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Ph. D. in Food Engineering

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UNIVERSITY OF GAZİANTEP
GRADUATE SCHOOL OF
NATURAL & APPLIED SCIENCES

PRODUCTION OF MODIFIED LIPID WITH INCORPORATION OF ESSENTIAL AMINO ACIDS INTO REFINED OLIVE POMACE OIL AND ITS CHARACTERIZATION

Ph. D. THESIS
IN
FOOD ENGINEERING

BY
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Supervisor
Prof. Dr. Sibel FADILOĞLU

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Hasene KESKİN ÇAVDAR

ABSTRACT

PRODUCTION OF MODIFIED LIPID WITH INCORPORATION OF ESSENTIAL AMINO ACIDS INTO REFINED OLIVE POMACE OIL AND ITS CHARACTERIZATION

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The objective of this thesis was to synthesize and characterize a novel modified lipid which is composed of mainly oleic acid at *sn*-2 position and hydrophobic essential amino acid derivatives at *sn*-1(3) or *sn*-1,3 positions. In the first part, 2-monoacylglycerol (2-MAG) with a high content of oleic acid at *sn*-2 position was synthesized by enzymatic ethanolysis of refined olive pomace oil (ROPO). Response surface methodology (RSM) was applied to optimize reaction conditions; time (4-10 h), temperature (45-60ºC), enzyme load (10-18 wt%) and ethanol:oil molar ratio (30:1-60:1). The highest 2-MAG yield (85%) was obtained at 45ºC using 10 (wt%) enzyme load and 50:1 ethanol:oil molar ratio for 5 h reaction time. The purity of the obtained 2-MAG was determined as higher than 96%. In the second part, method for the incorporation of *N*-acetyl-L-leucine and *N*-acetyl-L-valine into purified 2-MAG was developed. RSM was applied to enhance an empirical model for prediction and to optimize reaction conditions; reaction time (8–24 h), reaction temperature (40–55ºC), enzyme load (15–30 wt%). The optimum reaction conditions were determined as temperature 54ºC, time 8 h, enzyme load 15 (wt%) for the maximum *N*-acetyl-L-leucine incorporated product yield (29%). Besides, the temperature 55ºC, time 24 h, enzyme load 30 (wt%) were found for the maximum *N*-acetyl-L-valine incorporated product yield (16%). Some chemical and physical properties of the products were compared to ROPO and similarites and differences were introduced. The caloric values of the products were reduced 18% compared to that of ROPO.

Keywords: Enzymatic synthesis, *N*-acetyl-L-leucine, *N*-acetyl-L-valine, refined olive pomace oil, response surface methodology

ÖZET

RAFİNE EDİLMİŞ PİRİNA YAĞINA ESANSİYEL AMİNO ASİT BAĞLAYARAK MODİFİYE YAĞ ÜRETİLMESİ VE KARAKTERİZASYONU

ÇAVDAR KESKİN, Hasene
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Bu tezin amacı, *sn*-2 pozisyonunda temelde oleik asit, *sn*-1(3) veya *sn*-1,3 pozisyonlarında hidrofobik esansiyel amino asit türevlerini içeren yeni bir modifiye yağ elde etmektir. İlk aşamada, rafine edilmiş pirina yağının (ROPO) enzimatik etanoliziyle *sn*-2 pozisyonu oleik asitçe zengin 2-monoasilgliserol (2-MAG) sentezlendi. Reaksiyon koşullarının optimizasyonu için yüzey tepki metotu kullanıldı; süre (4-10 sa), sıcaklık (45-60ºC), enzim miktarı (10-18 Ağ.%) ve etanol:yağ mol oranı (30:1-60:1). En yüksek 2-MAG verimi (85%), 5 sa reaksiyon süresinde, 45ºC'de, %10 (Ağ.) enzim miktarı ve 50:1 etanol:yağ mol oranı kullanılarak elde edildi. Elde edilen 2-MAG'lerin saflık derecesi %96'nın üzerinde hesaplandı. İkinci aşamada, *N*-asetil-L-lösin ve *N*-asetil-L-valin'in saflaştırılan 2-MAG'e bağlanması için metot geliştirildi. Deneysel model tahmini ve reaksiyon şartlarının optimizasyonu için yüzey tepki metodu kullanıldı; reaksiyon süresi (8-24 sa), reaksiyon sıcaklığı (40-55ºC), enzim miktarı (15-30 Ağ.%). En yüksek miktarda *N*-asetil-L-lösin bağlı ürün (%29) için optimum reaksiyon şartları; sıcaklık 54°C, süre 8 sa ve enzim miktarı %15 (Ağ.) olarak belirlendi. Ayrıca, en yüksek miktarda *N*-asetil-L-valin bağlı ürün (%16) için sıcaklık 55°C, süre 24 sa ve enzim miktarı %30 (Ağ.) olarak belirlendi. Saflaştırılan ürünlerin bazı fiziksel ve kimyasal özellikleri ROPO ile kıyaslanarak benzerlikleri ve farklılıkları ortaya kondu. Elde edilen ürünlerin kalorik değeri ROPO'ya göre %18 oranında düşürüldü.

Anahtar Kelimeler: Enzimatik sentez, *N*-asetil-L-lösin, *N*-asetil-L-valin, rafine edilmiş pirina yağı, yüzey tepki metodolojisi

To my family

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LIST OF ABBREVIATIONS

Ac	Acetyl
ANOVA	Analysis of variance
BCAA	Branch chain amino acids
BOC	Tertbutoxycarbonyl
CCRD	Central composite rotatable design
DAG	Diacylglycerol
DATEM	Diacetyltartaric acid esters of monoacylglycerols
DDM	Dialkyl dihexadecylmularate
DSC	Differential scanning calorimeter
EPG	Esterified propoxylated glycerol
FAEE	Fatty acid ethyl esters
FDA	Food and Drug Administration
FFA	Free fatty acids
GC	Gas chromatography
GRAS	Generally Recognize as Safe
HPLC	High performance liquid chromatography
LC	Liquid chromatography
LDL	Low density lipoprotein
MAG	Monoacylglycerol
MS	Mass spectrophotmeter
PLM	Polarized light microscope
ROPO	Refined olive pomace oil
RSM	Response surface methodology
SCFA	Short chain fatty acids
SFC	Solid fat content
TAC	Trialkoxycitrate
TAG	Triacylglycerol
TATCA	Trialkoxytricaballylate

TLC	Thin layer chromatography
TLC/FID	Thin layer chromatography/flame ionization detector

CHAPTER 1

INTRODUCTION

High intake of fats causes high incidence of cardiovascular disease, hypertension, and obesity, especially in industrialized countries. However, it is difficult for individuals to change their dietary habits to reduce or minimize fat intake while enjoying their favorite foods. This problem and the interest shown by consumers in alternative fats and foods low in calories or without calories led to the search by the food industry and scientific community for the ‘‘ideal’’ fat substitute (Akoh and Min, 2002). So that, with increasing consumer awareness of the risks associated with high fat intake, a market for reduced calorie fats or fat replacers has opened up (Koh et al., 2010).

