triacylglycerols (TAG). Moreover, this lipase shows higher activity toward medium-chain than toward long-chain fatty acids (Mu and Porsgaard, 2005). The liberated medium-chain free fatty acids are directly absorbed because they can be solubilized in the aqueous phase of the intestinal contents, absorbed bound to albumin and transported to the liver by the portal vein (Decker, 1996). However LCFA which are incorporated into chylomicrons and transported through lymph (Figure 2.14).

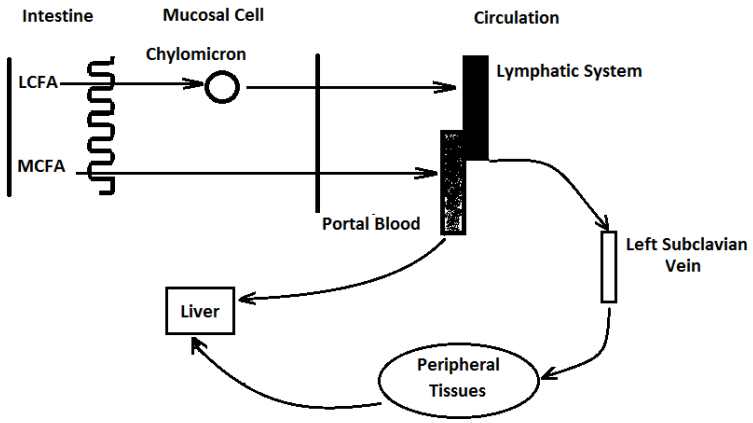

Figure 2.14. Differential MCFA and LCFA digestion, absorption and transport in the blood

MCFAs are not stored in the adipose tissues (Senanayake and Shahidi, 2002; Willis and Marangoni, 1998). They are frequently used in the diet of patients with maldigestion and malabsorption (Willis et al., 1998). They have also been employed in total parenteral nutrition and formulas for preterm infants. Moreover, calorie-reduced SL containing MCFA have recently been developed and represent exciting new application areas (Finley et al., 1997).

2.2.6 Safety and regulatory status for structured lipids

Governments have regulated food and drug products in countries. In general, these regulations focus on ensuring the quality and safety of food and drugs. Therefore, regulations has important role to protect public health by assuring the safety of foods and drugs. For example, the United States Food and Drug Administration (FDA or USFDA) is responsible for protecting and promoting public health through the regulation and supervision of food safety, tobacco products, dietary supplements, prescription and over-the-counter pharmaceutical drugs (medications), vaccines, biopharmaceuticals, blood transfusions, medical devices, electromagnetic radiation emitting devices, and veterinary products. Any food product is not available in United States unless approved for compassionate use by FDA. The compassionate-use approval process involves developing a study protocol which must go through the Institutional Review Board at the home medical center, getting all research participants Collaborative Institutional Training Initiative trained, and applying to the FDA.

In United States, enzymatic conversion of plant oils to produce SLs or tailored fats is a rapidly expanding area. There is a wealth of research and development going on in this area and much opportunity for commercialization. But it is necessary to take FDA-approval for their usage. Food and Drug Administration (FDA) has evaluated the GRAS status of a number of different triglycerides. "GRAS" is an acronym for the phrase **G**enerally **R**ecognized **A**s **S**afe. Products of some interesterification processes have been affirmed as GRAS by FDA. Examples are enzyme modified fats and cocoa butter substitute. Further, the fatty acids used in the interesterification process are commonly found in food and are approved for use in the manufacture of food components. The use of enzyme preparations as processing aids is accepted by

FDA so long as the source organisms are safe and suitable and potentially toxic by products are not produced.

For example, Salatrim, a reduced calorie fat, is licensed to Cultor Food Science, which established the brand name Benefat™ for manufacture and marketing. FDA accepted for filing in 1994 a GRAS affirmation petition submitted by Nabisco Foods Group (Akoh, 1998). Another example is olestra which is a fat substitute. Olestra is approved (FDA, 1996) for replacing up to 100 % of the conventional fat in savory snacks (i.e., snacks that are salty or piquant but not sweet, such as potato chips, cheese puffs, and crackers) and for frying of savory snacks. Olestra is not absorbed or metabolized (Grossman et al., 1994; Mattson and Nolen, 1972) and is non-caloric because the large size and number of the nonpolar fatty acid constituents prevent olestra from being hydrolyzed by digestive lipases.

With the help of biotechnology, researchers hope to design novel fats and oils. Such development would have an obvious impact on international fats and oil trade, if the substitutes were commercially viable and not banned by legislation.

In Canada, a lipase produced by *Aspergillus oryzae* has been approved for enzymatic interesterification for all food uses (Canada Gazette, 1994). The Food & Drug Regulations allows infant formula to contain BetapolTM.

In European Union, BetapolTM has been approved for use in preterm infant formulas in the United Kingdom (Advisory Committee on Novel Foods and Processes, 1997) and has been cleared by the Dutch Health Authorities for use in all types of infant formulas (Decision of the State Secretary for Public Health, Welfare and Sport 1995; Decision of the State Secretary for Public Health, Welfare and Sport 1997). As a result of these approvals, BetapolTM may be used as an infant formula ingredient throughout the European Union. BetapolTM is used in infant formulas for both term and preterm infants, with the predominant use being for term infant formula. In South Korea, BetapolTM (concentrate for further manufacture) is approved for use in infant formula.

2.3 Experimental Design

Experimental design and optimization are tools that are used to systematically examine different types of problems that arise within, e.g., research, development and production (Lundstedt, 1998). In an experiment, one or more process variables or factors are deliberately changed in order to observe the effect on one or more response variables. The statistical design of experiments is an efficient procedure for planning experiments so that the data obtained can be analyzed to yield valid and objective conclusions.

Response surface methodology is an effective statistical technique for the optimization of complicated systems, which enables the evaluation of effects of multiple parameters, alone or in combination, on response variables (Shieh et al., 1995; Xu et al., 1999). The advantages of using RSM are reported to be the reduction in the number of experimental runs needed to evaluate multiple variables, and the ability of the statistical tool to identify interactions (Chen et al., 2004; Lee et al., 2000). Therefore, it is less laborious and time consuming compared to one-variable at a-time. RSM has been widely applied for optimizing conditions and processes in various food studies (Junqueira et al., 2007; Liyana-Pathirana and Shahidi, 2005). The full factorial, partial factorial and central composite rotatable designs are the commonly used experimental design techniques. The choice of an appropriate experimental design technique is the first requirement to get adequate and reliable measurement of response of interest from experiments.

Central composite rotatable design (CCRD) is an effective alternative to factorial design. CCRD gives almost as much information as a three-level factorial, requires many fewer tests than the full factorial design and has been shown to be sufficient to describe the majority of steady-state process responses (Obeng et al., 2005; Cilliers et al., 1992; Crozier, 1992). Hence in this study, it was decided to use CCRD to design the experiments. CCRD have been reported as an applicable design for enzymatic esterification reactions in previous studies. CCRD for both STR and PBR systems have successfully applied to optimize reaction conditions. (Foresti and Ferreira, 2010; Zhou et al., 2001; Koh et al., 2008; Senanayake and Shahidi, 2002)

CHAPTER 3

MATERIALS & METHODS

3.1. Materials

Palmitic acid (purity ≥ 98 %), caprylic acid (purity ≥ 99 %) and some TAG standards; triolein (OOO), tripalmitin (PPP), trilinolein (LLL), 1,2-olein-3-palmitin (OOP), 1,3-palmitin-2-olein (POP), 1,2-linolein-3-palmitin (LLP), 1,2-linolein-3-olein (LLO) were purchased from Sigma–Aldrich (St. Louis, MO). Some other TAG standards; 1,3-caprylin-2-olein (COC), 1-palmitin-2-olein-3-caprylin (POC), 1,3-caprylin-2-linolein (CLC), 1-caprylin-2,3-olein (COO), 1-palmitin-2-linolein-3-caprylin (PLC) and 1-caprylin-2,3-linolein (CLL), were produced in our laboratory. Immobilized sn-1,3 specific lipase (Lipozyme IM, immobilized from *Mucor miehei*, 140 U/g), acetone, acetonitrile, *n*-hexane and silica gel (SG 60, 70-230 mesh) were also purchased from Sigma–Aldrich (St. Louis, MO). All solvents used were of HPLC grade. All other reagents and solvents were of analytical or chromatographic grade.

3.2 Extraction of Terebinth Fruit Oil

Harvested terebinth fruits first cleaned with an air screen cleaner to remove unwanted material like dust, leafs, stems etc. Then washed thoroughly in a large bowl and dried under sun light. After that fruit oil was extracted by hydraulic cold pressing. The terebinth fruit is compressed at 50 bar and 25 °C. The extracted virgin oil had some amount of solid particles so it was allowed to filtration and centrifugation to remove these fine particles. Crude oil was placed in a system in which oil was forced through a woven (fabric) filter from the bottom by a pump. The oil which is discharged from the filter was centrifuged for further purification. The purified oil was collected in amber colored glass bottles and stored at -20 ^{o}C until analysis.

3.3 Acidolysis Reaction in Stirred Tank Reactor System

Acidolysis of terebinth fruit oil (864 mg, MW: 864) was performed in 150 ml tightly closed screw capped flask. Terebinth fruit oil (TO) and the corresponding ratio of caprylic and palmitic acids were mixed in 25 ml of *n*-hexane. The specified amount of enzyme (wt%) was added, the values of time and temperature were set according to an experimental design (Table 4.4). The reactions were carried out in a rotary incubator (New Brunswick Scientific, Nova 40, USA) at 200 rpm. At the end of the reaction, the product was separated from the enzyme by decanting. The *n*-hexane was evaporated in a rotary vacuum evaporator (Heidolph Instrument GmbH & Co.KG. Schwabach, Germany) and the mixtures were stored at -20°C for subsequent analysis. All reactions were performed triplicate and average values were reported.

3.4 Acidolysis Reaction in Packed Bed Reactor System

3.4.1 Reactor design

Acidolysis reactions were carried out on a laboratory scale packed-bed bioreactor similar to the system of Çiftci et al. (2009). Figure 3.1 shows the process diagram of the reaction system. A jacketed glass column (Ildam, Turkey) was used to form the bed (i.d. 10 mm x length 100 mm). The column was packed with specified amount of Lipozyme IM and the upper and the lower ends of the column were layered with glass wool. The column temperature was maintained by a circulating water bath.

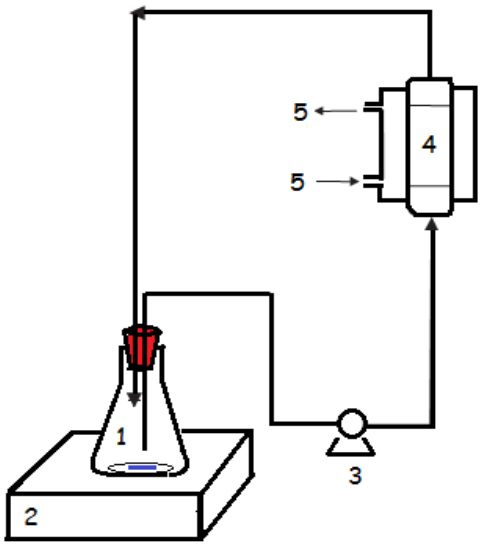

Figure 3.1. Immobilized lipase packed-bed reactor (1) substrate reservoir; (2) reactor temperature control (hot plate/magnetic stirrer); (3) peristaltic pump; (4) bed of immobilized lipase; (5) enzyme bed heating water

The substrate mixture were preheated to the reaction temperature in a reservoir and maintained at that temperature during the reaction on a hot plate / stirrer. The reaction mixture was pumped upward through the column using a peristaltic pump (Watson Marlow Bredel, model 505U, Falmouth, UK). The system was operated with product recirculation modes. The reaction mixture that leaves the bed directed into the substrate reservoir.

***Determination of the bed void fraction* (ε)**

Substrates were fed slowly into the newly packed column at room temperature. The column was gently shaken to remove bubbles in the liquid until the enzyme bed was completely filled with the substrates. The volume of the substrates (Vs) was measured and the volume of the enzyme bed (V) was calculated from the diameter of the column and length of the bed. The void fraction was then calculated as $\varepsilon = Vs/V$.

Determination of residence time in the bioreactor

The residence time was calculated as $V \times \varepsilon/V_f$, where V is the enzyme bed volume, ε is the void fraction, and V_f is the flow rate.

3.4.2 Acidolysis reaction

Terebinth fruit oil (1 mmol, MW: 864) and the corresponding ratio of caprylic and palmitic acids were mixed in 100 ml of *n*-hexane to prepare reaction mixture. The set values for enzyme load (wt%), substrate ratio, time and flow rate was set according to an experimental design (Table 4.4). Prepared reaction mixture was placed in 500-ml Erlenmeyer flask with silicone-capped stoppers. Then acidolysis was carried out by recirculation of the reaction mixture through the enzyme bed at the set variables. The reservoir content was continuously agitated at 200 rpm during the reaction by a magnetic bar. The system temperature was maintained at specific temperature (45 ^{o}C) by circulating hot water. Temperature of the reaction mixture in the substrate reservoir was preheated to the reaction temperature and also maintained at that temperature during the reaction on a hot plate/magnetic stirrer. This optimized reaction temperature was based on our previous study (Koçak et al., 2011). A new enzyme bed was used for each experimental point. At the end of the reaction *n*-hexane was immediately evaporated in a rotary vacuum evaporator (Heidolph Instrument GmbH & Co.KG. Schwabach, Germany) and the mixtures were stored at

-20°C for subsequent analysis. All reactions were performed triplicate and average values were reported.

3.5 Experimental Design and Optimization by Response Surface Methodology

A five-level, four factorial central composite rotatable design (CCRD) was employed to study the response patterns and to determine the optimum combination of variables. CCRD was composed of 30 experiments consisting of 16 axial points, 8 star points, and 6 center points (Table 4.4). The star points provide estimation of curvature of the models. Six replicate runs at the center point of the design were performed to allow the estimation of pure error. All 30 runs were performed in a totally random order to avoid bias.

The independent variables and their levels selected were as follows for STR system: reaction time (Ti; 12-20 h), enzyme load (En; 10-20 wt%), reaction temperature (Te; 45-60°C) and substrate mole ratios (Sb; 1:1.15-2.05). Although the substrate mole ratio of TO:FA varied from 1:1.15 to 1:2.05, the mole ratio of TO:PA:CA in the reaction mixtures varied from 1:2.3:1.15 to 1:4.1:2.05 due to the ratio of PA:CA which has been fixed as 2:1 with preliminary studies. These upper and lower limits of substrate ratio were generated by RSM design according to the defined star points. The minimum star point for substrate mole ratio was chosen as 0.7 due to prevent limiting substrate mole ratio of 2.0. In that case, the minimum mole ratio of TO:PA:CA was 1:1.4:0.7 and the amount of available substrate was 2.1 mole to bind in place of cleaved fatty acids.

The independent variables and their levels selected were as follows for backed bed reactor system: reaction time (Ti; 3.5-6.5 h), enzyme load (En; 10-20 wt%), substrate flow rate (Q: 4-8 ml/min) and substrate mole ratios (Sb; TO:FA, 1:1.22 –2.07). Although the substrate mole ratio of TO:FA varied from 1:1.22 to 1:2.07, the mole ratio of TO:PA:CA in the reaction mixtures varied from 1:1.83:1.22 to 1:3.07:2.05 due to the ratio of PA:CA which has been fixed as 1.5:1 with preliminary studies. This upper and lower limit of substrate ratio was generated by RSM design according to the defined star point. The minimum star point for substrate mole ratio was chosen as 0.8 due to prevent limiting substrate mole ratio of 2.0. In that case, the minimum mole ratio of TO:PA:CA was 1:1.2:0.8, there is 2 mole of substrate available to bind place of cleaved fatty acids.

3.6 Statistical Analysis

The experimental data obtained were fitted to a quadratic response surface model equation:

$$Y_{1,2} = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} B_{ii} {X_i}^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} B_{ij} X_i X_j + \varepsilon \qquad (1)$$

where Y_1 is the response for weight percent of total produced TAGs (CLC, COC, CLL, COL, PLC, COO, POC, OLLn, PLP, POP, PPP), Y_2 is the response for weight percent of desired TAGs (POP, POC, COC, CLC, PLP, PLC, and PPP). β_0, β_i, β_{ii}, β_{ij} were constant coefficients of intercept, linear, quadratic and interaction terms, respectively, x_i and x_j are independent variables and ε is the random error. The first- or second-order coefficients were generated by regression analysis with backward elimination.

The data from the experiments performed were analyzed using RSM (Stat-Ease, Design-Expert software, version 7). ANOVA, regression analysis, and model generation were used to evaluate the effects of factors and to optimize reaction conditions. The level of significance for model was set as 99% confidence level and all other tests were set at 95% confidence level. The goodness of the models established were determined using coefficient of determination, R^2, together with the absolute average deviation values and ANOVA (Arifin et al., 2010).

Solid fat content comparisons for produced SL and commercially available margarines were made using one-way analysis of variance and Duncan's test for multiple comparisons was used for all post hoc analyses. $P < 0.05$ was considered significant. The SPSS Statics 15.0, version 2.0 (2006), (SPSS Inc., Chicago) was used.

3.7 Methods of Analysis

3.7.1 Determination of chemical and physical properties of terebinth fruit oil

Some chemical and physical properties (free fatty acid content, peroxide value, fatty acid composition, triacylglycerols composition, total phenolic content, antioxidant

capacitiy and color of oil) were determined. Molecular weight of oil was calculated from the percentage of TAGs in oil composition.

Free fatty acid content and peroxide value were analyzed according to AOCS Official Method Ca 5a-40 (AOCS, 1989) and Cd 8-53 (AOCS, 1997), respectively. The color of extracted oil was measured by a Hunter-Lab ColorFlex, A60-1010-615 model colormeter. The L*a*b* color space was used to express the color.

3.7.1.1 Extraction and determination of total phenol content

Phenols were extracted following the method proposed by Kalantzakis et al. (2006). Five grams of terebinth oil were dissolved in 10 mL n-hexane to remove oil, extracted with 10 mL of a methanol:water mixture (60:40, v/v) and then shaken vigorously by means of a vortex and centrifuged at 3,500 rpm for 10 min.

The total phenols content (TPC) of the extracts from the method used above were determined using Folin–Ciocalteau reaction at 725 nm. Two hundred microliters of phenol extract were diluted with water to a total volume of 5 mL, followed by the addition of 0.5 mL Folin–Ciocal-teau reagent. After 3 min, 1.0 mL of sodium carbonate solution (35%, w/v) was added, mixed, and diluted with water to 10 mL. The mixture allowed standing for 1 h. The absorbance of the solution was measured after 1 h against a blank sample by UV–vis spectrophotometer at 725 nm. The results were expressed in mg caffeic acid/kg oil.

3.7.1.2 Determination of antiradical activity

The antiradical activity was measured by DPPH method in total terebinth oil samples and in methanol:water extracts from oil samples. Determination of antiradical activity of methanol/water extract used the following method. The extract solution (0.5 mL) was added to 3 mL of a 0.1 mM methanolic solution of DPPH and vortex-mixed. After 30 min, the absorbance was measured at 515 nm (Kıralan, 2009) and antiradical action toward DPPH radical was estimated from the difference in absorbance with or without sample (control) and the percent of inhibition was calculated from the following equation:

$$\%\ Inhibition = \left[\frac{(Absorbance\ of\ control - absorbance\ of\ test\ sample)}{Absorbance\ of\ control}\right] \times 100$$

Antiradical activities in total terebinth oil samples were determined as follows; One milliliter of oil solution (10% w/v) was added to 4 mL of a freshly prepared DPPH solution (0.1 mM) in a 20 mL test tube, which was immediately closed and vigorously mixed for 10 s in a vortex apparatus. Ethyl acetate was used as a solvent. Absorbance of the mixture was measured after 30 min at 515 nm and the percent of inhibition was calculated using above formula.

3.7.1.3 Analysis of fatty acid composition

The fatty acid composition of samples was determined after converting fatty acids into corresponding fatty acid methyl esters (FAME). After methylation, the fatty acid composition was determined with a Shimadzu GC17A gas chromatograph equipped with a flame ionization detector and a BPX capillary column (30 m × 0.22 mm × 0.25 µm film thicknesses). The temperatures of the injector and detector were set at 225 and 250 °C, respectively. The oven was heated to 60 °C for 1 min, then the temperature was increased to 170 °C at a rate of 10 °C/min and then from 170 to 230 °C at a rate of 3 °C/min and held at this temperature for 15 min. Nitrogen was used as a carrier gas, flowing at a rate of 1 ml/min. FAMEs were identified by comparison with relative retention times of standard mixtures.

Fatty acid composition at the *sn*-2 position was determined using the method developed by Brockerhoff (1965). The oil was hydrolysed with porcine pancreatic lipase, a lipase selective for *sn*-1,3 positions of TAGs. The products of lipolysis were separated by TLC plates that were developed with petroleum ether: diethyl ether: acetic acid (70:30:1, by volume) The band corresponding to *sn*-2 monoacylglycerol was scraped off and was extracted with diethyl ether and methylated for GC analysis as described above.

3.7.2 Analysis of triacylglycerol composition

3.7.2.1 Isolation of triacylglycerols

Neutralization

The produced TAGs were isolated in two steps. Firstly, the mixture obtained from the reaction was neutralized to remove free fatty acids by using the similar method as Çiftçi et al. (2009) with some modification. Reaction mixture (6-8 g) was mixed in 150 mL *n*-hexane and 1 mL phenolphtalein solution, and then required amount of 0.5

N KOH in 20 % (vol) ethanol (B mL) was added until the pink color was observed (neutralized). Then the mixture was transferred into a separatory funnel. The separatory funnel was shaken, and the upper phase was collected. Lower phase was washed again with 50 mL *n*-hexane and upper phase was collected. Then, 2.66*B mL of 0.5 N KOH in 20 % (vol) ethanol and 1.33*B mL of saturated NaCl solution were added to collected upper phase. After shaking, the upper phase was collected and *n*-hexane was evaporated by rotary vacuum evaporator to obtain the neutralized product containing TAGs, DAGs, and MAGs. The neutralized product was centrifuged in case of presence of soap traces. Then, the TAGs of neutralized product were separated from MAGs and DAGs by column chromatography as described below.

Purification by column chromatography

The TAGs of the neutralized product were separated from MAGs and DAGs by mini column chromatography on silica gel (SG 60, 70–230 mesh, Merck). 0.5- 1 g of the neutralized product was dissolved in 8 ml of elution solvent (light petroleum ether / diethyl ether, 90:10, v/v) and eluted through the mini silica column with elution solvent. Then the purified reaction product was obtained by evaporating the solvent (Dobarganes et al., 2000).

3.7.2.2 HPLC analysis

The TAG compositions of terebinth fruit oil and reaction product were determined by reversed phase HPLC using the method proposed by AOCS Official Method Ce 5b-89 (AOCS, 1993). The analyses were carried out isocratically with a mobile phase consisting of 64:36 (v/v) acetone/acetonitrile. Oil was diluted in acetone, filtered and auto-injected into the column (Supelcosil LC-18-DB, 5 μm, 250 x 4.6 mm; Supelco, USA), with an accompanying guard column (Supelguard LC-18-DB, Supelco, USA). It was eluted at a flow rate of 1.0 ml/min. The column temperature was set at 30°C and elution was monitored with a Schimadzu LC RID-10A refractive index detector. The total analysis time was 36 min. All triacylglycerol contents were expressed as weight percent of the total weight of the sample. All analyses were performed in triplicates, and average values were reported. Peak identification was performed by comparing the retention times of sample TAGs with those of TAG standards. Since there are no standards available for some new synthesized TAGs,

these were produced and isolated in our laboratory and were used in peak identification.

3.7.3 Separation of fat phase of margarine

10-15 g margarine was incubated at 55°C for 40 min in a separatory funnel. The upper phase was separated and used in DSC analysis.

3.7.4 Determination of thermal characteristics: SFC and melting profile

The melting profiles and solid fat content (SFC) of margarine fats and structured lipids were analyzed by DSC (Perkin Elmer DSC-6, Norwalk, CN, USA). The DSC instrument was calibrated with indium (m.p. 156.6°C, ΔHf = 28.45 J/g). Nitrogen was used as purge gas and flowed at 40 ml/min. A sample was completely melted at 80°C before being weighed (5-10 mg) into an aluminum pan which was then sealed. An empty, hermetically sealed aluminum pan was used as reference. The previous thermal history of the sample was erased by heating the sample to 80°C in the DSC instrument and holding it for 10 min. The sample was then cooled to -60°C at a rate of 40 °C/min and waited at -60°C for 10 min. At the end of the cooling, the sample was heated at 5°C/min to 80°C. The SFC was calculated at various temperatures from the DSC heating thermogram data by partial integration according to Tieko and Aparecida (1995). All DSC values reported are the average of two scans.

3.7.5 Microstructure

Microstructure of the SLs, terebinth fruit oil and commercially available margarines were examined by using a polarized light microscope (PLM) (Olympus BX51, Olympus Optical Co., Ltd., Tokyo, Japan) equipped with a Pixera color video camera (model PVC 100C, Los Gatos, CA, USA). A crystallization method similar to the method of Ahmadi et al. (2008) was used. Samples were molten at 80 ^{o}C for 15 min in order to erase the crystal memory and 20 µL of melt placed on a glass microscope slide which was heated to the same temperature. A cover slip at the same temperature of the sample was then gently laid over the fat drop to remove air and spread the fat. Samples were then allowed to crystallize for 48 h at room temperature (21–23 ^{o}C). Images were captured under polarized light with 40X magnification on the grayscale.

3.7.6 Calculation of caloric value

The theoretical energy values of TO and produced SLs were calculated using the approach of Livesey (1984). The energy values for the FA were estimated by calculation using the method proposed by Taguchi et al. (2001).

$$-\Delta H_c(fatty\ acid) = 0.653n - 0.166d - 0.421 \quad (2)$$

where $-\Delta H_c$ is the heat of combustion in MJ/mol, n is the number of carbon atoms/molecule of fatty acid, and d is the number of double bonds/FA. The energy value that was used for glycerol was 18.0 kJ/g. The heat of esterification was neglected because it amounts to only 3.8 kJ per mole of ester bond as determined with methyl stearate. In case of tristearin, this value corresponds to 13 J/g.

Then the heat of combustion of each fatty acid was used to calculate the heat of combustion of each produced TAG according to the equation:

$$-\Delta H_c(triacylglycerol) = 1.66 \sum_{FA_1}^{FA_\infty} \frac{B'}{3D} + \sum_{FA_1}^{FA_\infty} \frac{B'}{D} I \quad (3)$$

in which B' is g fatty acid /100 g TAG total fatty acid, D is molecular weight of the corresponding fatty acid and I is -ΔHc of the corresponding fatty acid. The constant 1.66 is heat of combustion (MJ/mol) for glycerol. -ΔHc*(triacylglycerol)* is the potential metabolizableenergy of the TAG. To give -ΔHc*(triacylglycerol)* in MJ/gTAG, -ΔHc*(triacylglycerol)* was divided by the molecular weight of the triacylglycerol per grams of fatty acids in 100 g TAG. Caloric values of SLs produced for each run were calculated according to their TAG composition.

3.7.7 Determination of oxidative stability

The oxidative stability of terebinth oil, produced SLs and margarine samples was determined by a Perkin Elmer differential scanning calorimeter (DSC-6, Norwalk, Conn., U.S.A.). Instrument was calibrated with indium standard. Fat samples of 5 ± 0.5 mg were weighed into open aluminum pans, with an empty pan as reference, and placed in the sample chamber of DSC. The isothermal temperature program was programmed at 140 ◦C and oxygen was passed through the sample chamber at 100 mL/min flow rate at atmospheric pressure. Similar to Tan et al. (2002), the oxidation induction time of the oxidation reaction was determined by taking the time value

corresponding closely to the intersection of the extrapolated baseline and the tangent line of the exotherm (Figure 3.2.).

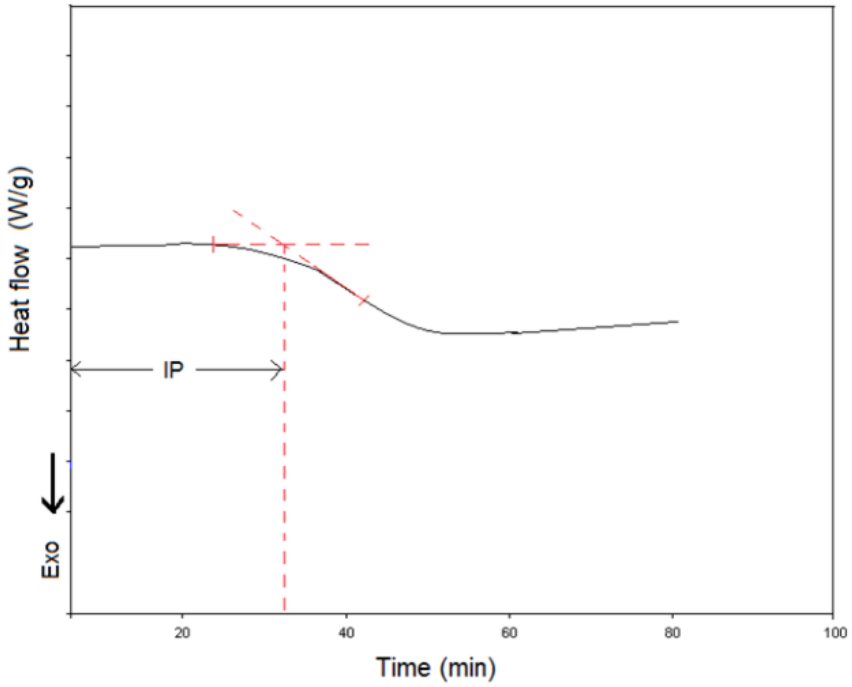

Figure 3.2. Determination of the oxidation induction time for oxidation reaction in DSC

3.7.8 Determination of lipase reusability

Reusability of Lipozyme RM IM in the acidolysis of TO with CA and PA were determined at optimized conditions of STR and PBR system. For both, STR and packed bed reactor systems, after completion of each reaction the enzyme was washed with fresh hexane, dried and reused in the next reaction. The activity was calculated by percentages of desired TAGs in the SLs.

CHAPTER 4

RESULTS & DISCUSSION

4.1 Chemical and Physical Properties of Terebinth Fruit Oil

Terebinth fruit oil was composed of TAGs (96%) in majority and free fatty acids, pigments, mono-diacylglcerols in minor amounts. For the identification of TAGs constituents the TAGs of terebinth oil were separated on RP–HPLC column (Supelcosil LC-18-DB) according to their degree of polarity. The overall polarity of TAGs changes with the number of double bonds in the fatty acid, their position along the triacylglycerol backbone, and the number of carbon atom. The TAGs which has a greater polarity will elute faster from the C18 column while the elution of low polar TAGs takes longer time. The HPLC chromatogram in Figure 4.1 shows the TAGs composition of terebinth fruit oil. Seventeen components with concentrations more than 0.1 % were detected and identified in TAG fractions.

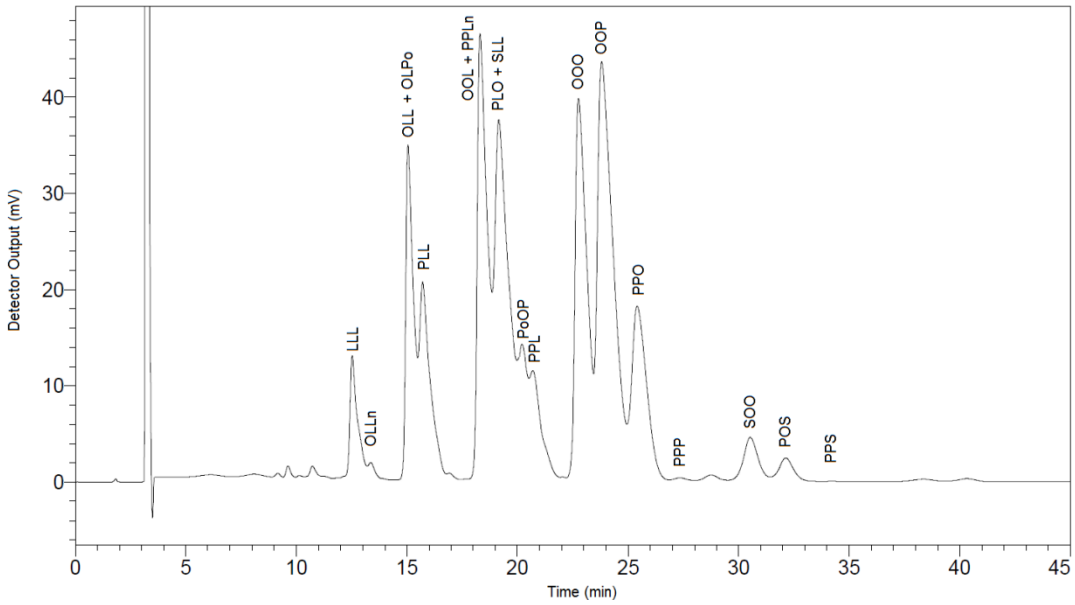

Figure 4.1. The reversed-phase high-performance liquid chromatogram of terebinth fruit oil. Ln, linolenic acid; L, linoleic acid; P, palmitic acid; O, oleic acid; Po, palmitoleic acid: S, stearic acid

Peak identification for some of these TAGs was performed by comparing the retention times of sample TAGs with those of TAG standards (LLL, LLO, LLP, OOO, POP, POO and PPP). These TAGs standards were also used as reference point to compare relative polarity of other TAGs species.

The properties and TAGs with composition (>1%) of terebinth fruit oil are shown in Table 4.1. The TAGs composition is one of the most important chemical characteristic that determines the physical properties of fats and oils (Ciftci et al., 2010). The predominant TAGs are OOP, PLO+SLL and OOO and their percentages are 23.30 %, 15.03% and 13.88%, respectively. According to HPLC analysis, the percentages of TAGs containing three unsaturated FA are around 37 % of the total TAGs. Although the oleic and linoleic acids are the major fatty acids in the TAG composition of sunflower, corn, peanut, soybean, hazelnut, walnut, sesame, olive oil and TO, TAG composition of terebinth fruit oil is quite different than that of sunflower, corn, peanut, soybean, hazelnut, walnut, sesame, olive oil.