Recognized strategies for developing a poorly or nondigested, and poorly or nonabsorbed fat-like substance are to reengineer, redesign, chemically alter, synthesize, or structure conventional triglyceride components of fats and oils to retain conventional functional properties in foods while reducing or removing susceptibility to hydrolysis and/or absorption in the intestine. Suggested design strategies for fat substitutes include (Hamm, 1984; Mascioli et al., 1988; Matthews and Kennedy, 1990; Singhal et al., 1991):

1. Replacement of the glycerol moiety of triglycerides with alternative polyols or sugars,
2. Reversal of the ester linkage of fatty acids to glycerol,
3. Reduction of the ester linkage of the glycerol moiety to an ether linkage.

Being different from these approaches, the aim of this thesis was to produce a modified lipid whose calorie is reduced, nutritional value is increased and oil properties are conserved with incorporation of hydrophobic and essential amino acid derivatives into refined olive pomace oil (ROPO) by enzymatic methods. While mainly oleic acid is present at *sn*-2 position of this product, hydrophobic and

essential amino acids (*N*-acetyl-L-leucine and *N*-acetyl-L-valine) were present at *sn*-1,3 positions. In current literature, any research related with incorporation of amino acid into oil and investigation of its properties evaluating of this product as an oil has not been met.

A strategy consisting of two steps were performed to achieve the mentioned modification. In the first step, 2-monoacylglycerols (2-MAG) were produced from refined olive pomace oil with enzymatic ethanolysis. The effect of the parameters (temperature, enzyme load, time, ethanol:oil molar ratio) on the enzymatic ethanolysis were investigated using four factors five levels face centered central composite design response surface methodology to obtain maximum yield of 2-MAG. In the second step, *N*-acetyl-L-amino acids were incorporated into 2-MAG with enzymatic methods. For this aim, first of all, effects of enzyme type and solvent system on reaction were studied to fix the conditions at which maximum incorporation occurs. Afterwards, reaction conditions at which maximum incorporation occurs were optimized by studying the effects of other independent parameters (temperature, enzyme load, time) using three factors three levels face centered central composite design. New methodologies were improved for the synthesis, purification and characterization since this system has ever been studied. The physicochemical and crystallographic similarites and differences of the newly handled product and refined olive pomace oil were also exhibited.

Olive pomace oil is a good potential source for the targeted products because of its high *sn*-2 position oleic acid content, low cost and highly abundance. Olive pomace is a byproduct of olive oil production and olive pomace oil is the oil obtained by the extraction of oil from olive pomace. Olive pomace contains 4–12% oil depending on the extraction technology (Meziane and Kadi, 2008). Olive pomace oil is considered as a low quality oil, because of long storage periods of pomace and high temperature drying applications. Thus, olive oil producers seek alternative uses for olive pomace such as extraction of antioxidant compounds, conversion to a renewable fuel source and extraction of residual oil. Synthesis of the mentioned novel products rich in oleic acid at *sn*-2 position from olive pomace oil can be a good potential way for valorization of olive pomace. In this way, value added product may be produced from an inexpensive raw material.

CHAPTER 2

LITERATURE REVIEW

2.1 Fats and Oils

Fats and oils are one of the major food sources for humans. The major animal sources include butter, lard, tallow and fish and plant sources include sunflower, olive, corn, soy, cotton, palm, palmkernel, coconut, rape, groundnut and linseed. Most of these oil and fat sources are consumed as human food (Gunstone, 2001). Natural oils are mainly triacylglycerols (TAG) which differ in their physical and nutritional properties and do not always meet all nutritional recommendations or possess desirable physical properties. Fats and oils are used in human diet directly as natural products or, more often, after appropriate modifications (Adamczek, 2004; Gunstone, 2004).

Fats and oils contribute to the physical and functional properties of most food products, affecting sensory and nutritional aspects of food. It is difficult to obtain most of these properties without adding fats and oil. The quantity and sort of fat in a food affect the quality of the food and acceptance by consumers (Akoh and Min, 2002). Fats and oils are the esters of glycerol and three fatty acids and they are commonly called as triacylglycerol. Triacylglycerols have the same glycerol unit. Thus, the chemical and physical properties of fats are regulated by the type and position of the fatty acids that they contain.

2.2 Lipids and Health

Fat is an important macromolecular component of plant and animal tissues. Proper growth and development and maintenance of good health can be achieved by fats and oils. Fats and oils aid the absorption of fat soluble vitamins A, D, E, and K in the intestine. They are the only source of the essential fatty acids linoleic and linolenic acids. Fats and oils provide energy for many adults and for infants and toddlers (Khetarpaul et al., 2014). Fat (~9 kcal/g) gives higher energy than carbohydrate or

protein (~4 kcal/g) (Gunstone, 2001). Fatty acids have a role in the synthesis of signaling molecules like steroids and prostanoids (AICR, 1996).

Despite the fact that fats and oils have so many benefits in human health, diets high in fat emerges several problems. Diets high in fat causes weight gain and obesity since they have high calories. Risk of heart diseases is increased by consumption of high amounts of saturated and trans fat (Insel et al., 2014; NRC, 1989).

2.2.1 Obesity

Obesity is the excessive body fat accumulation of body fat resulting in a body weight in relation to height that is higher than body mass index standarts. High consumption of dietary fat contributes to this obesity (Insel et al., 2014). Obesity is one of the crucial public health problem in USA and Europe and is coming to be progressively vital in many countries of the world (Hausman and Grossman, 2008). According to the World Health Organization, obesity is one of the main public health problems in the world. It is predicted that by 2025, around 2.3 billion adults will be overweight and >700 million, obese (ABESO, 2015).

2.2.2 Heart and artery disease

The risk of cardiovascular diseases is invited by person who has a diet rich in saturated or trans fats. Blood cholesterol and low density lipoprotein cholesterol is raised by consumption of high saturated and trans fat intake. High blood cholesterol is one of the crucial reasons of atherosclerosis which is a type of heart diesase in arteries become progressively clogged with accumulation of fatty material (Insel et al., 2014).

2.2.3 Cancer

In different researches, decreasing fat in the diet reduces the incidence of carcinogenic breast and colon tumors while increasing dietary fat consumption has a supplementary effect on cancer in rodent models. However, a direct extrapolation can not be done for human from the results from animal experiments. The other relation of fat hypothesis for cancer bases on some reasonable mechanisms in cell membrane. For instance, alterations of the lipid composition in cell membrane

influence membrane permeability, which controls movement of fats and other chemicals accessing inside of the tissues and leaving out of the tissues. Fats and oils can also contribute to carcinogenesis influencing the immune system and synthesis of prostaglandins which may increase tumor growth. Moreover, the consumption of fats and oil indirectly causes carcinogenesis since it contributes to obesity which itself increases risk for some cancer types such as endometrium, kidney, breast and colon (AICR, 1996).