Table 4.1. Properties of terebinth fruit oil and TAGs composition of terebinth fruit oil

Property	**Value**
Free fatty acid (% oleic acid)	0.592
Peroxide value (m_{eq} O_2/kg)	1.81
Total Phenols content (mg CA/kg oil)	46.5
Antiradical activity (% inhibition of DPPH)	54.85 %
Color	L^*: 80.04
	a^*: 19.58
	b^*:131.79
Monoacylglycerol+Diacylglycerol (%)	3.39
Molecular weight (g/mol)	864
TAGs (%)	96
TAGs composition (%)	
OOP	23.30
PLO+SLL	15.03
OOO	13.88
OOL+ PPLn	12.52
POP	9.64
OLL+ OLP_O	6.76
PPL	4.34
P_OOP	3.77
PLL	2.68
LLL	1.98
SOO	1.70
POS	1.08
OOP_O	1.80

Ln: linolenic acid, L: linoleic acid, S: stearic acid, P: palmitic acid, O: oleic acid, Po: palmitoleic acid

The fatty acid composition of terebinth fruit oil in TAGs, at *sn*-2 and *sn*-1,3 positions were analysed by GC and given in Table 4.2. Oleic acid (18:1), palmitic acid (16:0)

and linoleic acid (18:2) constituted the majority of the fatty acids with percentages of 54.50%, 21.30% and 16.60%, respectively. Palmitoleic (16:1), stearic (18:0) and linolenic acids (18:3) were seen in minor amounts and myristic acid (14:0), margaric acid (17:0) and eicosenic acid (20:1) were present as traces with a percentage of 0.1%. This result is in agreement with results of Özcan (2004). Terebinth fruit oil is very high in unsaturated (16:1, 18:1, 18:2 and 18:3) fatty acids and their percentages around 75 % of total FAs. The proportions of oleic and linoleic acids in the sn-2 position of the terebinth fruit oil were 67 and 23.6%, respectively. It can be concluded that the sn-2 position was mainly acylated by unsaturated FA. Additionally high oleic content especially in sn-2 position is an added value for this oil due to its health benefits (Mensink and Katan, 1987). Palmitic acid is the second most abundant fatty acid which preferentially esterified at the sn-1and sn-3 positions in the whole TAG.

Table 4.2. Fatty acid composition of terebinth fruit oil

Fatty acid composition			
Fatty Acid	FA % in TAG	FA % in *sn*-2	FA % in *sn*-1,3
14:0	0.10	0.30	-
15:0	-	-	-
16:0	22.60	4.50	31.65
16:1	3.30	2.50	3.70
17:0	0.10	-	0.15
17:1	0.10	0.10	0.10
18:0	2.00	1.20	2.40
18:1	54.50	67.00	48.25
18:2	16.60	23.60	13.10
18:3	0.60	0.70	0.55
20:1	0.10	0.10	0.10

The color parameters, L*, a*, b* for TO was measured as 80.04, 19.58 and 131.79, respectively. This means that TO exhibits a quite bright yellow color. Total phenolic content and antiradical activity for oils show differences with respect to plant cultivar. Total phenol content was found as 46.5 mg caffeic acid/kg oil for terebinth fruit oil. The antiradical activity was measured as % reduction in concentration of DPPH after a 30 min reaction. It was determined as 54.85 %. TPC and antiradical activity for different olive oil samples were determined by **Kıralan et al. (2009)** and reported between 38-495 mg CA/kg oil for phenolic and 70-95 % for radical scavenging activity for different olive cultivar.

4.2. Identification of Unknown Peaks

A preliminary study was performed in order to identify the TAGs molecules which are produced after the acidolysis reaction of terebinth fruit oil with caprylic and palmitic acid. The determination of TAGs constituents of terebinth oil was explained in section 4.2 and most of them were determined by comparing their retention time with TAG standards. Since there is no standard for some new synthesized TAGs (CLC, CLL, COL, COC, COO, POC, PLC) these were produced and isolated in our laboratory and were used in peak identification.

A acidolysis reaction was performed with standard OOO and CA at a mole ratio of 1:2 in n-hexane in order to produce COC and COO. The possible products of this reaction are OOO, COO and COC. The produced TAGs were separated on RP–HPLC column according to their degree of polarity. OOO peak in mixture was identified by comparing the retention time of standard OOO. Then amount of CA was increased (1:6) in reaction system and acidolysis performed again to distinguish which one is COC which one is COO. Following, standard OOO was allowed to react with CA and PA to identify the POC. Similar procedures were followed and retention time for this TAG molecule was determined.

In order to identify the CLC and CLL, acidolysis reaction was performed with standard LLL and caprylic acid at a mole ratio of 1:2 in n-hexane. Standard LLL was used as a reference in this identification. The same procedure explained above for COO and COC was followed to distinguish CLC and CLL. Then LLL was allowed to react with CA and PA to identify PLC.

After the identification of all unknown peaks, these produced TAGs were separated with HPLC and each eluted TAG species were collected in a separate tube. Then those isolated TAGs were added in to the orginal terebinth oil to check their position in overall HPLC chromatogram.

4.3. Production of Low Caloric and Spreadable Lipid

4.3.1. Acidolysis reaction in stirred tank reactor system

Batch system production was achieved in a lab scale STR within the first part of the study. The interesterification reaction of terebinth fruits oil (TO) with caprylic acid (CA) and palmitic acid (PA) was performed. The effect of reaction conditions and

relationship among them were analyzed by response surface methodology (RSM) with a four-factor five-level central composite rotatable experimental design.

4.3.1.1 Preliminary studies

Preliminary study was implemented prior to the RSM work to understand the effect of different PA:CA mole ratios in reaction mixture**.** The levels of other independent variables (reaction temperature, reaction time, and enzyme load) were determined according to the literature survey (Arifin et al., 2010; Shuang et al., 2009; Koh et al., 2008; Zhou et al., 2001; Linder et al., 2005; Senanayake and Shahidi, 2002).

Mole ratio determination

In this preliminary study the mole ratios of palmitic to caprylic acid was studied to understand the effect of different PA:CA mole ratios in reaction mixture**.** For this purpose some preliminary acidolysis reactions were performed and the triacylglycerol content, caloric value and SFC percentages of produced SLs were analyzed. The aim was to get maximum incorporation of FAs and to reach desired melting profile with possible low calorie. First of all, the reaction were performed at constant temperature (45 ^{o}C), enzyme load (10 %) and time (12 h) with changing molar ratio of TO:PA:CA from 1:1:1 to 1:6:6. Figure 4.2 shows the change of percentages of produced desired TAGs within these mole ratios. Desired TAGs mean TAGs which are produced with incorporation of both PA and CA into the terebinth fruit oil. These are POP, POC, COC, CLC, PLP, PPP, PLC.

As seen in Figure 4.2 the contents of target TAGs increased with increasing substrate mole ratio. Among the studied substrate mole ratios (1:1:1, 1:2:2, 1:3:3, 1:4:4, 1:5:5, 1:6:6) sharp increase was observed from 1:1:1 to 1:2:2. Further increase in substrate mole ratio did not cause a significant effect on target TAG species. Therefore the ratio 1:2:2 was obtained as optimum between these studied ranges. Other than that, high substrate ratios are economically not feasible, because purification of the products would require a cost increasing extra separation steps.

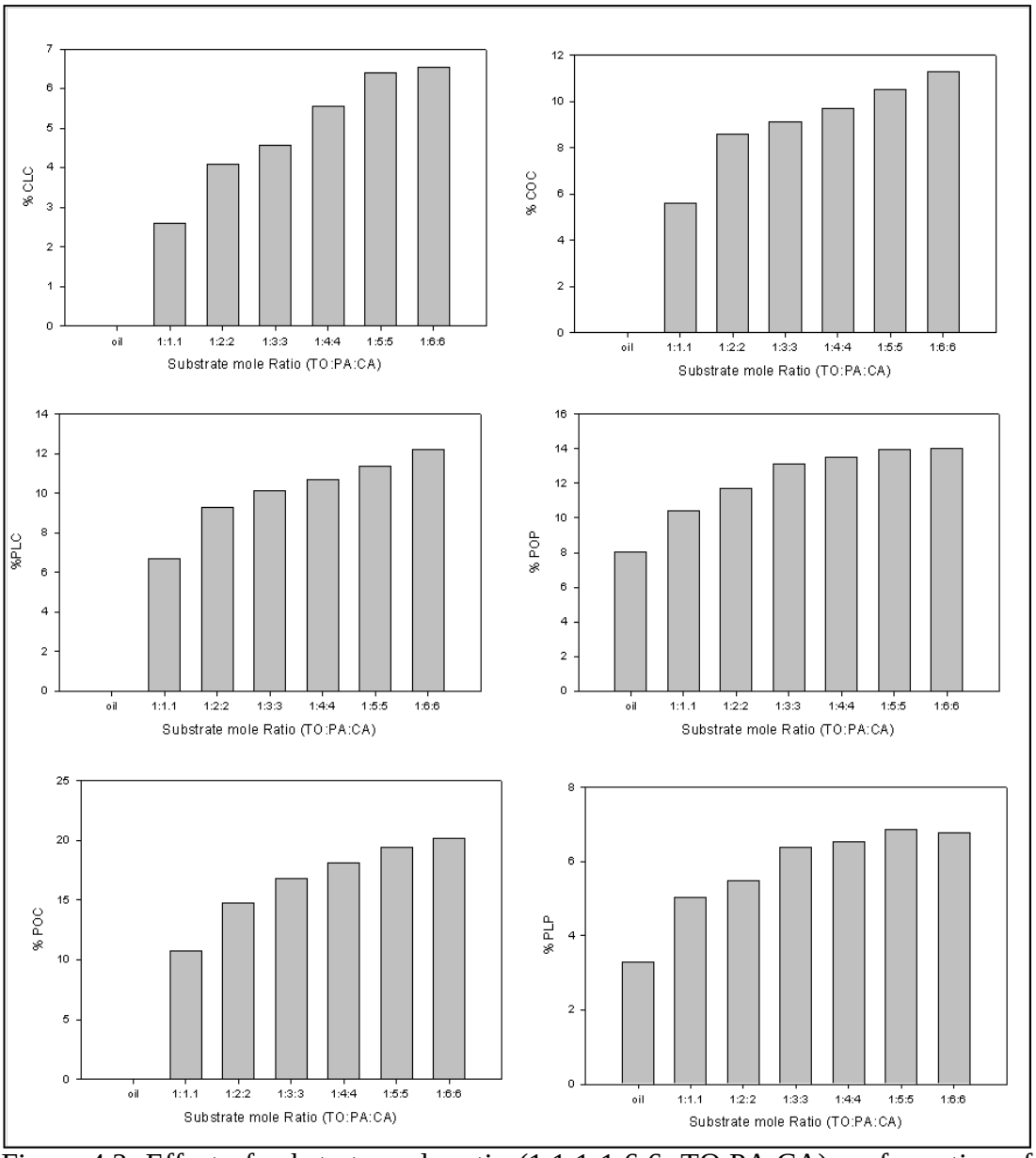

Figure 4.2. Effect of substrate mole ratio (1:1:1-1:6:6; TO:PA:CA) on formation of desired TAGs (POP, POC, COC, CLC, PLP, PLC and PPP)

After that the upper and lower mole ratios for PA:CA in 1:2:2 combination were studied. Figure 4.3 shows the changes in desired TAGs among these studied mole ratios (1:2:1, 1:2:1.5, 1:2:2, 1:1:2, 1:1.5:2). It has been observed that higher PA in ratio (1:2:1, 1:2:1.5) caused to increase in conversion of TAGs into POP and PLP in which PA incorporated to both side of TAGs. On the other hand increasing the mole of CA in ratio increased the conversion of TAGs into CLC, COC. But the study has been focused to produce a SL which contains all of these produced TAGs to reach the ultimate purpose of having low calorie and spreadability. From this view point the mole ratio 1:2:1 seems to be best combination to get maximum incorporation of both PA and CA. The SFC of the upper and lower mole ratios of 1:2:2 combination

has also been investigated to decide whether the produced SLs had desired thermal properties or not. The SFC of produced SLs was compared with some margarine samples purchased from local supermarkets.

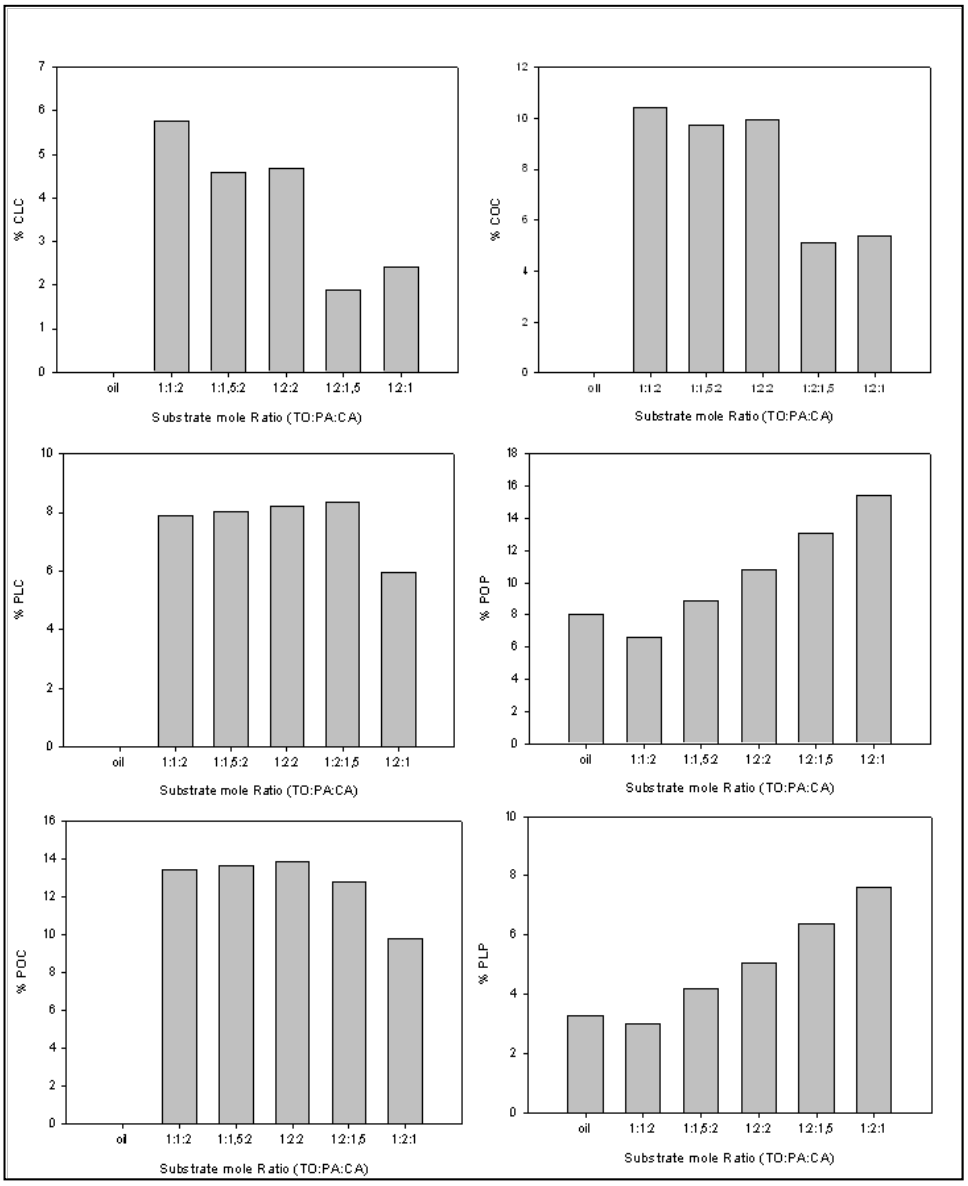

Figure 4.3. Effect of substrate mole ratio (1:2:1,1:2:1.5, 1:2:2, 1:1:2, 1:1.5:2; TO:PA:CA) on formation of desired TAGs (POP, POC, COC, CLC, PLP, PLC and PPP)

Table 4.3 shows the SFC of produced SL, fat extracts of margarines and TO at different temperature. They were compared to decide what should be the ratio of PA to CA prior to the experimental design. The SL obtained at 1:2:1 substrate mole ratio gave similar SFC with soft type margarines (Table 4.3). The theoretical caloric values were calculated as 38.76, 38.89, 39.01 and 39.08 (kj/g) for 1:1:2, 1:2:2, 1:2:1 and 1:1.5:1, respectively. Although the 1:2:1 ratio for TO:PA:CA gave the lowest

energy, it was not proper for spreadability. Therefore when the SFC %, maximum incorporation of PA and CA, and calorie value were considered the ratio of PA:CA was fixed as 2:1 and this ratio was kept constant in experimental design by RSM.

Table 4.3. Solid fat content of structured lipids and fat extracts of commercial margarines

Samples	SFC[a]			
	Temperature (^{o}C)			
	10	21	25	33
TO[b]	26.03	0.42	0	0
SL[c](1:1:2)	18.90	2.34	0.65	0
SL[c](1:2:2)	38.98	16.15	5.72	0.04
SL[c](1:2:1)	54.37	28.00	14.74	2.61
SL[c](1:1.5:1)	40.01	15.74	7.88	0.76
A[d]	25.82	22.34	17.96	8.52
B[e]	24.88	19.32	14.73	6.27
C[f]	24.69	20.34	16.12	6.27
D[g]	33.88	26.50	21.11	11.23
E[h]	69.13	53.31	41.26	18.75
F[i]	77.01	52.68	34.47	9.09

[a] Solid fat content; [b] Terebinth fruit oil; [c] Structured lipids produced at various substrate mole ratios; [d,e,f,g] Fat extracts of soft margarines; [h,i] fat extracts of hard margarines

4.3.1.2 Experimental design and model fitting

Modeling and optimization of the acidolysis reactions was performed by RSM, a four-factor five-level central composite rotatable experimental design was used for this purposes. The four major factors chosen were enzyme load (10-20 wt %), reaction time (12-20 h), reaction temperature (45-60 ^{o}C) and substrate mol ratio (TO:PA:CA, 1:2.3-4.1:1.15–2.05). Experiments were conducted under the designed conditions and responses were obtained. RSM was implemented to model the three responses, namely, total produced TAGs (%), desired TAGs (%) and energy (kj/g). Table 4.4 lists the settings of factors and responses. The best fitting quadratic model was determined by regression and backward elimination by means of elimination of insignificant factors and interactions in the models. Similar model was reported by several authors who determined the optimization of lipase-catalyzed acidolysis reactions (Carrín and Crapiste, 2008; Koh et al., 2008; Sharma et al., 2009; Foresti and Ferreira, 2010). The model coefficients and probability values were given in Table 4.5. It is seen that some linear, quadratic or interaction terms were not eliminated by backward elimination to maintain the hierarchy of the model although they were statistically insignificant. Presence of insignificant interaction terms in Table 4.5 were the reason of this elimination principle. The models predicted for all responses were significant at the 99% confidence level and showed statistically

insignificant ($P<0.05$) lack of fit with high coefficients of determinations (R^2) between 0.95 and 0.99.

Table 4.4. Treatment schedule for four-variable five-level CCRD and experimental data for acidolysis of terebinth fruit oil with caprylic and palmitic acids

Run	Level of variables				Total Produced	Desired	Energy	SFC[e]
	Sb[a]	En[b]	Te[c]	Ti[d]	TAGs (%)	TAGs (%)	(kj/g)	(%)
1	1.15	10	45	12	48.72±0.53[f]	38.79±0.07	39.07±0.01	17.25±0.46
2	2.05	10	45	12	60.29±0.06	49.69±0.13	38.95±0.01	13.24±0.11
3	1.15	20	45	12	49.17±0.54	34.84±0.09	39.08±0.0	31.11±0.23
4	2.05	20	45	12	61.51±0.34	45.15±0.40	38.94±0.01	39.68±0.82
5	1.15	10	60	12	49.88±0.22	37.19±0.07	39.08±0.01	25.65±0.26
6	2.05	10	60	12	62.66±0.24	47.59±0.25	38.95±0.01	40.98±0.37
7	1.15	20	60	12	51.79±0.14	33.04±0.33	39.08±0.01	46.03±0.51
8	2.05	20	60	12	63.57±0.33	39.22±0.42	39.02±0.02	54.37±0.70
9	1.15	10	45	20	49.28±0.28	36.34±0.80	39.09±0.01	26.76±0.18
10	2.05	10	45	20	61.32±0.08	47.56±0.57	38.94±0.01	26.26±0.09
11	1.15	20	45	20	51.51±0.44	33.29±0.07	39.09±0.02	39.32±0.27
12	2.05	20	45	20	63.39±0.26	40.97±0.60	38.93±0.01	43.90±0.90
13	1.15	10	60	20	51.01±0.21	34.60±0.14	39.10±0.00	39.81±0.36
14	2.05	10	60	20	63.68±0.55	42.32±0.38	38.98±0.01	40.05±0.52
15	1.15	20	60	20	56.01±0.33	33.35±0.07	39.08±0.01	60.80±1.10
16	2.05	20	60	20	68.33±0.92	37.92±0.23	38.96±0.01	60.02±0.70
17	0.70	15	52.5	16	37.37±0.24	25.07±0.37	39.22±0.01	29.83±0.21
18	2.50	15	52.5	16	65.18±0.31	44.47±0.17	38.93±0.00	49.98±0.82
19	1.60	5	52.5	16	54.80±0.09	45.39±0.42	39.02±0.01	20.81±0.36
20	1.60	25	52.5	16	60.09±0.21	36.81±0.52	39.01±0.01	58.39±0.63
21	1.60	15	37.5	16	56.02±0.47	43.38±0.60	39.00±0.01	32.45±0.38
22	1.60	15	67.5	16	60.66±0.42	36.59±0.90	39.03±0.00	61.16±0.53
23	1.60	15	52.5	8	54.75±0.16	42.85±0.14	39.00±0.00	24.46±0.26
24	1.60	15	52.5	24	59.77±0.17	37.38±0.31	39.02±0.01	52.83±0.70
25	1.60	15	52.5	16	57.77±0.28	39.24±0.22	39.02±0.00	36.82±0.18
26	1.60	15	52.5	16	57.50±0.13	39.71±0.47	39.02±0.00	41.01±0.30
27	1.60	15	52.5	16	57.69±0.15	40.26±0.33	39.01±0.02	38.91±0.05
28	1.60	15	52.5	16	57.15±0.21	40.13±0.53	39.03±0.01	37.80±0.17
29	1.60	15	52.5	16	57.75±0.37	39.90±0.27	39.01±0.02	40.80±0.32
30	1.60	15	52.5	16	58.47±0.08	39.13±0.25	39.04±0.00	36.50±0.26

[a] Substrate ratio (mol/mol; Terebinth oil : Fatty acids, 1:1.15-2.05); [b] Enzyme load (wt.%, based on the amount of substrates); [c] Reaction temperature (^oC); [d] Reaction time (h); [e] Solid fat content at 25 ^oC; [f] Mean ± SD, n=3

4.3.1.3 Effects of reaction parameters

Yield of the Total Produced TAGs

According to the results of statistical analysis performed; the enzyme load, temperature, time and substrate mole ratio were significant ($P<0.0001$) and had positive effect on TAGs yield (Table 4.5). Substrate mole ratio had the most significant effect while the other variables had relatively small effects. Similar results were reported by Zhou et al., (2001); Lumor and Akoh (2005) in their lipozyme-catalyzed esterification reaction. All the interactions were found not significant except the interaction of En and Ti. In addition, the second order of substrate mole ratio was found significant. The negative coefficient of second order of substrate mole ratio implies that this variable has optimum value within the studied range.

To evaluate the effect of the independent variables three dimensional response surface plots were constructed by varying two variables and holding others at middle level. Figure 4.4 shows the effects of the experimental factors on total TAGs yield and the interactions between the independent variables. The trends of response surface plots were in parallel with the results of model coefficient. The increasing enzyme load, substrate mole ratio, time and temperature improved the incorporation. As expected, the incorporation increased with increasing mole ratio until the reaction reaches equilibrium. Figure 4.4e shows the increased effect of enzyme load when the time interaction is considered on yield of TAGs or vice versa.

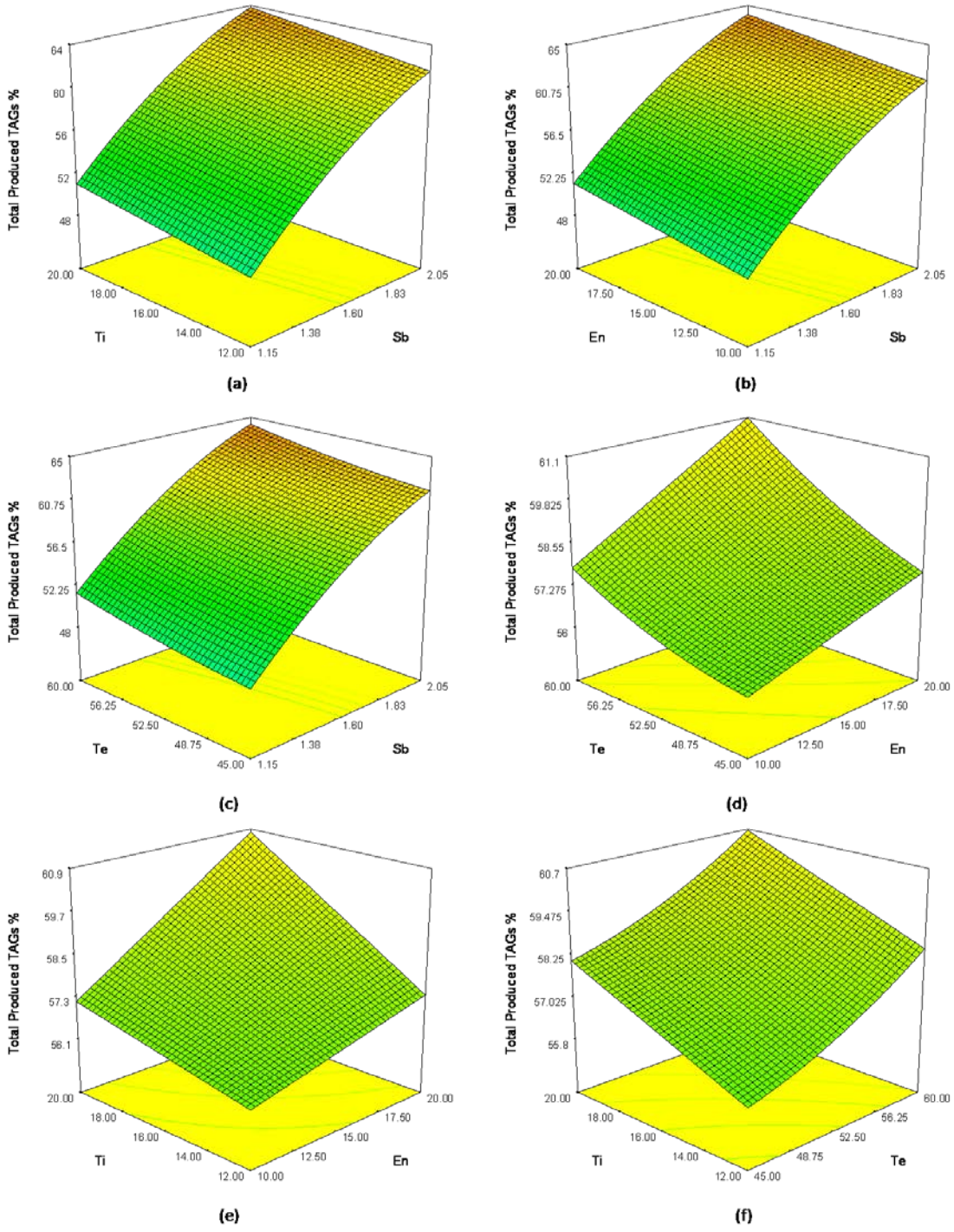

Figure 4.4. Response surface plots for percentage of total produced TAGs: (a) time vs. substrate molar ratio; (b) enzyme load vs. substrate molar ratio; (c) temperature vs. substrate molar ratio; (d) temperature vs. enzyme load; (e) time vs. enzyme load (f) time vs. temperature

Yield of the desired produced TAGs

Desired TAGs are the TAGs produced by the incorporation of both palmitic and caprylic acids in sn-1,3 position of original TAG species of terebinth oil. These TAGs are POP, POC, COC, CLC, PLP, PLC, and PPP. The coefficients and *P*-values for the yield of desired TAGs were given in Table 4.5. Results of ANOVA test indicate that only substrate mole ratio had positive effect and it was the most significant factor as it was in the case of production of total TAGs. The negative and small coefficients for other parameters (time, temperature and enzyme load) mean that they were inversely and less affected. The coefficients of some of the interactions (Sb*En; Sb*Te) were negative and significant in the model of response. Moreover, the second order of substrate mole ratio and enzyme load was also found significant.

Figure 4.5 shows the response surface plots for the production of desired TAGs. Desired TAGs increased with an increase of Sb. Interestingly, the response surface plots show that Ti, Te, and En hinder the yield of desired TAGs in contrast to that of produced total TAGs. Therefore when the desired TAGs are the goal, being high Ti, Te and En seem to be undesirable. The possible reason of this is the effective production of other TAGs such as OLO, COL, COO and PPO. Production of these TAGs could be explained with re-esterification of some released *sn*-1,3 fatty acids. They are probably palmitic, oleic and linoleic acids which are originally present at *sn*-1,3 position of TO. Therefore dominantly increasing amount of such TAGs in the percentage of total TAGs caused to cover up the increase in the amount of desired TAGs. Figure 4.5b and 4.5c show the interactions between Sb*En and Sb*Te, respectively. The interaction between them also had negative effect on the yield of desired TAGs.

Table 4.5. Estimated coefficient for the fitted second order polynomial representing the relationship between the response and process variables

Total Produced TAGs				Desired TAGs			
Variables	Estimated Coefficients	Standard error	*P*- value Prob>F	Variables	Estimated Coefficients	Standard error	*P*-value Prob>F
Sbstrate Ratio (Sb)	6.374	0.166	< 0.0001[a]	Sbstrate Ratio (Sb)	4.491	0.165	< 0.0001[a]
Enzyme Load % (En)	1.209	0.166	< 0.0001[a]	Enzyme Load % (En)	-2.229	0.165	< 0.0001[a]
Temperature (Te)	1.293	0.166	< 0.0001[a]	Temperature (Te)	-1.457	0.165	< 0.0001[a]
Time (Ti)	1.124	0.166	< 0.0001[a]	Time (Ti)	-1.254	0.165	< 0.0001[a]
En*Te	0.406	0.203	0.0584[b]	Sb*En	-0.720	0.203	0.0021[c]
En*Ti	0.592	0.203	0.0082[c]	Sb*Te	-0.703	0.203	0.0026[c]
Sb^2	-1.474	0.152	< 0.0001[a]	Sb*Ti	-0.412	0.203	0.0560[b]
Te^2	0.294	0.152	0.0672[b]	En*Ti	0.357	0.203	0.0939[b]
				Sb^2	-1.197	0.152	< 0.0001[a]
				En^2	0.385	0.152	0.0201[d]

[a] Significant at 0.001; [b] Not significant even at 0.05; [c] Significant at 0.01; [d] Significant at 0.05

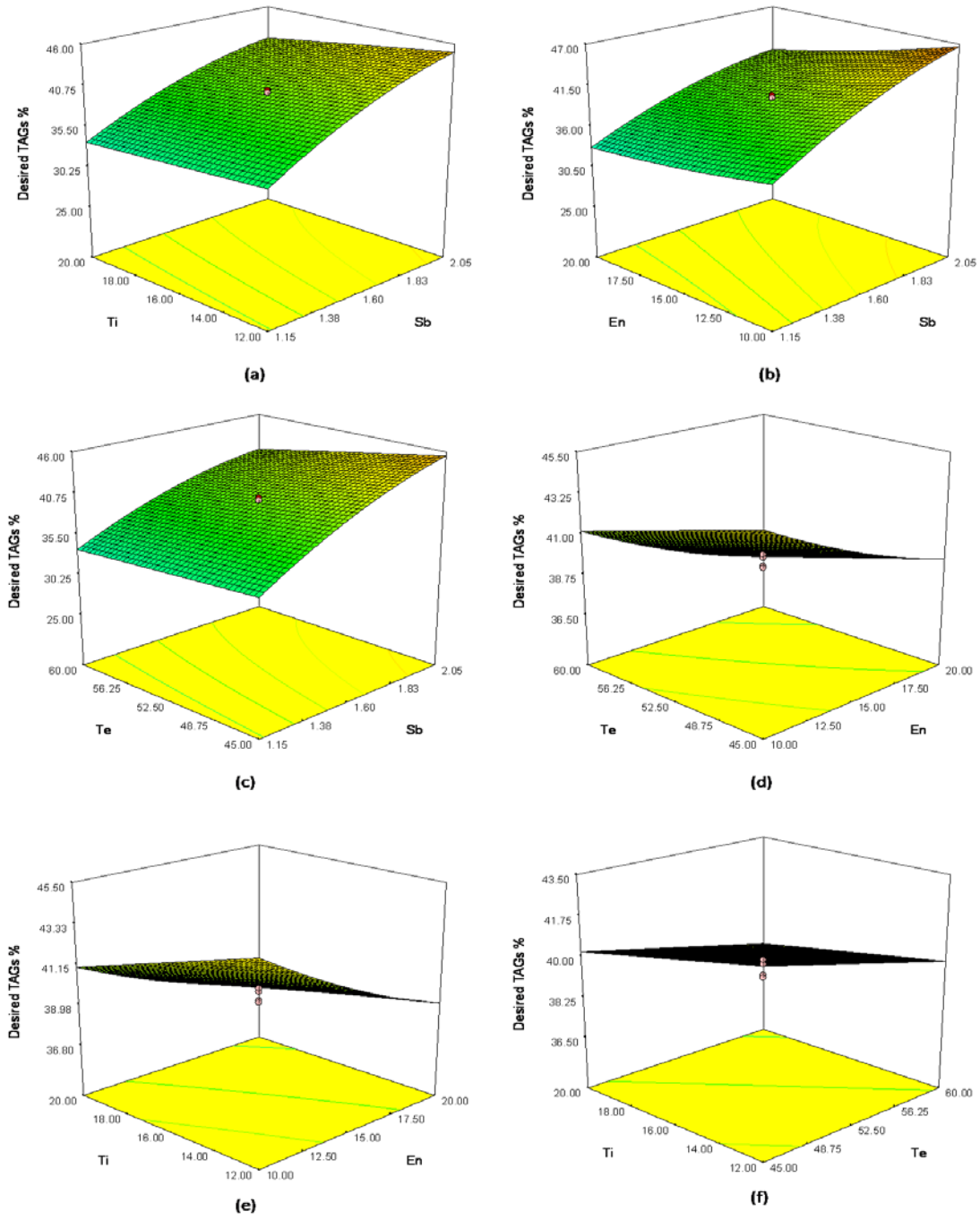

Figure 4.5. Response surface plots for percentage of produced desired TAGs: (a) time vs. substrate molar ratio; (b) enzyme load vs. substrate molar ratio; (c) temperature vs. substrate molar ratio; (d) temperature vs. enzyme load; (e) time vs. enzyme load (f) time vs. temperature

As a consequence, increased value of temperature, time, enzyme load and substrate mole ratio would be more convenient for the highest yield of produced TAGs in the studied range. However, the highest yield for desired TAGs was observed at the

lower values of given parameters except substrate mole ratio. So optimization has been performed in order to decide optimum reaction conditions for obtaining target structured lipid.