2.3 Fat Replacers

Although high intake of fats causes health problems such as cardiovascular disease, hypertension, and obesity, their good taste and smoothness make them difficult to be circumvented (Akoh and Min, 2002; Houde et al., 2004). Changing the dietary habits and reducing the fat consumption is difficult for people while enjoying their foods. Because of these problems and the curiosity shown by the public, low calorie or calorie free alternative fats and foods has been started to be produced (Koh et al., 2010). Different substances are produced which offer partial solutions that are mentioned above. These substances are called as fat replacers and are categorized into two groups depending on their origin:

- natural (fat mimetics)
- synthetic (fat substitutes)

Fat replacers are any compound used for replacing fats, having less calories than fat and may/not supply nutritional value. Fat substitutes are synthetic compounds designed for weight-by-weight replacement of fats with a normally similar structure to that of fats with the exception of being resistant to hydrolysis by digestive enzymes. Fat mimetics are fat replacers which mimic at least one of the organoleptic and physical functions of fat requiring high water content to realize its functionality (Clavier, 2008).

2.3.1 Classification of Fat Replacers

Fat replacers give sensory and functional qualities provided by fat in foods. It is nearly not possible to replace fats in the food with only one compound or process

since they have different functions in foods. Type of fat replacers is very critical and should be selected considering how the food product is consumed or prepared. For example, a heat sensitive fat replacer should be used for its stability at high temperatures (Khetarpaul et al., 2014).

Fat replacers are useful in decreasing the quantity of fat in foods and by this way decreasing the fat intake of people. Some group of fat replacers may be used as fat substitutes and may be replaced with fat in the food. Some group of fat replacers may be used as fat mimetic and may partially be replaced with fat (Khetarpaul et al., 2014).

2.3.1.1 Fat Mimetics

The chemical structure of fat mimetics is definetly different from those of fats. Fat mimetics are generally carbohydrate or protein based fat replacers (Ognean et al., 2006). Fat mimetics absorb a considerable amount of water and give the characteristic properties and functionalities of fat. However, because of binding excessive water, fat mimetics obtained from polysaccharides or proteins are not stable and can not transfer heat at high temperature operations such as cooking and frying. Fat mimetics caramelize or denature at high temperatures. On the other hand, many of them may be used in baking or retorting. Fat mimetics can not replace with fats in the food on a one to one (or gram for gram) basis. Larger or less concentrations of fat mimetics are required than those of the fats. The caloric values of the fat mimetics are in the range of 0-4 kcal/g although caloric contribution of the fat mimetic depends on the concentration, digestion and absorbtion in the body (Swanson, 2006).

2.3.1.2 Fat Substitutes

The chemical structure and physiochemical properties of fat substitutes are similar to fats. Fat substitutes are generally indigestible and have lower caloric value per gram. Fat substitutes resemble triglycerides physically and chemically. They can replace the fat in food products on a one-to-one, gram-for-gram basis. Fat substitutes can be obtained chemically or enzymatically. Fat substitutes usually show stability at cooking and frying temperatures (Adamczak and Bednarski, *2*010; Ognean et al., 2006).

2.3.2 Strategies for Production of Low-Calorie Substitutes for Fats and Oils

The nutritional and functional properties of fats may be altered by using physical, chemical and biotechnological methods (Adamczak and Bednarski, 2010). The strategies, rationales, and examples are given for the production of fat replacers similar to structure of triacylglycerols:

1. Changing the glycerol moiety of triacylglycerols with different alcohols.
2. Changing the long-chain fatty acids with different acids to give steric protection to the ester bonds.
3. Reversion of the ester linkage in triacyglycerols by replacing the glycerol with a polycarboxylic acid, amino acid, or other polyfunctional acid and esterify with a long-chain alcohol.
4. Reduction of the ester bond of the glycerol moiety to ether bond. Lipases can not hydrolyze ether bonds as rapidly as ester bonds (Emadzadeh and Ghorani, 2015; Jones, 1996).

2.3.3 Types of Fat Replacers

There are different fat replacers with diverse chemical types and physicochemical and sensory properties. Thus, a simple classification is not easily provided. Fat replacers may be categorized as carbohydrate-based, protein-based, or lipid-based replacers (Chung and Min, 2004). The protein-based and carbohydrate-based replacers are considered as fat mimetics and lipid-based replacers are considered as fat substitutes.

2.3.3.1 Lipid-based Fat Replacers

The chemical structure of lipid based fat replacers is close to triacylglycerols. The caloric value of lipid based fat replacers is reduced or zero since they can not be fully hydrolyzed by digestive enzymes. Table 2.1 shows some examples for lipid-based replacers (Chung and Min, 2004).

Olestra is composed of hexa-, hepta- and octaesters of sucrose. Olestra may be assembled by chemical trans/interesterification of sucrose with 6-8 carbon fatty acids (Ognean et al., 2006). The physical properties of olestra are very close to those of a

TAG which has the similar fatty acid composition (Crites et al., 1997). Gastric or pancreatic enzymes can not hydrolyze olestra. Hydrolysis of olestra by digestive lipases can be prevented by steric hindrance since it has large size of fatty acids in its structure (Cooper et al., 1997; Peters and Lawson, 1997). Thus, olestra has no caloric contribution in human diets since it is not absorbed in gastrointestinal tract (Cooper et al., 1997; Thomson et al., 2000). The Food and Drug Administration (FDA) allows olestra to be employed as a replacement for fats and oils (Akoh, 1998a, 1998b; Cooper et al., 1997; Ognean et al., 2006; Peters and Lawson, 1997).

Caprenin is a structured lipid that contains caprylic, capric acids and behenic acid in its structure (Gunstone, 2001). Caprenin has a caloric value of 5 kcal/g (Akoh, 2008). Behenic acid is poorly absorbed in the intestine. Capric and caprylic acids are more readily metabolized than other longer chain fatty acids. Caprenin has functional properties that are similar to those of cocoa butter (Ognean et al., 2006). Caprenin was commercially unsuccessful because of its tempering difficulties although FDA was allowed its use in USA in 1991 (Akoh, 2008).

Salatrim (Benefat), is a structured triglyceride comprising mixture of at least one short chain fatty acid (C2:0, C3:0, or C4:0) and one stearic acid (C18:0) randomly attached to the glycerol (Kosmark, 1996). Salatrim has a caloric value of 5 kcal/g (Adamczak and Bednarski, 2010; Ognean, 2006; Swanson, 1996). The calorie reduction of Salatrim is a result of having short-chain fatty acids providing fewer calories and stearic acid is not fully absorbed by human body (Ognean et al., 2006). Salatrim should not be used for frying because of short chain fatty acids, which are hydrolysed easily at high temperatures during resulting with the development of off-flavors, in its structure (Clavier, 2008). FDA accepted Salatrim in 1994 as a GRAS (Generally Recognize as Safe) (Akoh, 1998a; Kosmark, 1996; Ognean et al., 2006).

Trialkoxytricarballylate, trialkoxycitrate, and trialkoxyglyceryl ether are polycarboxylic acids. They contain 2-4 carboxylic acids esterified with alcohols having 8–30 carbon. They can not be hydrolysed completely by lipases (Ognean et al., 2006).

Dialkyl dihexadecylmalonate is a fatty alcohol dicarboxylic acid ester of malonic acid and alkylmalonic acid. It is produced by the reaction of a malonyl dihalide and a

fatty alcohol. Dialkyl dihexadecylmalonate does not contain calorie since it is nondigestable. However, high temperature usages are not suitable for dialkyl dihexadecylmalonate (Akoh, 2008; Chung and Min, 2004; Ognean et al., 2006).

Esterified propoxylated glycerol is produced with the reaction of glycerol and propylene oxide in which a polyether polyol is formed. This polyether polyol is esterified with fatty acids. Esterified propoxylated glycerol shows an oil like property. The caloric value of esterified propoxylated glycerol is 0 kcal/g (Chung and Min, 2004). Oxypropylene group between the glycerol and fatty acids differs this product from triacylglycerols (Akoh, 1998a).

Sorbestrin is produced by esterification of sorbitol and sorbitol anhydrides with fatty acids of different chain length and saturation (Shahidi and Senanayake, 2006). Sorbestrin gives a caloric value of is 1.5 kcal/g. Sorbestrin shows thermally stability. Thus, it can be used at frying temperatures (Akoh, 1998a).

Emulsifiers have been in wide use in fat replacement systems. Incorporating emulsifiers such as mono and diacylglycerols (DAG) into the formulation of fat reduced products is appealing because these compounds are familiar ingredients with an established record of safety. Emulsifiers perform as fat replacers most optimally when used in combination with other emulsifiers and as part of a fat replacement system with other ingredients such as water or flavorings (Finley et al., 1997).

Table 2.1 Types of lipid based fat replacers

Trade name	Composition	Application
Olestra	Sucrose with 6-8 fatty acids	Cooking/frying oil, baked products, margarine, processed meats, chips, crackers
Caprenin	Caprocaprylobehenin Structured lipid	Chocolate, baked goods, margarine
Salatrim/Benefit	Structured triacylglycerol	Chocolate, baked goods, fillings, dressings
TATCA	Trialkoxytricarballylate	Mayonnaise, margarine, dressings

2.3.3.2 Carbohydrate-based Fat Replacers

An association of water with the carbohydrate gives fat-mimicking properties to carbohydrates. Final structure presents a sensory and rheological property close to

fat. Table 2.2 shows the examples for carbohydrate based fat replacers (Chung and Min, 2004). Carbohydrate-based fat substitutes have a caloric value of 0-4 kcal/g. Some of them are partially digestible while some of them are nondigestable (Khetarpaul et al., 2014).

Table 2.2 Types of carbohydate based fat replacers

Trade name	Composition	Application
Amalean I	Modified high amylose starch	Salad dressings, baked goods, souces
Avicel	Microcrystalline cellulose and carboxymethylcellulose	Baked goods, salad dressings, souces, ice cream, spreads
C*Pur 01906	Potato maltodextrin	salad dressings, souces, margarine
Fibruline	Inulin	Dressing, meat products
Keltrol	Xanthan	Baked goods, salad dressings, margarines
Maltrin M040	Hydrolyzed corn starch	Baked goods, dairy products, spreads, salad dressings, dips
Litesse	Polydextrose	Dressingsü, spreads, bakery fillings
N-Oil	Tapioca dextrin	Frozen desserts, soups, sauces, puddings
Paselli SA 2	Maltodextrin from potato starch	Baked goods, dips, spreads, mayonnaise
Raftiline	Inulin	Baked goods, desserts, beverages, ice cream
Slendid	Pectin from citrus peel	Spreads, mayonnaise, dressings, frozen deserts, baked goods
Staslim	Modified tapioca/potato starch	Salad dressings, soups, baked goods
Stellar	Corn starch	Baked goods, fillings, souces
Oatrim	B-glucans from oat flour	Dairy products, confectionary, frozen desserts, meat products

Paselli is a potato starch-based fat replacer. It may be used instead of fats and oils in different food products. Paselli contributes 3.8 kcal/g in diets. Paselli constructs a smooth thermostable gel with a fat-like texture under proper temperature (Shahidi and Senanayake, 2006).

N-Oil contains tapioca dextrin and may take the place of fats and oils in food products, giving the illusion of high fat content. Commercial N-Oil is available since 1984. N-Oil contributes 1 kcal/g in the diets. It shows stability under high temperature, shear, and low pH conditions (Shahidi and Senanayake, 2006).

2.3.3.3 Protein-based Fat Replacers

The protein-based fat replacers contain microparticulated protein. They are not suitable for frying temperatures since the protein is denatured at these temperatures (Chung and Min, 2004). Some examples of protein-based fat replacers are given in Table 2.3.

Simplesse had a GRAS approval for use frozen desserts in 1990 by the FDA (Singer and Moser, 1993). Simplesse is prepared from milk and/or egg white protein, sugar, pectin, and citric acid and is an all-natural versatile product. Microparticulation is used in the production of Simplesse. 1 μm in diameter microparticulated protein is produced using by homogenization and pasteurization at high temperatures (Chung and Min, 2004). Simplesse has a caloric value of 4 kcal/g, while its hydrated gel gives 1.3 kcal/g. Protein gel and its texture are not stable at high temperatures (Chung and Min, 2004; Clavier, 2008).

Table 2.3 Types of protein based fat replacers (Chung and Min, 2004)

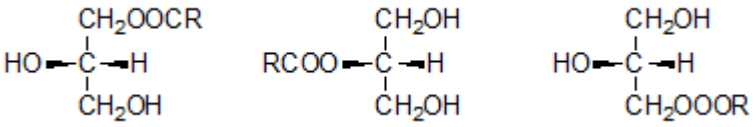
Trade name	Composition	Application
Simplesse	Microparticulated egg white and whey protein concentrate	Dairy products, spreads, baked goods, frozen desserts, salad dressings, mayonnaise
Dairy-Lo	Partially denaturated whey protein concentrate	Dairy products, spreads
Dairylight	Partially denaturated whey protein coagulate	Frozen desserts, sour cream, dairy products, salad dressings
Trailblazer	Egg white and xanthan gum	Frozen desserts, dressings, sauces, baked goods

2.3.4 Potential Effects of Fat Substitutes on Health Status

The effects of fat substitutes on health depend on type of fat substitutes. In literature, little or no adverse effect of carbohydrate or protein based fat substitutes on digestion, absorption and metabolism of other nutrients. Fat-based substitutes (Caprenin, salatrim, mono/di-acylglycerol) produced using different length and number of fatty acids also seems to have no adverse effect (Finley et al., 1997).