4.3.1.4 Optimization of reaction parameters

Optimization function of the Design Expert Software was successfully applied in order to optimize the reaction conditions for the lipase catalyzed acidolysis reaction of TO with CA and PA. Evaluation of more than one parameter is a general case to get desired products in such optimization processes. It has been needed also to use more than one parameter to get target product in our study. However, increasing the amount of variables included decreases the desirability function because it is difficult to find the condition that simultaneously fit all the criteria (Foresti and Ferreira, 2010). So the yield of desired TAGs and caloric value of produced SL were evaluated to produce a target SL from TO. SFC values have not been included in optimization processes even it has been aimed to produce low caloric and spreadable SL in this study. However, SFC values have been evaluated finally for the target product obtained by optimization process.

Fatty acid and TAG compositions of the terebinth fruit oil and optimized SLs are given in Tables 4.6 and 4.7, respectively. The chromatogram of optimized SL is also given in Figure 4.6 to show TAG profile.

Table 4.6. Fatty acid composition of terebinth fruit oil and optimized structured lipids

Fatty acid	Terebith fruit oil (%)	Optimized SL STR (%)	Optimized SL PBR (%)
8:0	-	6.96	7.59
14:0	-	-	-
16:0	21.76	42.51	42.06
16:1	1.43	-	-
17:0	-	-	-
17:1	-	-	-
18:0	1.03	0.41	0.38
18:1	53.49	32.13	32.51
18:2	21.89	17.12	16.88
18:3	0.40	0.87	0.58
20:1	-	-	-

Table 4.7. TAG composition of terebinth fruit oil and optimized structured lipids

TAG	Terebinth fruit oil (%)	Optimized SL STR (%)	Optimized SL PBR (%)
CLC	-	0.74	1.10
COC	-	0.93	2.12
CLL	-	0.95	0.77
COL	-	2.97	2.33
PLC	-	8.12	8.06
COO	-	3.18	2.83
POC	-	14.37	14.54
LLL	2.20	-	-
OLLn+PoLL	1.21	2.93	1.73
OLL + OLPo	8.66	0.72	0.67
PLL	4.47	2.92	2.96
OOL+PPLn	14.43	1.35	1.35
PLO+SLL	12.98	9.26	9.12
PoOP	4.27	-	-
PPL	3.45	12.92	13.57
OOO	14.75	1.21	1.29
OOP	21.80	9.90	10.16
PPO	8.43	23.5	24.06
PPP	0.06	1.96	1.88
SOO	1.98	0.23	0.24
POS	1.03	0.85	0.86
PPS	-	0.12	-
Unknowns	0.27	0.29	0.34

C: caprylic acid, Ln: linolenic acid, L: linoleic acid, S: stearic acid, P: palmitic acid, O: oleic acid, Po: palmitoleic acid

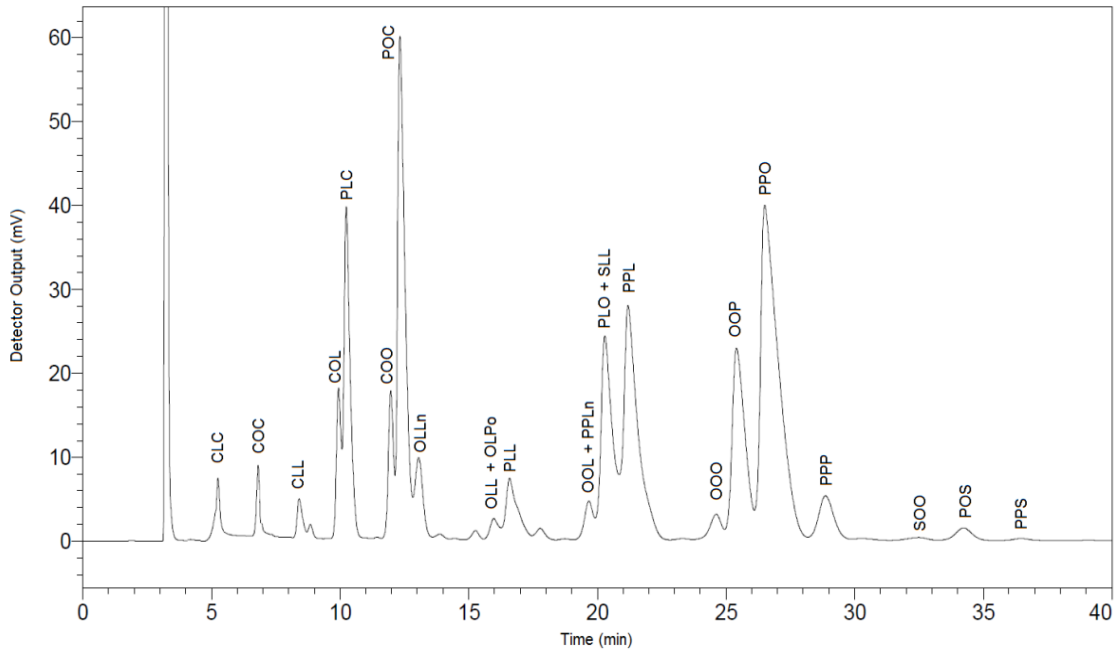

Figure 4.6. The reversed-phase high-performance liquid chromatogram of optimized structured lipid (STR). C, Caprylic acid; Ln, linolenic acid; L, linoleic acid; P, palmitic acid; O, oleic acid; Po, palmitoleic acid: S, stearic acid

Table 4.8 shows first five solutions predicted for the maximum percent desired TAGs and minimal energy for optimized conditions. Optimum condition for enzyme load was set at minimum due to cost consideration, while the other variables were set in the range (Koh et al., 2008). The optimum combination, which satisfied the above requirements with high desirability (0.99) would give 60.41 % of total produced TAGs, 50.86 % of desired TAGs and 38.94 kj/g energy.

Table 4.8. Solutions of optimum condition generated by design expert software

Solutions	Independent variables				Responses		
	Sb[a]	En[b]	Te[c]	Ti[d]	Total produced TAGs (%)	Desired TAGs (%)	Energy (kj/g)
1	2.05	10.00	45.00	12.00	60.41	50.86	38.94
2	2.05	10.00	45.00	12.03	60.41	50.84	38.94
3	2.04	10.02	45.00	12.00	60.35	50.77	38.94
4	2.05	10.05	45.06	12.00	60.42	50.81	38.94
5	2.05	10.00	46.74	12.00	60.48	50.34	38.94

[a] Substrate ratio(mol/mol; Terebinth oil : Fatty acids, 1:1.15-2.05); [b] Enzyme load (wt.%, based on the amount of substrates); [c] Reaction temperature (^{o}C); [d] Reaction time (h)

4.3.1.5 Caloric values of structured lipids

The theoretical energy values of TO and produced SLs were calculated using the approach of Livesey (1984) and Taguchi et al. (2001). Taguchi et al. (2001) were reported that the calculated energy values were in agreement with the energy values measured by bomb calorimeter in their study. First of all the heat of combustion for 1 g of each TAG species present in produced SL was calculated. Then total theoretical energy value was calculated from the TAGs composition of SL.

As it was expected, the medium chain fatty acid, caprylic acid (8:0), is most affective on the reduction of caloric value. TAG species which contain caprylic acids in their structure have low calorie value than other TAG species. For instance, the calculated energy value for 1 g of CLC, COC CLL, COL and PLC were 35.4, 36.84, 38.29, 38.41 and 36.82 kj, respectively. Palmitic acid (16:0) is a saturated fatty acid and has less energy contribution than unsaturated fatty acid, oleic acid (18:1), because of its short chain length. As an example the calculated calorie value for COL were 38.41 kj/g however it was 36.82 kj/g for PLC. The total theoretical energy value of TO was calculated as 39.53 kj/g. The theoretical energy value for produced SLs is given in Table 4.4. They were used in optimization of reaction parameters. The predicted energy value of SLs at the optimized process set-up would be 38.94 kj/g. In

optimized conditions, there is 1.5 % reduction in caloric value of terebinth oil by incorporation of caprylic and palmitic acids.

4.3.1.6 Solid fat content of structured lipids

Differential scanning calorimetry (DSC) is a thermoanalytical technique used in oil research to determine the thermal properties of the oils which have been found to be affected by their chemical compositions. DSC application has been proposed as a tool for oil characterization in various studies (İlyasoglu and Özcelik, 2010; Kowalski et al., 1997). Solid fat content is a good indicator of melting behavior of fats. SLs from gram-scale synthesis were analyzed for SFC by DSC. The experimental data for SFC at 25^{o}C for all produced SLs were given in Table 4.4. Their high solid fat content at workable temperature (25 ^{o}C) indicated that they could be suitable to produce functional shortening and margarine.

The SFC values of some commercially available margarine fat extracts, terebinth oil and SL which was produced at optimized process set-up were given in **Table 4.9.** Noor Lida and Ali, (1998) were reported that a SFC not greater than 32% at 10°C is essential for good spreadability at refrigeration temperature and SFC not less than 10% at 20°C is essential for product's stability and resistance to oil exudation at room temperature. Margarines without a waxy mouth feel have less than 3.5% solid fat at 33.3 ^{o}C and melt completely at body temperature. Commercial margarines served as target criteria to give SFC profile of desired consistency and plastic texture to that of produced SLs. Average melting completion values of soft, hard margarines and SL and TO are 42, 48, 33 and 22 ^{o}C. As it is seen SFC values of SL have been found significantly different compared to the TO. Even if they are also different than those of a particular margarine type, they are in a range of soft and hard margarines. The SFC of SL at the optimized process set-up was comparable with that of commercial fats.

Table 4.9. Solid fat content of structured lipid and fat extracts of commercial margarines

Samples	SFC[a]			
	Temperature (°C)			
	10	21	25	33
TO[b]	26.03[A]	0.42[A]	0[A]	0[A]
SL[c]	60.90[B]	38.80[B]	13.24[B]	0[A]
A[d]	25.82[A]	22.34[C]	17.96[C]	8.52[B,D]
B[e]	24.88[A]	19.32[C]	14.73[B,C]	6.27[B]
C[f]	24.69[A]	20.34[C]	16.12[B,C]	6.27[B,D]
D[g]	33.88[C]	26.50[D]	21.11[D]	11.23[C]
E[h]	69.13[D]	53.31[E]	41.26[E]	18.75[C]
F[i]	77.01[E]	52.68[E]	34.47[F]	9.09[D]

[a] Solid fat content; [b] Terebinth fruit oil; [c] Structured lipid produced at optimized conditions; [d,e,f,g] Fat extracts of soft margarines; [h,i] fat extracts of hard margarines, [A-F] Means within each column with different letters are significantly ($P<0.05$) different.

4.3.1.7 Enzyme reusability

Ever since the industrial use of enzymes began almost many years ago, people have discussed whether and how it would be possible reuse them due to running costs. Immobilized enzymes are currently in industrial use and stabilization of enzymes is still one of the major challenges in bio-catalytic processes. Reusability of a biocatalyst is an important factor that can determine the economic feasibility. Therefore, most researchers directed their efforts for reusing enzymes. In this study, the repeated use capability of Lipozyme IM from *Rhizomucor miehei* was also investigated. It was detected by measuring the residual activity towards the acidolysis reaction with respect to the number of its reuses. Reaction yield, the production of desired TAGs, with repeated use of lipase is shown in Figure 4.7. The enzyme was almost stable during the 10 batch cycles.

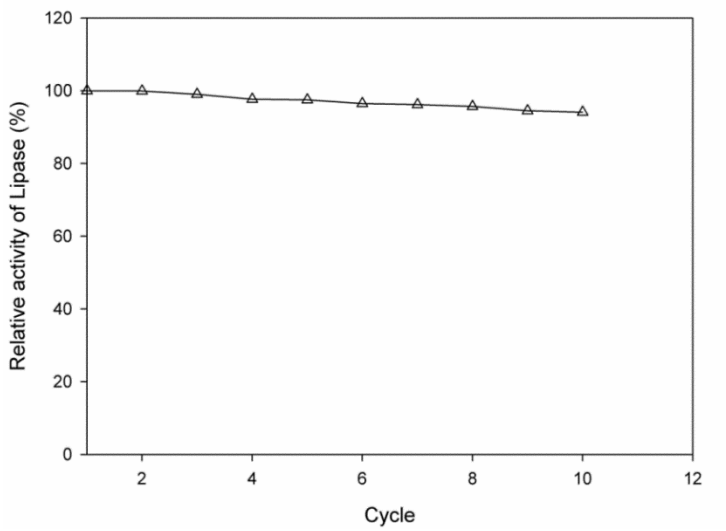

Figure 4.7. Operational stability of stirred tank reactor system

From the above results, the immobilized lipase 94 % keeps its activity after 10 repeated usage. It can be concluded it was an effective biocatalyst for the acidolysis reaction. The significant reusability for *Rhizomucor miehei* was reported in some previous studies (Yang et al., 2003; Michaux et al., 2010)

4.3.2 Acidolysis reaction in packed bed reactor system

The interesterification reaction of TO with CA and PA was also investigated for a recirculating PBR system. The effect of reaction conditions and relationship among them were analyzed by RSM with a four-factor five-level central composite rotatable experimental design.

4.3.2.1 Preliminary studies

In this preliminary study, experimental ranges and levels of some independent variables (substrate mole ratio, reaction time) used in RSM were determined. The range of substrate flow rate and enzyme load for reaction system was selected according to the literature survey. The amount of solvent used in reaction was also studied to maintain the reaction condition. Selection of temperature as 45 ^{o}C based on results obtained from STR system study.

Mole ratio determination

In this preliminary study the mole ratios between PA and CA was fixed for reaction mixture prior the RSM application. For this purpose preliminary acidolysis reaction was performed with a mole ratio of 1:2:1 (TO:PA:CA) using 10 wt % of enzyme, 45°C of reaction temperature and 12 h of reaction time on the basis of STR system study. But the SFC value of SL obtained at these conditions was too high when compared that of STR system. Therefore the mole ratio of PA was reduced in reaction mixture and the reaction repeated with 1:1.5:1. In this case, reaction gave a product having similar properties to that of optimized STR system. Therefore the mole ratio 1.5:1 (PA:CA) was chosen as the best to study in the further experiments.

Determination of solvent amount

In this preliminary study the required minimum amount of solvent to maintain the reaction was determined. Acidolysis reactions were performed at various solvent volume (100, 200, and 300 mL of *n*-hexane) while keeping the other reaction

parameters (T: 45 ^{o}C, t:12 h, Q: 7.5 mL/min, En: 10 %, TO:PA:CA (1:1.5:1)) constant in PBR system. Figure 4.8 shows the change of percentages of produced desired TAGs within these solvent ranges.

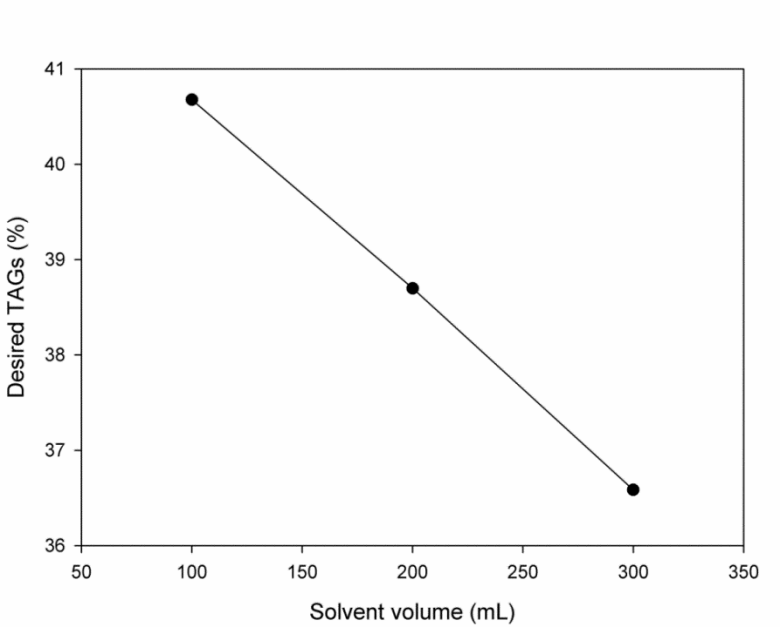

Figure 4.8. Effect of solvent amount on formation of desired TAGs (POP, POC, COC, CLC, PLP, PLC and PPP)

As seen in Figure 4.8 the incorporation of substrate to produce desired TAGs increase with decreasing solvent amount. Therefore it can be concluded that there is no any solubility problem even if in use of 100 mL of *n*-hexane. Additionally, using of minimum amount of solvent would improve the contact chance of substrate with enzyme per unit time and cause to increase in production of desired TAGs. Furthermore, high solvent volume is not economically feasible, because evaporation of high amount of solvent would cause extra cost. As a result, 100 mL of solvent was used for subsequent reactions in PBR system.

Determination of reaction time

The time course of reaction was studied between 1- 12 hours prior the experimental design by RSM to decide about time range. For this purpose some preliminary acidolysis reactions were performed at 45 ^{o}C, 7.5 mL/min flow rate, 10 % enzyme load, and TO:PA:CA (1:1.5:1) mole ratio. Reaction was followed by analyzing the TAGs content of reaction mixtures at predefined time intervals. Figure 4.9 shows the change of percentages of produced desired TAGs within these time intervals. The

incorporation of fatty acids increased with increasing time and after 6 hours reaction reach to equilibrium and the percentage of desired TAGs remained constant. Therefore the time interval was decided between 3.5 and 6.5 hours for RSM studies.

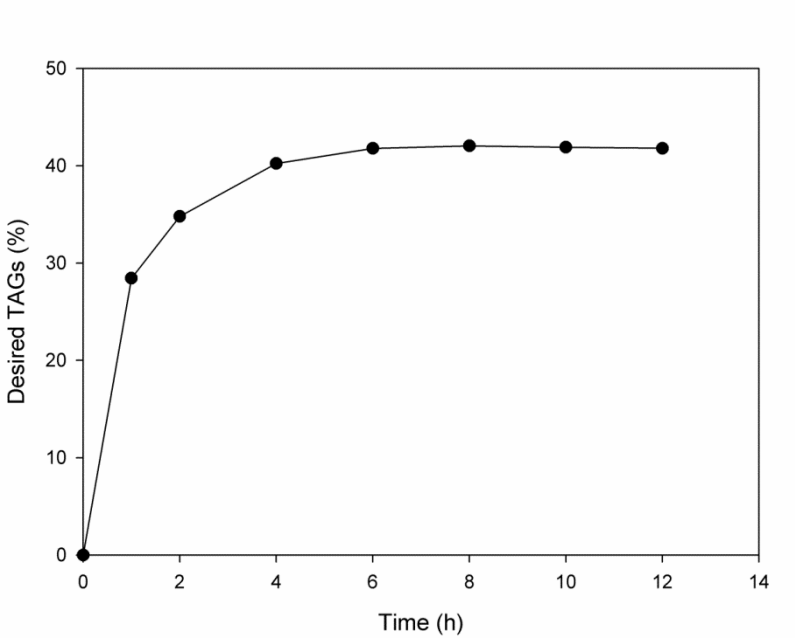

Figure 4.9. Effect of reaction time on formation of desired TAGs (POP, POC, COC, CLC, PLP, PLC and PPP)

4.3.2.2 Experimental design and model fitting

RSM was also used for experimental design in PBR system. Four-factor five-level central composite rotatable design was used for this purpose. Reaction time, temperature, enzyme load, substrate mole ratio and flow rate were independent variables and their levels are as follows: Ti; 3.5-6.5 h, En; 10-20 wt%, Q: 4-8 ml/min and Sb; TO:FA, 1:1.22 –2.07. RSM was implemented to model the experimental data of three responses, namely, total produced TAGs (%), desired TAGs (%) and energy (kj/g). Table 4.10 lists the factors settings and responses. The experimental data were best fitted to quadratic models by using multiple regression analysis. Insignificant factors and interactions were eliminated by backward elimination in the models. The model coefficients and probability values were given in Table 4.11. Even if some linear, quadratic or interaction terms were statistically insignificant they were not eliminated by backward elimination to maintain the hierarchy of the model. Presence of insignificant terms in Table 4.11 was the reason of this elimination principle. The models predicted for all responses were significant at the 99% confidence level and

showed statistically insignificant ($P<0.05$) lack of fit with high coefficients of determinations between 0.92 and 0.98.

Table 4.10. Treatment schedule for four-variable five-level CCRD and experimental data for acidolysis of terebinth fruit oil with caprylic and palmitic acids

No	Factors				Total Produced TAGs %	Desired TAGs %	Energy (kj/g)	SFC[e] at 25 °C
	Sb[a]	En[b]	Q[c]	Ti[d]				
1	1.23	10	4	3.5	39.99±0.17[f]	33.99±0.14	39.10±0.01	2.11±0.06
2	2.08	10	4	3.5	54.08±0.32	48.22±0.28	38.96±0.03	6.01±0.12
3	1.23	20	4	3.5	46.16±0.26	39.05±0.17	39.10±0.01	5.17±0.26
4	2.08	20	4	3.5	57.84±0.27	51.02±0.37	38.96±0.01	11.17±0.35
5	1.23	10	8	3.5	37.69±0.15	30.43±0.43	39.16±0.03	1.87±0.47
6	2.08	10	8	3.5	50.55±0.43	44.10±0.32	39.04±0.02	6.84±0.52
7	1.23	20	8	3.5	49.37±0.38	43.10±0.23	39.05±0.01	6.15±0.38
8	2.08	20	8	3.5	60.52±0.62	54.15±0.41	38.91±0.01	10.77±0.42
9	1.23	10	4	6.5	-	-	-	-
10	2.08	10	4	6.5	60.18±0.42	51.92±0.12	38.91±0.01	15.05±0.25
11	1.23	20	4	6.5	52.74±0.21	43.57±0.25	39.00±0.02	10.92±0.13
12	2.08	20	4	6.5	61.90±0.35	52.88±0.27	38.93±0.02	21.34±0.28
13	1.23	10	8	6.5	47.17±0.37	39.48±0.38	39.06±0.01	5.57±0.12
14	2.08	10	8	6.5	59.65±0.53	51.71±0.19	38.93±0.01	15.23±0.22
15	1.23	20	8	6.5	53.21±0.19	43.46±0.13	38.97±0.01	12.03±0.33
16	2.08	20	8	6.5	62.38±0.65	52.73±0.35	38.86±0.01	18.15±0.42
17	0.80	15	6	5.0	41.66±0.15	32.90±0.09	39.10±0.01	5.20±0.18
18	2.50	15	6	5.0	63.46±0.28	56.90±0.42	38.87±0.01	13.82±0.70
19	1.65	5	6	5.0	46.23±0.42	39.12±0.20	39.05±0.02	4.69±0.35
20	1.65	25	6	5.0	58.60±0.35	49.50±0.28	38.90±0.01	13.84±0.28
21	1.65	15	2	5.0	52.59±0.10	46.59±0.52	39.08±0.03	12.19±0.51
22	1.65	15	10	5.0	57.17±0.23	47.97±0.11	38.93±0.01	13.12±0.32
23	1.65	15	6	2.0	42.00±0.08	34.90±0.14	39.11±0.01	2.81±0.25
24	1.65	15	6	8.0	52.01±0.45	46.33±0.05	39.23±0.01	22.97±0.32
25	1.65	15	6	5.0	52.40±0.27	45.62±0.33	39.07±0.01	9.42±0.07
26	1.65	15	6	5.0	51.63±0.58	44.30±0.47	39.01±0.02	5.46±0.48
27	1.65	15	6	5.0	52.20±0.43	44.30±0.26	39.04±0.00	6.72±0.37
28	1.65	15	6	5.0	51.00±0.10	44.12±0.38	39.05±0.01	8.30±0.39
29	1.65	15	6	5.0	52.70±0.29	45.30±0.22	39.06±0.00	7.20±0.42
30	1.65	15	6	5.0	51.17±0.23	45.80±0.37	39.00±0.02	7.70±0.17

Abbreviations: En, Enzyme load (wt.%. based on the amount of substrates); Ti, reaction time (h); Te, reaction temperature (°C); Sb, substrate mole ratio (mol/mol. Trebinth oil : Fatty acids. 1:1.15-2.05. *Palmitic acid: Caprylic acid. fixed as 1.5:1

4.3.2.3. Effects of reaction parameters

Comparison of main effects of variables on total produced TAGs and desired TAGs

The coefficients and *P*-values for both the yield of desired TAGs and the percentages of total produced TAGs were given in Table 4.11. According to the results of statistical analysis; the enzyme load, time and substrate mole ratio were significant ($P<0.0001$) and had positive effect on these responses. However, the substrate flow rate was not significantly affected. In addition substrate mole ratio had the most significant effect while the other variables have relatively small effect. This observation was in agreement with previous reports (Xu et al., 2000; Arifin et al., 2010; Hamam and Budge, 2010) also reported the effect of these parameters on enzymatic esterification in a PBR system.

Some of the interaction terms; Sb*En, En*Q and En*Ti were also found significant on the yield of total TAGs. The interaction between Sb*En and En*Ti was found negatively affected. However, the interaction between En and Q had positive effect. The interactions between En*Ti and En*Q was also found to be significant on the yield of desired TAGs. While the En*Ti was found negatively affected, the interaction between En*Q had a positive effect. Moreover, the second order of substrate flow rate and time was found significant on both yield of total TAGs and desired TAGs. The negative coefficient of second order for time implies that this variable has an optimum value within the studied range.

Table 4.11. Estimated coefficient for the fitted second order polynomial representing the relationship between the response and process variables

Total Produced TAGs				Desired TAGs			
Variables	Estimated coefficients	Standard error	*P*- value Prob>F	Variables	Estimated coefficients	Standard error	*P*- value Prob>F
Substrate mole ratio (Sb)	5.735	0.260	< 0.0001[a]	Substrate mole ratio (Sb)	5.853	0.243	< 0.0001[a]
Enzyme Load % (En)	3.033	0.260	< 0.0001[a]	Enzyme Load % (En)	2.488	0.243	< 0.0001[a]
Substrate flow rate (Q)	0.419	0.260	0.1244[ns]	Substrate flow rate (Q)	0.004	0.243	0.9860[ns]
Reaction time (Ti)	2.826	0.260	< 0.0001[a]	Reaction time (Ti)	2.321	0.243	< 0.0001[a]
Sb*En	-0.732	0.324	0.0360[c]	Sb*En	-0.579	0.303	0.0725[ns]
En*Q	0.798	0.324	0.0236[c]	Sb*Ti	-0.585	0.303	0.0698[ns]
En*Ti	-0.945	0.324	0.0089[b]	En*Q	1.033	0.303	0.0032[b]
Q^2	0.817	0.232	0.0023[b]	En*Ti	-1.389	0.303	0.0002[a]
Ti^2	-1.152	0.232	< 0.0001[a]	Q^2	0.711	0.216	0.0041[b]
				Ti^2	-0.956	0.216	0.0003[a]

[ns] not significant even at 5% level; [a] Significant at 0.001; [b] Significant at 0.01; [c] Significant at 0.05

Comparison of response 3D plots for total produced TAGs and desired TAGs

In order to evaluate the effect of the independent variables and interactions between them three dimensional response surface plots were constructed. Figure 4.10 and Figure 4.11 show the effects of the experimental factors on the yield of total TAGs and desired TAGs, respectively. The trends of all response surface plots in Figure 4.10 and Figure 4.11 were in parallel with the results of model coefficients. It can be seen that the increasing enzyme load, substrate mole ratio and time improved the incorporation the production of desired TAGs. As expected the incorporation of fatty acids increased with increasing time and equilibrium has been established at around 6 hours. Figure 4.10 b and Figure 4.10c shows the interaction between En*Sb and En*Ti, respectively. While the increase in Sb, En and Ti individually had a significant effect on incorporation, decreasing effect was observed in case of combination. Figure 4.10d shows the decreased effect of flow rate when the enzyme interaction is considered on yield of TAGs or vice versa. However, individually Q was not found significantly affected. Figure 4.11 c and Figure 4.11d shows the interaction between En*Ti and En*Q on the yield of desired TAGs, respectively. Their effect was found similar to that of produced total TAGs. However interaction between En*Sb was not found significant in contrast to that of produced total TAGs.

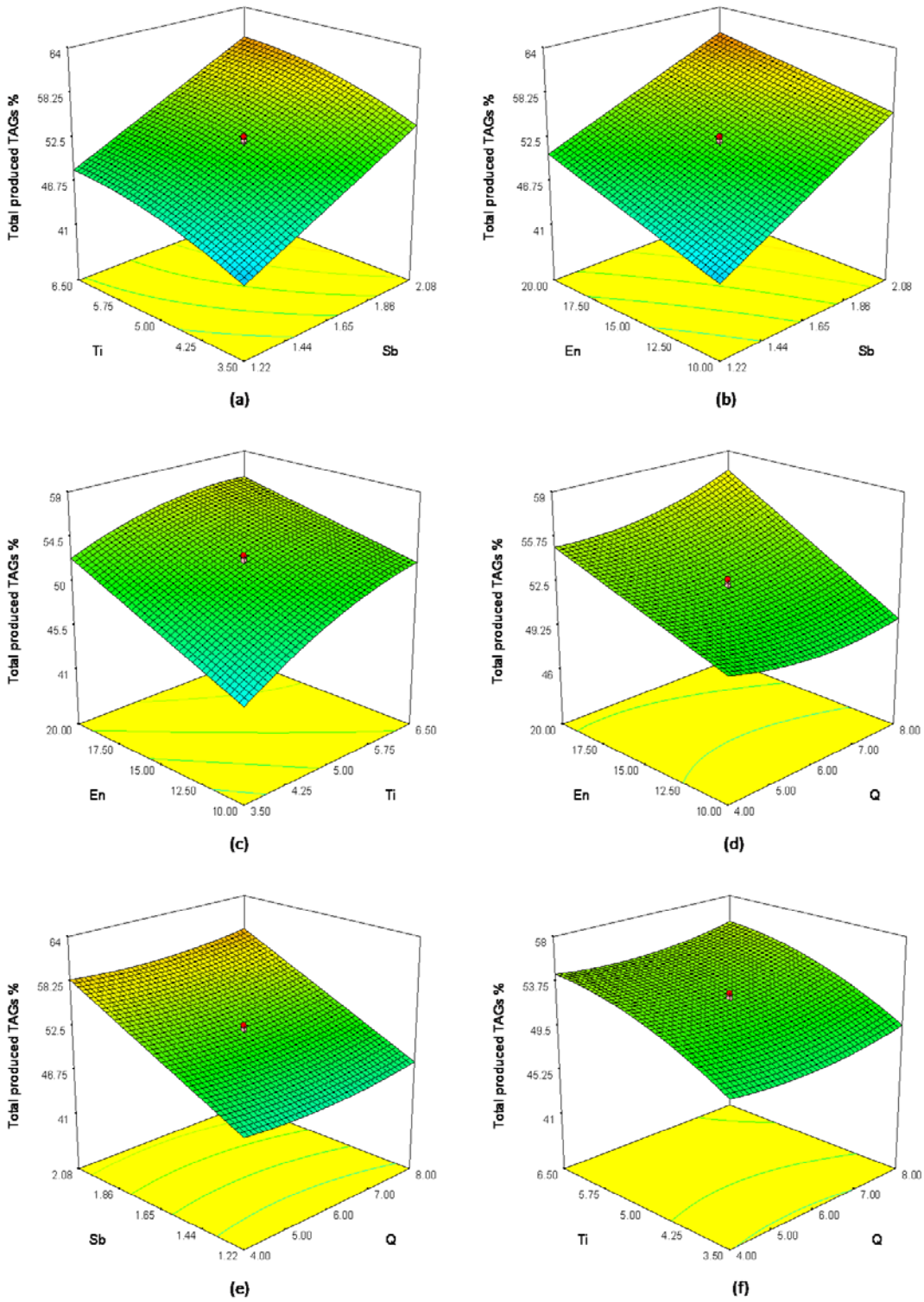

Figure 4.10. Response surface plots for percentage of total produced TAGs: (a) time versus substrate mole ratio; (b) enzyme load versus substrate mole ratio; (c) flow rate versus substrate mole ratio; (d) flow rate versus enzyme load; (e) Time versusenzyme load; (f) time versus flow rate