2.4 Monoacylglycerol (MAG)

Monoacylglycerols are defined as esters of the glycerol in which one of the hydroxyl groups is esterified with a fatty acid (Figure 2.1).



sn-1-monoacylglycerol *sn*-2-monoacylglycerol *sn*-3-monoacylglycerol

Figure 2.1 Three isomers of monoacylglycerol. R is a saturated or an unsaturated hydrocarbon chain.

MAG contains 3 isomers, which are regioisomeric (*sn*-2) and enantiomeric (*sn*-1 and *sn*-3). 1-/3-isomers are not differentiated from each other and are termed as 'α-monoacylglycerols'. 2-isomers are named as β-monoacylglycerols (Christie, 2014). 1(3)-MAG consist of 92–95% of total MAG at low temperatures and nearly 70% of the total MAG at high temperatures in the MAG mixtures (Zhong et al., 2014). However, 2-monoacylglycerol are not accepted as suitable for being a commercial product since it easily isomerizes to 1-MAG by acyl migration. However, it is an important product for the production of some lipid products such as symmetric structured triacylglycerols which can be synthesized from 2-MAG (de Freitas et al., 2010).

Monoacylglycerols are nonionic surfactants and contains hydrophilic and hydrophobic groups. MAG and their mixtures with diacylglycerols provide nearly 75% of the world's annual emulsifier production. They have a wide use in food products and in pharmaceutical industry as drug carriers (de Freitas et al., 2010; Zhong et al., 2014).

2.4.1 Production of Monoacylglycerol

MAG is produced industrially by continuous chemical glycerolysis of fats and oils under nitrogen. The restrictions of this chemical synthesis are high energy consumption, development of unwanted by-products, partial product degradation,

and high investment capital. Alternative to the chemical glycerolysis, MAG can be produced employing commercial lipases (de Freitas et al., 2010).

2.4.1.1 Chemical Production of Monoacylglycerols

Commercial MAG and DAG production is carried out by chemical glycerolysis of oils and fats at 220-250°C with alkaline catalysts such as KOH and NaOH under inert atmosphere. The chemical glycerolysis results with a mixture including 35-60% MAG, 35-50% DAG, 1-20% TAG and 1-10% free fatty acid (FFA) depending on oil and glycerol mass ratio and unwanted by-products like alkali metal salts (Singh and Mukhopadhyay, 2012).

Chemical reaction occurs randomly with the formation of mono-, di-, and triacylglycerol. The formation of isomers of *sn*-1-monoacylglycerol and of *sn*-1,3-diacylglycerol, *sn*-2-monoacylglycerol and *sn*-1,2-diacylglycerol can also occur (Figure 2.2). Thus, extensive purification of the product is required (Monteiro et al., 2003). Molecular distillation is required to get highly pure MAG for food industry (Bornscheuer, 1995).

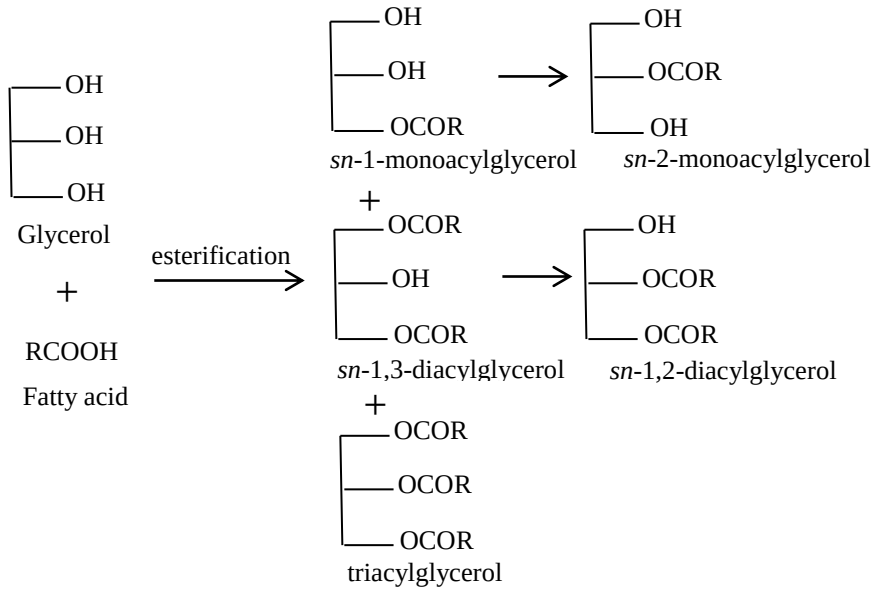


Figure 2.2 Chemical esterification of glycerol and fatty acid (Monteiro et al., 2003)

Furthermore, a high temperature chemical glycerolysis is not desirable for the production of heat-sensitive MAG consisting of polyunsaturated fatty acids. Polyunsaturated fatty acids, whose beneficial health and functional properties should be conserved, are destroyed at high temperature application. However, synthesis of heat-sensitive MAG is great commercial interest because of their nutrition value, which is desirable in food and pharmaceutical industry (Pawongrat et al., 2007; Zeng et al., 2010).

2.4.1.2 Enzymatic Production of Monoacylglycerol

The use of enzymes as catalysts is an alternative method as well as practical considerations because of the disadvantages of the chemical production. Enzymatic production of MAG may overcome the limitations of the chemical process in terms of the low MAG yield, low specificity, undesirable by-products, high energy consumption and the high capital investment.

The enzymatic production is generally performed at milder conditions. The lower temperature enhances MAG quality and makes synthesis of heat-sensitive MAG feasible (Gupta et al., 2012; Pawongrat et al., 2007; Yang et al., 2005). The enzymatic synthesis of monoglycerides may be performed by; (1) selective hydrolysis or alcoholysis of oils and fats; (2) direct esterification of glycerol with fatty acids or esters; (3) glycerolysis of fats and oils.

2.4.1.2.1 Hydrolysis or Alcoholysis of Oils and Fats

Monoacylglycerol may be produced through hydrolysis or alcoholysis of both position-specific and nonspecific lipases (Sellappan and Akoh, 2005). Hydrolysis or alcoholysis of TAG catalysed by *sn*-1,3 specific lipase yields 2-Monoacylglycerol (Figure 2.3). The other methods produce an equilibrium mixture of MAG, from which 1(3)-MAG may be separated in a good yield (Gupta et al., 2012). However, acyl migration restricted the 2-MAG yield by forming 1(3)-MAG which was subjected to further hydrolysis. Alcoholysis is an alternative to hydrolysis. Alcoholysis is performed in nonpolar solvents in which acyl migration occurs slower. Thus, alcoholysis of triglycerides yields higher amount of 2-MAG than that of hydrolysis. Moreover, alcoholysis reaction is faster than hydrolysis since pH is stable in the reaction. Hence, lipase is not inhibited by free fatty acids. However, the

yield of MAG is very low in both of the methods, although triacylglycerol is completely converted to monoacylglycerol. For hydrolysis and alcoholysis, one mole of monoacylglycerol is synthesized from 1 mole of triacylglycerol. The enzymatic hydrolysis is rarely used for MAG production (Yang et al., 2005).

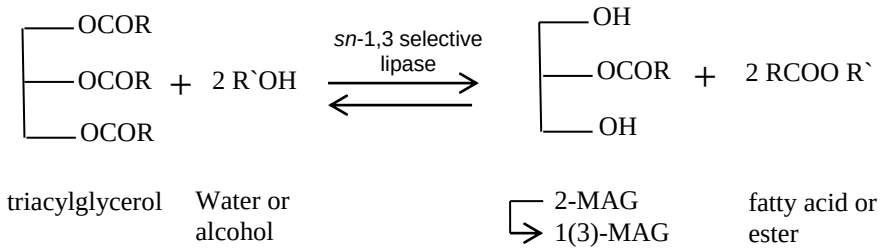


Figure 2.3 Hydrolysis or alcoholysis of a triacylglycerol to produce 2-MAG (Bornscheuer, 1995)

2.4.1.2.2 Direct Esterification of Glycerol with Fatty Acids or Esters

Glycerol may be esterified with free fatty acids using lipases (Figure 2.4). In esterification reactions, low water content and water activity are required for the high yield MAG production (Bornscheuer, 1995; Yang et al., 2005). Water, a by product in esterification reaction, should be separated from the reaction medium to drive the reaction toward. Adsorption, azeotropic distillation, inclusion of molecular sieves, and salts may be used to overcome this problem. Direct esterification is the least desirable method for the production of MAG because of the difficulties in removing the water (Sellappan and Akoh, 2005).

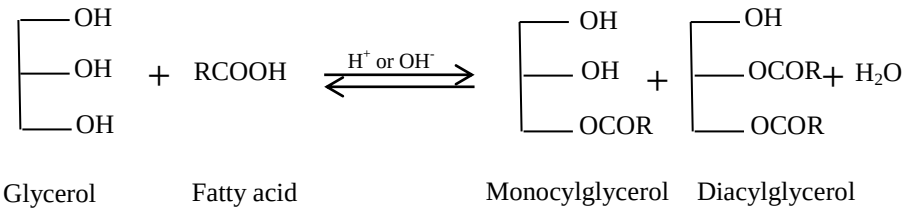


Figure 2.4 Monoacylglycerol synthesis through direct esterification (Hasenhuettl, 2008)

2.4.1.2.3 Glycerolysis of Fats and Oils

Glycerolysis is the reaction of glycerol and a triacylglycerol to yield MAG, DAG, TAG (Willis and Marangoni, 2008) (Figure 2.5). For food MAG, researcher and industry are interested in lipase catalyzed glycerolysis of oils and fats in solvent free

medium at low temperatures. Reaction products may be easily separated using vacuum distillation. This method had the advantage of low energy demands, possible reusability of immobilized enzymes, high yield (3 moles of monoacylglycerol is formed from 1 mole of triacylglycerol). Thus, production of monoacylglycerol and diacylglycerol may be produced by enzymatic glycerolysis which is an important alternative method (Yang et al., 2005; Singh and Mukhopadhyay, 2012).

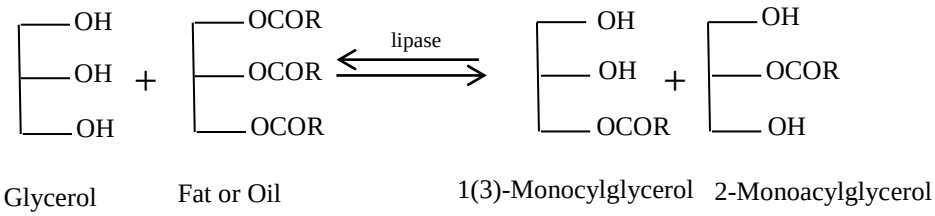


Figure 2.5 Monoacylglycerol synthesis through glycerolysis (Bornscheuer, 1995)

Glycerolysis is the most economic method if it is compared to hydrolysis or alcoholysis since all fatty acids of the triacylglycerols are converted into product in glycerolysis. Vegetable oils are used as raw material sources (Damstrup et al., 2007). Additionally, converting glycerol, which an important byproduct in biodiesel plants, into value-added products supplies an alternative for highly produced glycerol disposal and for its surplus problems (Pagliaro et al., 2007; de Freitas et al., 2010).

2.4.1.3 Strategies to Obtain High Content of 2-Monoacylglycerols

Enzymatic/chemical hydrolysis or alcoholysis or chemoenzymatic methods can be used for the synthesis of 2-MAG. Wang et al. (2014) mentioned that 2-monoolein produced using chemoenzymatic methods is not suitable for human consumption since toxic chemicals are used. Moreover, alcoholysis is desirable than hydrolysis for the synthesis of 2-MAG since acyl migration can be reduced in alcohol rather than in water as mentioned above (Gibon and Kellens, 2014; Diks and Bosley, 2000). Köse et al. (2002) also reported that lipase catalyzed alcoholysis was advantageous in the development of new processes since purer products can be obtained. For these reasons, in this thesis, the alcoholysis of refined olive pomace oil was investigated and performed to synthesize 2-monoacylglycerols.

2.4.1.4 Recovery of MAG from Reaction Mixture

Winterization of solvent is used for purification of MAG from a mixture consisting of high quantities of free fatty acids (Watanabe et al. 2006). Monoacylglycerol can be purified by temperature induced crystallization to obtain high yields in lipase catalysed monoacylglycerol synthesis. *n*-hexane is used to dissolve the oil layer and this solution is kept at 0°C for a specific time. The resulting precipitates were separated by using centrifugation (Watanabe et al., 2006; Watanabe and Shimada, 2011).

Monoglycerides may also be removed from di- and triglycerides by molecular distillation. To produce distilled monoglyceride, glycerolysis product with 40-60% 1-monoglyceride is submitted to thin film molecular distillation using either multi-stage centrifugal short path distillation procedure or a one stage wiped falling film cylindrical type of still. The distilled product contains about 95% monoglyceride along with diglyceride (about 3%), triglyceride (<1%) and free fatty acids (<1%) (Young et al., 1994).