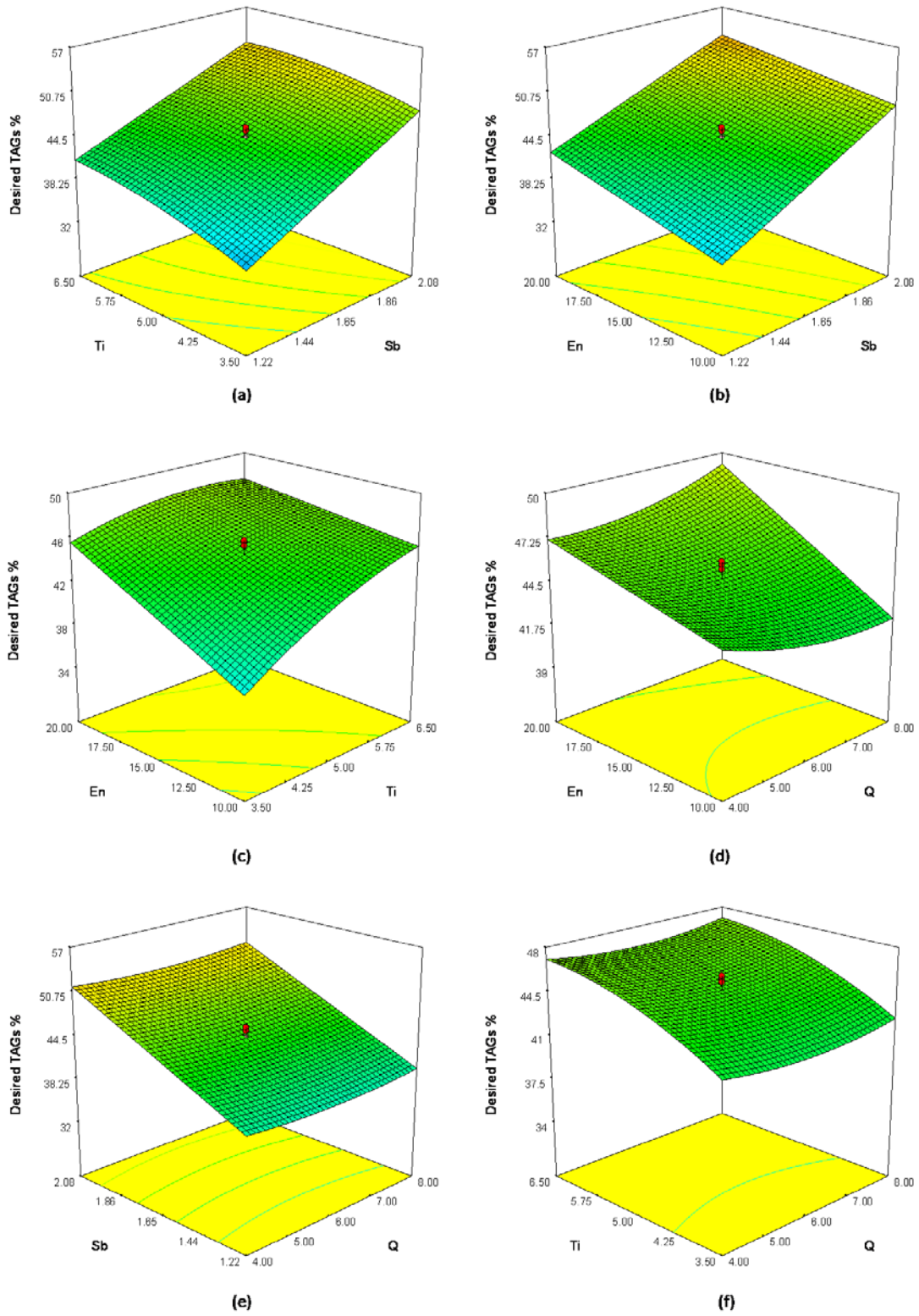

Figure 4.11. Figure 6. Response surface plots for percentage of produced desired TAGs: (a) enzyme load versus substrate mole ratio;(b) flow rate versus substrate mole ratio; (c)time versus substrate mole; (d) flow rate versus enzyme load; (e) Time versusenzyme load; (f) time versus flow rate.

4.3.2.4. Optimization of reaction parameters

Optimization function of the Design Expert Software was also applied in order to optimize the reaction conditions in PBR system. The yield of desired TAGs and caloric value of produced SL were evaluated to produce a target SL. In optimization process SFC values have not been considered even it has been aimed to produce low caloric and spreadable SL. However, SFC values have been evaluated finally for the target product obtained by optimization process. Table 4.12 shows first five solutions predicted for the maximum percent desired TAGs and minimal energy for optimized conditions.

Table 4.12. Solutions of optimum condition generated by design expert software

Nu.	Sb	En%	Q(mL/min)	Ti(h)	Total (TAGs) %	Desired TAGs %	Energy (kj/g)	D*
1	2.07	10.00	4.00	5.90	58.90	52.23	38.96	0.84
2	2.07	10.01	4.00	5.63	58.44	51.84	38.96	0.84
3	2.07	10.00	4.12	5.80	58.63	51.95	38.96	0.84
4	2.07	10.00	4.00	6.24	59.39	52.63	38.97	0.84
5	2.07	10.00	4.02	5.49	58.15	51.59	38.96	0.84

En, enzyme load; Q, Substrate flow rate; Ti, time; Sb, substrate mole ratio. * Desirability

Optimum condition for enzyme load was set at minimum due to cost consideration, while the other variables were set in the range as it was done in STR system production (Koh et al., 2008). The optimum combination, which satisfied the above requirements with high desirability (0.84) would give 60.41 % of total produced TAGs, 50.86 % of desired TAGs and 38.94 kj/g energy.

Fatty acid and TAG compositions of the terebinth fruit oil and optimized SLs are also given in Tables 4.6 and 4.7, respectively for PBR system. Additionally the chromatogram of optimized SL is given in Figure 4.12 to show TAG profile.

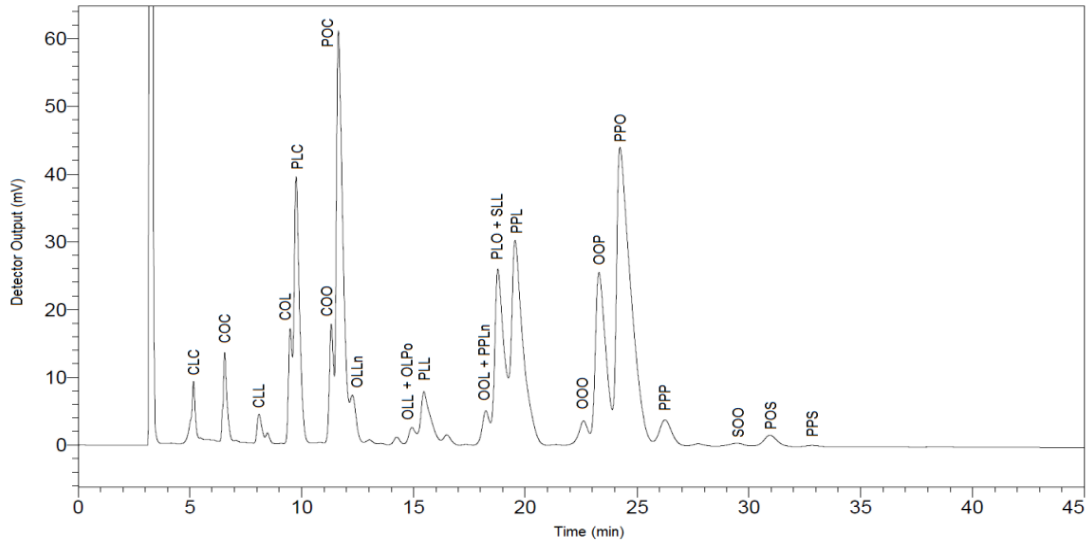

Figure 4.12. The reversed-phase high-performance liquid chromatogram of optimized structured lipid (PBR). C, Caprylic acid; Ln, linolenic acid; L, linoleic acid; P, palmitic acid; O, oleic acid; Po, palmitoleic acid: S, stearic acid

4.3.2.5 Caloric values of structured lipids

The theoretical energy values of TO and produced SLs were calculated. The total theoretical energy value of TO was calculated as 39.53 kJ/g. The theoretical energy value for produced SLs was given in Table 4.10 and they were used in optimization of reaction parameters. The predicted energy value of SLs at the optimized process set-up would be 38.96 kJ/g. In that case, there is 1.44 % reductions in caloric value of terebinth oil by incorporation of caprylic and palmitic acid in a PBR system.

4.3.2.6 Solid fat content of structured lipids

The SFC is the percentage of the lipid that is solid at various temperatures, and it is responsible for many properties of the margarine, such as general appearance, ease of packing, organoleptic characteristics (flavor release, coolness and thickness), and ease of spreading and oil exudation (Lai et al., 1998). Table 4.13 shows the SFC values of terebinth oil, some commercially available soft and hard margarine fat extracts, and SL which was produced at optimized process set-up. The SFC profile of produced SL was compared with that of commercial margarines. The SFC of SL at the optimized process set-up was higher at 10 ^{o}C than the commercial soft margarines and it was close to hard margarines. However it was comparable at other test temperatures with soft margarines.

Table 4.13. Solid fat content of structured lipid and fat extracts of commercial margarines

Samples	SFC[a]			
	Temperature (°C)			
	10	21	25	33
TO[b]	26.03[A]	0.42[A]	0[A]	0[A]
SL[c]	65.10[B]	35.70[B]	15.05[B]	2.10[A]
A[d]	25.82[A]	22.34[C]	17.96[C]	8.52[B,D]
B[e]	24.88[A]	19.32[C]	14.73[B,C]	6.27[B]
C[f]	24.69[A]	20.34[C]	16.12[B,C]	6.27[B,D]
D[g]	33.88[C]	26.50[D]	21.11[D]	11.23[C]
E[h]	69.13[D]	53.31[E]	41.26[E]	18.75[C]
F[i]	77.01[E]	52.68[E]	34.47[F]	9.09[D]

[a] Solid fat content; [b] Terebinth fruit oil; [c] Structured lipid produced at optimized conditions; [d,e,f,g] Fat extracts of soft margarines; [h,i] fat extracts of hard margarines, [A-F] Means within each column with different letters are significantly ($P<0.05$) different.

4.3.2.7. Enzyme reusability

As mentioned earlier, enzyme reusability is major factor which determines economical potentiality to be involved in industrial sector for bio-catalytic processes. Therefore, reusability of Lipozyme RM IM in PBR system were also studied at optimized conditions (Sb: 2.07, En: 10 %, Q: 4 mL/min, Ti: 5.9 h). After completion of each reaction, the enzyme bed was washed with fresh hexane and reused for the next reaction. The relative activity of enzyme was followed from the amount of desired TAG species within the total TAGs of produced SL. Figure 4.14 shows the amount of desired TAGs with repeated usage of lipase. It shows that there was negligible loss in lipase activity even after repeated use for 10 times. It can be concluded it was well maintained (97%) in repeated reaction cycles.

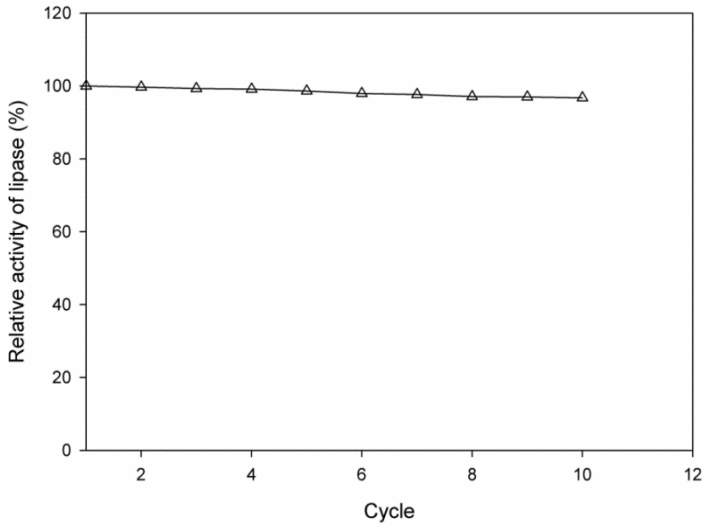

Figure 4.13. Operational stability of bioreactor (PBR)

4.3.3 Comparison of STR and PBR system and properties of target SL

In a basic sense, this study is objected to develop a biotechnological process to produce a low-caloric, spreadable structured lipid from terebinth fruit oil. As it was planned, the interesterification reaction of TO with CA and PA was performed in two different reaction systems. Batch system production was achieved in a lab scale STR. A recirculating PBR system was also designed and operated in discontinuous mode to produced target SL. The effect of reaction conditions and relationship among them were analyzed by RSM for both of these systems. The optimum conditions for STR and PBR systems to produce a SL with desired properties are presented in Table 4.14 and they were compared with each other.

Table 4.14. Comparison of STR and PBR systems

	STR system	PBR system
Reaction conditions		
Temperature (^{o}C)	45	45
Time (h)	12	6
Enzyme load (%)	10	10
Substrate mole ratio (TO:PA:CA)	1:4.1:2.05	1:3.1:2.07
Properties of optimized SLs		
Total produced TAGs (%)	60.73	58.9
Desired TAGs (%)	47.67	52.2
Energy (kj/g)	38.94	38.95

From Table 4.14 it is clear that the optimized SLs for both systems have close properties. Target SL can be produced with lower amount of substrate and within the shorter reaction time in PBR system when compared with that of STR system (Table 4.14). In general the ratio between enzyme and substrate is much higher in a PBR than in conventional STR reactors, this is the reason of high effectiveness of PBR system. Low amount of substrate and shorter reaction time can be concluded as advantageous for PBR system due to economic considerations. Short reaction time was also reported as an advantage in terms of prevention of acyl migration and for production of purer structured lipids (Akoh et al., 2002; Laudani et al., 2007). Additionally, PBR system has advantages because enzyme loss could be reduced by the absence of collisions between enzyme particles in this system (Zhao et al., 2012). So PBR system was decided a more appropriate method for this study. After that, some properties such as oxidative stability, solid fat content and microstructure of SLs which produced at optimized condition in PBR system were analyzed and discussed in the following sections.

4.3.3.1 Melting profile

DSC was used to determine melting profile of terebinth fruit oil, margarines and optimized SL. DSC has been used in lipid chemistry in the characterization of melting and crystallization of pure edible oils (Kaiserberger, 1989). DSC thermograms are characterized by endothermic and/or exothermic peaks whose area is proportional to the enthalpy gained or lost by the material undergoing phase transition. The phase transitions take place over a specific temperature range depending on the sample compositions and physical properties (Wonderlich, 1990). Melting profile is of key importance for both consumer perception and product stability. The fatty acid composition and/or the regiospecific distribution of the fatty acids in the TAGs can be changed by modification of an oil or fat. So it could be affected on physical properties of oils and fats such as melting point, melting profile or melting behavior.

Figure 4.15 shows the melting curves of terebinth fruit oil, margarine and produced SLs. The differences in the melting profiles are obvious. Two melting components appears in terebinth oil, these are two endothermic peaks; one major endothermic peak at -2°C (T<0 °C) with a small shoulder onset at around -5°C and one minor endothermic peak at around 14 °C (T>0 °C). This profile shows similarity with the melting profile of olive oil which has a major endothermic peak at -3.5 °C (T<0 °C) and one minor endothermic peak at 8 °C (T>0 °C) (Chiavaro et al., 2009). It can be concluded that terebinth oil consists high amount of low melting fraction TAGs like olive oil. TO can be considered within classes of oils melt at lower temperature ranges. It is clear that intereseterification altered this melting profile and induced the formation of a plastic fat. As expected the incorporation of saturated fatty acids (CA and PA) broadened the melting range. SL has also two major endothermic regions as that of TO however these peaks were much smaller and broader than TO. Especially the peaks at the lower melting ranges seemed to be reduced. Disappearing or diminishing of these peaks could be due to a decrease in TAGs that consisted of unsaturated fatty acid. In the melting curve of SL the first region (T<0 °C) can be considered as the low melting fraction TAGs which highly contain oleic, linoleic and caprylic acids. The second region can be considered as the transition region of high melting fraction TAGs such as POP, POS and PPP.

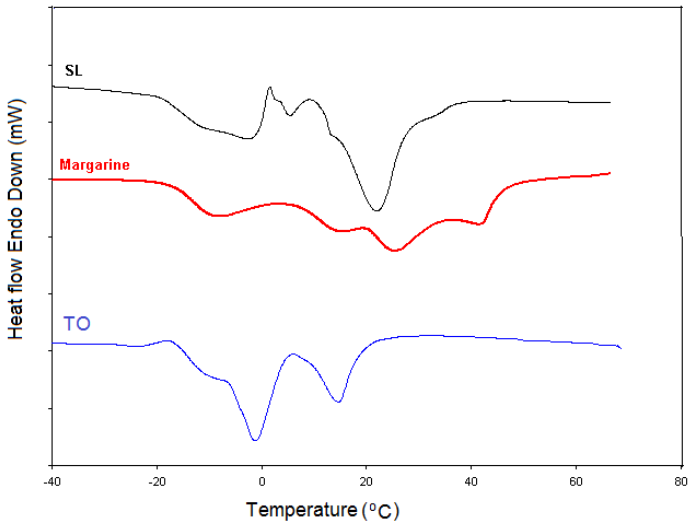

Figure 4.14. Melting thermograms of TO, margarine and SL

As expected the trend of melting curve were in parallel with the structured modification. The increasing number of peaks in the melting curve indicated presence of various TAGs with different melting ranges in produced SLs.

4.3.3.2 Microstructure

In the food industry, the texture of fat-containing products strongly depends on the macroscopic properties of the fat network formed within the finished product. These properties include the spreadability of margarine, butter and spreads (Narine and Marangoni, 1999). Even if fats have similar thermal properties they may have different rheological properties due to their microstructurer differences (Çiftçi et al., 2009b). Predicting the macroscopic properties of fat networks is therefore of importance. The concept of microstructure comprises information on the state, quantity, shape, size, spatial relations, and interaction among all the components of the crystalline network. Microstructure influences fats' macroscopic properties enormously. Polarized light microscopy is the technique used to visualize the microstructural network of fats (Ribeiro et al., 2009).