2-monoacylglycerols may also be separated from alcoholysis reaction by solvent extraction method (del Mar Munio et al., 2008; Irimescu et al., 2002). Alcoholysis final reaction mixture includes major amounts of 2-monoacylglycerol and fatty acid ethyl esters (FAEE) and minor amounts of diacylglycerol and unreacted triacylglycerol. This separation can be achieved with the development of the biphasic system ethanol–water and hexane. Here, MAG is dissolved in the hydroalcoholic phase while FAEE and TAG are dissolved in the hexane phase (del Mar Munio et al., 2008).

2.4.2 Derivatives of Monoacylglycerols

Derivatives of monoglycerides containing organic acids such as acetic acid, lactic, diacetyltartaric, or citric acids have different properties than those of monoglycerides in terms of crystalline behaviour and surface activity (polarity) (Krog, 1997a). The properties desired in a monoacylglycerol may be improved by incorporation of different organic acids into one of the free hydroxyl groups on monoacylglycerol (Gunstone, 2004).

Organic acid esters are in principle made by reacting mono-diglycerides or distilled monoglycerides with anhydrides of the organic acid on a mole/mole basis yielding a mixture of components where the organic acid is esterified to one of the free hydroxyl groups on the monoglyceride molecule (Krog, 1997b).

2.4.2.1 Acetylated Monoacylglycerols

Acetylated monoacylglycerol may be synthesized by replacement of an acetyl (Ac) group with the hydroxyl group of monoacylglycerol. Production of acetylated monoacylglycerols may achieved with two different methods: (1) Reaction of monoacylglycerols with acetic anhydride to synthesis the acetate ester and one equivalent of acetic acid using strong mineral or organic acids as catalysts, (2) Reaction of monoacylglycerols with glyceryl triacetate using an alkaline catalyst. The second method is more advantageous than the first one as being less corrosive and less flammable (Hasenhuettl, 2008).

2.4.2.2 Lactylated Monoacylglycerols

A lactylated monoacylglycerol may be produced by addition of lactic acid into monoacylglycerol. By this way, hydrophilic effect of the molecule is increased while its non-ionic character is not changed. Synthesis of lactylated monoacylglycerol is carried out in two steps: (1) Synthesis of the mono or diacylglycerol or distilled monoacylglycerol, (2) Reaction of lactic acid with these intermediates (Hasenhuettl, 2008; Woods, 1961).

2.4.2.3 Succinylated Monoacylglycerols

The reaction of succinic anhydrate with monoacylglycerol is similar to the reaction of acetic anhydride and monoacylglycerols. However, succinic acid contains two carboxyl groups in its carbon chain and the second carboxyl group is retained in the surfactant molecule rather than expelled as an acid by-product. This product is more hydrophilic than monoacylglycerol and anionic at the appropriate pH (Freund, 1968; Hadeball, 1986; Hasenhuettl, 2008).

2.4.2.4 Citrate Esters of Monoacylglycerols

Citrate esters are produced with the reaction of acylglycerol with citric acid with the help of an acid catalyst such as acetic acid. Citrate esters of monoacylglcerols are derivatives with diverse functional groups. The hydrophilic head group is larger than monoacylglycerol in size and polarity (Bade, 1978; Hasenhuettl, 2008).

2.4.2.5 Diacetyltartaric Acid Esters of Monoacylglycerols

Diacetyltartaric acid esters of monoacylglycerols are the products produced by the reaction of a monoacylglycerol and a polycarboxylic acid. Self-condensation of tartaric acid is prevented by acetate esters since they behave as protecting groups. The hydrophilic head group of diacetyltartaric acid esters of monoacylglycerols are anionic at pH greater than its pKa. Synthesis of this derivative may be carried out in two or three stages: (1) Acetic anhydride and tartaric acid is reacted to synthesis diacetyltartaric acid using sulfuric acid as catalyst (Gladstone, 1960). (2) Optionally, the diacetyltartaric acid may be converted to its anhydride. (3) Diacetyltartaric acid or its anhydride and monoacylglycerol is reacted (Hasenhuettl, 2008).

2.4.2.6 Monoacylglycerol Phosphate

Conversion of a free hydroxyl group on monoacylglycerols with a phosphate ester introduces four (1P + 3O) additional electronegative heteroatoms into the molecule. The surfactant is anionic at pH values higher than pKa. Synthesis is performed by the reaction of a monoacylglycerol with phosphoric acid, polyphosphoric acid, or phosphorous pentoxide (Cawley and O'Grady, 1969; Hasenhuettl, 2008; Kazyulima, 1986).

These monoacylglycerol derivatives which are mentioned above are widely used as food emulsifiers. Another new approach for monoacylglycerol derivative is the synthesis of monoacylglycerol amino acid conjugates as food emulsifiers. The chemical design and structure of the monoacylglycerol amino acid conjugates are virtually same with those of the product which were synthesized in this thesis. Because of this reason, detailed information was given about the synthesis of monoacylglycerol amino acid conjugates in the following sections.

2.4.3 Monoacylglycerol Amino Acid Conjugates

Amino acid glyceride conjugates are a novel group of lipoamino acids, which are analogous of partial glycerides and phospholipids. They contain one or two aliphatic chains and one polar head, the amino acid, linked together through a glycerol moiety. These products are similar to the monoacyglycerol acid esters such as lactic, citric, tartaric and succinic glyceride esters (Clapés and Infante, 2002; Krog, 1997a; 1997b).

Amino acids contain two functional groups: carboxylic and amino group. Esterification of hydroxyl group of glycerol with α-carboxyl group of the amino acid may be performed chemical, enzymatic, and chemoenzymatic methods, although chemical processes have been prevalent due to their relatively low cost of production. In recent decade, more researchers have focused on the use of enzymatic methods to synthesize amino acid based surfactants. The enzymatic approach would be more attractive to manufacturers in the near future (Hasan et al., 2006; Rodrigues and Lafuente, 2010).

Preparation of monoacylglycerol amino acid derivatives composed of two steps:
1. Synthesis of amino acid glyceryl esters namely 1-O-(Nα-protected-aminoacyl) glycerols.
2. Acylation of the free hydroxyl groups of the glyceryl moiety with fatty acid using lipases as catalyst (Morán et al., 2001).

2.4.3.1 Chemical or Chemoenzymatic Synthesis of Monoacylglycerol Amino Acid Conjugates

In literature, there are several studies describing methods for the acylation of the glycerol employing chemical catalysts at approximately 100°C. However, hydrolysis of the ester bond between the amino acids and the glycerol occur at these temperatures (Pérez et al., 2004). Pérez et al. (2004) suggested a new reaction system in which the esterification of the two glycerol hydroxyl groups with fatty acyl chloride in pyridine as solvent at room temperature avoiding the hydrolysis of the ester bonds. However, this chemical synthesis does not satisfy the demands of pharmaceutical, food or cosmetic industry (Valivety et al., 1997).