In this study polarized light microscopy was used to investigate the crystal network of samples. Figure 4.16 shows the crystal morphology of terebinth fruit oil, a commercially available soft margarine and SL. Microstructural differences exist in the fat crystal networks of TO, SL and margarine. While more loosely packed needle-like shape crystals are present in terebinth fruit oil, large symmetrical

spherulites crystals were observed after interesterification. This could be due to the production of TAGs structures such as COC, POC, and POP etc. by the incorporation of saturated fatty acids (caprilic and palmitic acid). Figure 4.16 demonstrates also that SL contained a few number of large fat crystals than those of margarine where small spherulites are overlapped with each other in a denser network.

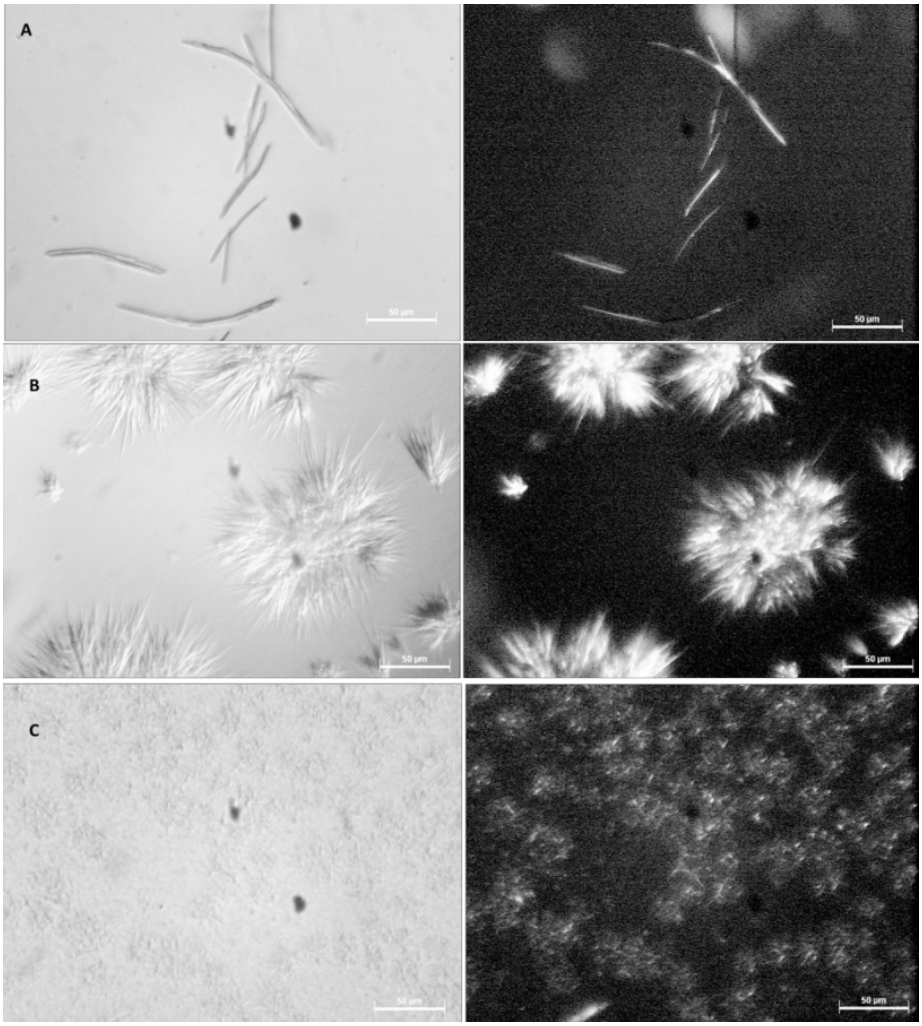

Figure 4.15. Polarized light micrograph grayscale images of (A) margarine (B) structured Lipid (C) terebinth oil

4.3.3.3. Oxidative stability

Oxidative stability is one of the most important indicators of the storage quality of vegetable oils. In this study DSC technique has been successfully applied to obtain the oxidative stability of terebinth fruit oil, a commercially available soft margarine and SL. In this method the thermal changes occurring during oxidation of the oil are recorded. A rapid increase in the amount of evolved heat is observed during initiation of the oxidation reaction. The stability of the oil can be evaluated on the basis of the

length of induction time. Previous findings indicate that DSC is a reliable technique for assessing the oxidative stability of various edible oils (Tan et al., 2002; Pardauil et al., 2011; Kowalski et al., 2004). Figure 4.17 shows the exotherms obtained for the oxidation of terebinth fruit oil, margarine and SL at 140 ◦C. The induction time of terebinth fruit oil, margarine and SL were measured as 37.6, 22.6 and 53.8 min, respectively at 140 ^{o}C. Generally, edible oils with higher degree of unsaturation are more susceptible to lipid oxidation (Tan et al., 2002). However, terebinth fruit oil found more resistant than margarine to oxidative deterioration, even though it contains high amount of unsaturated fatty acids such as oleic and linoleic acids. The possible reason could be its high phenolic and tocopherol content (Durmaz and Gökmen, 2011). Similar result was reported by Tan et al. (2002); Pardauil et al. (2011), and Albuquerque et al. (2003) in their oxidative stability studies. As expected, terebinth fruit oil showed lower induction time than that of produced SL. This result implies that incorporation of saturated fatty acids (CA and PA) into the TO improve the stability of oil.

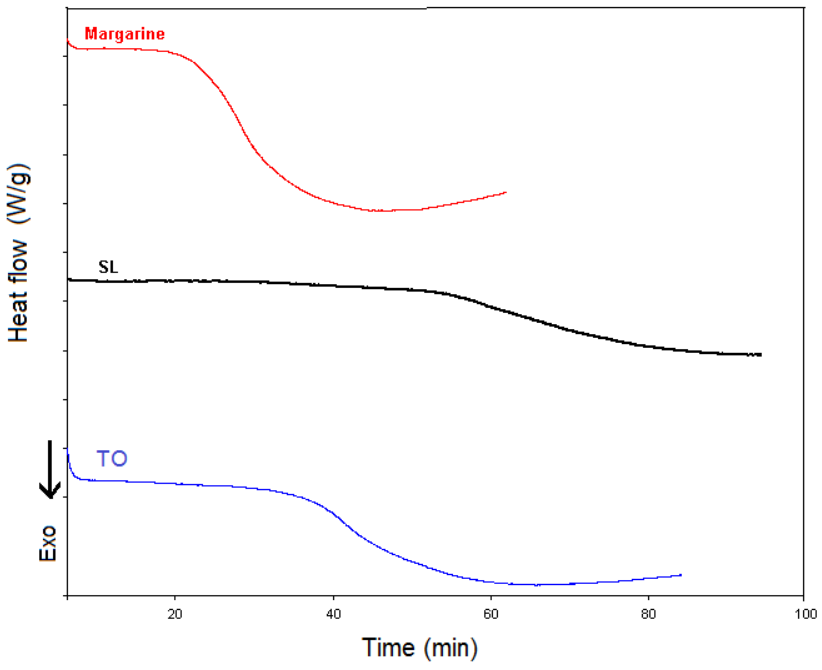

Figure 4.16. DSC exotherms for oxidation of terebinth oil, margarine and structured lipid at 140 ◦C. Atmospheric oxygen pressure, oxygen flow 100 mL/min.

CONCLUSIONS

In a basic sense, two main objectives have been aimed in this study. Firstly it is objected to characterize the properties of terebinth fruit oil in detail. In this sense total phenolic content, oxidative stability, antioxidant activity, fatty acid composition, TAGs composition and melting profile of terebinth fruit oil was investigated. The OOP, PLO+SLL and OOO were found as predominant TAGs with 23.30 %, 15.03% and 13.88%, respectively. Oleic acid (18:1), palmitic acid (16:0) and linoleic acid (18:2) constituted the majority of the fatty acids with percentages of 54.50%, 21.30% and 16.60%, respectively. The sn-2 position was mainly acylated by unsaturated FA and the proportions of oleic and linoleic acids in the sn-2 position of the terebinth fruit oil were 67 and 23.6%, respectively. The total phenolic content was found as 46.5 mg caffeic acid/kg oil. The antiradical activity was measured as 54.85 % reduction in concentration of DPPH.

Within the framework of this study, the development of biotechnological processes to produce a low-calorie and spreadable structured lipid from terebinth fruit oil was studied in stirred tank and recirculating packed bed reactor systems. Modeling and optimization of the acidolysis reactions of terebinth fruit oil with caprylic and palmitic acids in solvent medium were successfully performed by RSM for both of these systems. The analysis of the effects of selected reaction parameters on diverse response variables showed that substrate ratio, enzyme load, reaction time and temperature are key factors for modulating the distribution of reaction products. Investigations of the results have showed that the production of a target SL succeeded with both of these systems. However, PBR system has been found most effective. Target SL can be produced with lower amount of substrate and within the shorter reaction time in PBR system when compared with those in STR system. In PBR system, a SL with desired properties could be obtained using 10 % of enzyme load, 45°C of reaction temperature, 5.9 h of reaction time, and 1:3.1:2.07 of substrate mole ratio (TO:PA:CA). The maximum yield of desired TAGs obtained at these

optimum conditions was 52.2 %. The caloric value of this product was 1.5 % lower than the terebinth fruit oil (39.53 kJ/g).

The study showed that the commercial immobilized form of the 1,3-specific lipase from R. miehei (Lipozyme RM IM) can be used for obtaining structured lipids with a desired stereochemistry in the acidolysis of terebinth oil, caprylic and palmitic acid in a recirculating PBR system. Investigation of physicochemical characteristics of produced SL showed that a SL with melting characteristics and microstructure similar to those of commercial margarine fat extracts was produced. The melting characteristics of produced SLs showed its suitability to be used as a spreadable fat. Produced SL showed maximum oxidative stability.

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CURRICULUM VITAE
DERYA KOÇAK YANIK

Address: *Mailing address*:
Güneykent Mah.
221. Cad. No:28
Demet Apt. Kat:4 Daire:7
27310, Gaziantep, TURKEY
Phone: +90-5303403470
E-mail: dkocak@gantep.edu.tr

EDUCATION

M.Sc.	2009	Food Engineering Department, University of Gaziantep, Graduate School of Natural & Applied Sciences, Turkey
B.Sc.*	2006	Food Engineering Department, University of Gaziantep, Turkey

*** graduated with honors**

WORK EXPERIENCE

2006-	Research Assistant, University of Gaziantep, Faculty of Engineering, Food Engineering Department, Gaziantep, Turkey

STUDENT SCHOLARSHIPS AND AWARDS

2012	The Council of Higher Education, YÖK, Ph.D Student Scholarship, (4 months), University of YORK, England, (2012).
2011	Erasmus Teaching Staff Mobility, (one week), University of Algarve, Faro, Portugal.
2009	Erasmus Ph.D. Exchange student (5 months), Warsaw University of Life Science, Warsaw, Poland.
2006	European Dairy Week, Invited Student, at a one week course 'Frontiers in dairy science and technology'. Wageningen University, Wageningen, Netherlands.

PROFESSIONAL AFFILIATIONS

2011-	Euro Fed Lipid, European Federation for the Science and Technology of Lipids
2009-	YABİTED, The Turkish Lipid Group

SCIENTIFIC PUBLICATIONS

Peer-Reviewed Journals

2012 **Koçak, D.**; Keskin, H.; Fadıloğlu, S.; Göğüş, F. Acidolysis Reaction of Terebinth Fruit Oil with Palmitic and Caprylic Acids to Produce Low

Caloric Spreadable Structured Lipid. ***J Am Oil Chem Soc***, DOI: 10.1007/s11746-013-2250-z, (in press).

2012 **Kocak, D**., Özel, M.Z., Göğüş, F., Hamilton, J.F., Lewis, A.C. Determination of Volatile Nitrosamines in Grilled Lamb and Vegetables Using Comprehensive Gas Chromatography - Nitrogen Chemiluminescence Detection. ***Food Chemistry***,135, 2215–2220

2012 Keskin, H.; **Koçak, D**.; Fadıloğlu, S.; Göğüş, F. Enzymatic Acidolysis of Triolein with Palmitic and Caprylic Acids: Optimization of Reaction Parameters by Response Surface Methodology. ***Grasas y Aceites,*** 63 (3), 274-283.

2011 **Koçak, D.**; Keskin, H.; Fadıloğlu, S.; Kowalski,B.; Göğüş, F. Characterization of Terebinth Fruit Oil and Optimization of Acidolysis Reaction with Caprylic and Stearic Acids. ***J Am Oil Chem Soc***, 88:1531–1538.

2011 Göğüş, F., Özel, M.Z., **Kocak, D**., Hamilton, J.F., Lewis, A.C. Analysis of roasted and unroasted Pistacia terebinthus volatiles using direct thermal desorption-GCxGC–TOF/MS. ***Food Chemistry***, 129, 1258–1264

Book Chapters

2013 Göğüş, F., Koçak Yanık D. Chapter 4 Yağ Teknolojisi, In "Heryönüyle Gıda" (Ed. Özkaya, F.D., Coşansu, S., Ayhan, K.), Sidas, pp.121-134.

Conference Proceedings

2013 **Koçak, D**.; Keskin, H.; Fadıloğlu, S.; Göğüş, F. Acidolysis Reaction of Terebinth Fruit Oil with Palmitic and Caprylic Acids in a Recirculating Packed Bed Reactor: Optimization using response surface methodology, Submitted to be presented in Euro Fed Lipid Congress, Antalya.

2012 Özel, M.Z, Gogus, F.. **Kocak, D**., Hamilton, J.F., Lewis, A.C. Et ve Sebzelerin Mangalda Pişirilmesi Esnasında Oluşan Nitroz Aminlerin Azot Kemilüminesans Dedektörlü İki Boyutlu Gaz Kromatografisi ile Tayinleri. Ulusal Analitik Kimya kongresi, Hatay, Türkiye.

2012 Keskin, H.; **Koçak, D**.; Fadıloğlu, S.; Göğüş, F. Trioleinin enzimatik asidoliz metoduyla modifikasyonu. 1. Bitkisel yağ kongresi, 12-14 Nisan, YABİTED, Adana, Türkiye.

2012 **Koçak, D**.; Keskin, H.; Fadıloğlu, S.; Göğüş, F. Menengiç meyve yağının palmitik ve kaprilik asit ile enzimatik asidolizi: Tepki yüzey metodu ile reaksiyon koşullarının optimizasyonu. 1. Bitkisel yağ kongresi, 12-14 Nisan, 2012, YABİTED, Adana, Türkiye.

2011 Keskin, H.; **Koçak, D**.; Fadıloğlu, S.; Göğüş, F. Interesterification of Triolein with Palmitic and Caprylic Acids: Optimization of Reaction Parameters by Response Surface Methodology. 9th Euro Fed Lipid Congress, "Oils, Fats and Lipids for a Healthy and Sustainable World", 18-21 September, Rotterdam, The Netherlands.

2011 **Koçak, D**.; Huub Lelieveld and Göğüş, F. TRACK_FAST: traınıng requırements and careers for knowledge- based food science and technology in europe- food industry flash animations . 2nd Intarnational ISEKI_Food Conference, August 31th-September2nd, 61, Milan, ITALY.

2011 Gogus, F.. Özel, M.Z, **Kocak, D**., Hamilton, J.F., Lewis, A.C. Analysis of roasted and unroasted Pistacia terebinthus volatiles using direct thermal desorption-GCxGC–TOF/MS . International Food congress, Novel Approches in Food Industry, May 26-29, 509-514, İzmir, Turkey.

2010 **Koçak, D**.; Eren, S.; Göğüş, F. The effect of air drying on shrinkage and color characteristics of mulberry *(Morus alba*). 1st International Congress on Food Technology; Nowember 03-06, Antalya, Turkey.

2010 Keskin, H.; **Koçak, D**.; Fadıloğlu, S.; Göğüş, F. New developments in the synthesis of reduced calorie fats. 1st International Congress on Food Technology; Nowember 03-06, Antalya, Turkey.

2010 **Koçak, D**.; Fadıloğlu, S.; Kowalski,B.; Göğüş, F. Characterization of Terebinth (*Pistacia terebinthus L.*)Fruit oil and Enzymatic modification by incorporation of Caprylic and stearic acid. The 4th International Student Conference on Advanced Science and Technology; May 25-26, Ege University, İzmir, Turkey.

Completed projects

1. Production of Low Caloric and Spreadable Lipid by Enzymatic Interesterification of Terebinth Fruit (*Pistacia terebinthus* L.) Oil. ScientificResearch Projects Governing Unit **(BAPYB)** of University of Gaziantep. (2010 - 2011). Researcher.

PRESENT ACTIVITIES/PROJECTS

Ongoing activities

1. Training Requirements and Careers for Knowledge-based Food Science and Technology in Europe, **FP7 project,** Researcher.

2. Portakal işleme atıklarından yeşil ekstraksiyon teknikleri ile limonene ektraksiyonu, research project, ScientificResearch Projects Governing Unit **(BAPYB)** of University of Gaziantep. (2012-2013). Researcher.

Papers in preparation

1. Acidolysis Reaction of Terebinth Fruit Oil with Palmitic and Caprylic Acids in a Recirculating Packed Bed Reactor: Optimization using response surface methodology