Valivety et al. (1997) introduced the first chemoenzymatic synthesis methodolgy of amino acid glyceride conjugates. The first step is the synthesis of amino acid glyceryl esters namely 1-O-(Nα-protected-aminoacyl) glycerols. Reaction is performed using an acid catalyst such as BF_3 at about 60°C which served as solvent and reactant. In that situation, cumbersome orthogonal protection of polyfunctional amino acids is necessary (Morán et al., 2001). The synthesis was followed by the acylation of the free hydroxyl groups of the glycerol with fatty acid with the aid of lipases.

2.4.3.2 Enzymatic Synthesis of Monoacylglycerol Amino Acid Conjugates

Enzymatic synthesis of monoacylglycerol amino acid conjugates is more advantagous. Enzyme catalyzed reactions are performed under mild reaction temperatures (<70°C). By that way, high energy consumption, and the use of strong acids and bases may be avoided. Enzymatic reactions results in less impurities than chemical methods since they require mild conditions and are selective. Thus, enzymatic production facilitates product purification and improves yields. Also, formation of hazardous by-products because of strong chemical conditions, and toxic starting materials is avoided (Clapés and Infante, 2002).

The other particular advantages provided by enzymes are specificity and selectivity and reduced waste producing oils or fats with desired composition. Moreover, the desired products may be controlled and unwanted side reactions may be minimized by choosing the specific enzyme (Clapés and Infante, 2002; Hasan et al., 2006; Rodrigues and Lafuente, 2010).

The suggested method for the synthesis includes two steps: (1) Enzymatic synthesis of the amino acid glyceryol ester derivatives, (2) Enzymatic acylation of the hydroxyl groups of the amino acid glyceryl ester by fatty acids (Morán et al., 2002). *N*-protected amino acid glyceryl esters may be synthesized by enzymatic esterification of hydroxyl groups of glycerol with α-carboxyl group of the amino acid. Glycerol behaves both as a reactant and solvent at elevated temperature (Adlercreutz and Hatti Kaul, 2010; Moran et al., 2004; 2001).

Hydrolases may be used in the synthesis of monoacylglycerol aminoacid conjugates (Clapés and Infante, 2002; Mitin et al., 1997; Moran et al., 2001). Proteases and

lipases may also be used as biocatalysts in the production of monoacylglycerol amino acid conjugates. Proteases and lipases may be found in large quantities commercially. They are cheap and do not need cofactors (Clapés ve Infante, 2002).

Solvent free systems are uncommonly suitable for the production of lipoamino acids because of the problems in detecting a solvent for both amino acid and hydrophobic tail (Clapés and Infante, 2002). Despite the fact that the researches includes the use of organic solvents to increase the solubility of the reactants and products, reseachers recently has focused on the progressing solvent free reactions. Solvent free reactions are safer, more environmental friendly, supply higher space-time yields. Thus, they are more attractive for commercial applications (Clapés et al., 1999**)**.
Polarity of solvents, type of biocatalyst, time, substrate mole ratio, pH and temperature are the most important parameters that must be controlled in enzymatic reaction to control the final product composition.

2.4.3.2.1 Lipases as biocatalyts

Lipases catalyze hydrolysis, esterification and transesterification. Transesterification may be divided into three subclasses as alcoholysis, acidolysis and interesterification. Alcoholysis is the reaction with an ester and an alcohol. Interesterification occurs between two different esters, where alcohol and acid moiety is swapped (Pham and Pham, 2012).

Lipases do not need cofactors and may be used in free or immobilized forms. There are commercial cheap lipases which have high stability. Lipases work at the lipid-water interface. Thus, they do not require water-soluble substrates. This property differs lipases from other hydrolytic enzymes (Zarevúcka and Wimmer, 2008).

Three dimensional conformations of lipases determine their catalytic activity and stability. Since hydration state of the enzyme can change the 3-dimensional conformation of the lipase, the activity and stability of the enzyme is also changed. The active site of the lipase contains a movable lid-region (Sharma et al., 2001; Secundo et al., 2006). The enzyme is active only at the interface between a hydrophobic solvent and an aqueous medium since this interaction is associated with the opening of the lid (Cajal et al., 2000; Saifuddin and Raziah, 2008; Tejo et al., 2004).

Kinds of lipase selectivity:

1. Regio-selectivity
    - *sn*-1,3-Regio-selectivity
    - Non-selectivity
2. Fatty acid selectivity
    - Long chain fatty acids
    - Saturated fatty acids
    - cis- ω-9 unsaturated fatty acids
    - Short chain fatty acids
3. Acylglycerol selectivity
    - Monoacylglycerol
    - Monoacylglycerol and Diacylglycerol
    - Triacylglycerol

Acylglycerol combinations which may not be produced by conventional chemical methods may be obtained with the help of specific lipases. For example, *sn*-1,3 specific lipases can change the *sn*-1 and *sn*-3 positions of triacylglycerols while can not act on acyl groups at *sn*-2 positions (Pham and Pham, 2012).

Moreover, in literature there are some studies in which different lipases were used in the transesterification of N-blocked-L-amino acids and sugars as catalysts. For example, lipases from *Candida rugosa*, *Mucor javanicus*, *Pseudomonas cepecia* and *Pseudomonas fluorescens* catalysed the transesterification of N-blocked-L-phenylalanine ester and D-glucose regioselectively and that too preferably at the primary hydroxyl groups (Divakar, 2013). According to Valivety et al. (1997) who firstly performed a chemoenzymatic synthesis in monoacylglycerol amino acid conjugates, Novozyme and Lipozyme is successfully employed as biocatalysts.

2.4.3.2.2 Proteases as Biocatalysts

Proteolytic enzymes are widely used in food and other industries. They consist of approximately 60% of the industrial market in the enzyme technology (Pandey and Ramachandran, 2005). Proteases could catalyse hydrolysis of esters or amides of carboxylic acid, esterification or transesterification reactions or amide bond

formation. However, esterases and lipases are more efficient catalysts than proteases in esterification reactions since they have wider substrate specificities (Ward et al., 2009).

In contrast to lipases, proteases do not catalyse direct esterification with fatty acids as acyl donors, but proteases have proven to be efficient catalysts of transesterification reactions with carbohydrates (Pedersen et al., 2009). Proteases are used in the synthesis of glycoconjugates where subtilisin is the dominant enzyme used. Subtilisin is active in the synthesis of glycoconjugate through either catalysis of a new peptide or through direct acylation of carbohydrates **(**Ward et al., 2009**)**.

2.4.3.2.3 Solvents

A proper solvent has to dissolve sufficient amounts of substrates, amino acids and 2-monoacylglycerol in the enzymatic reaction system studied in this thesis. The solubilities of *N*-acetyl-L-amino acids and monoacylglycerol in organic solvents are different. Moreover, the solvent should not adversely impact the stability of the enzyme and its activity. The three dimensional conformation of the enzyme is changed due to the kind of organic solvent. The change in three dimensional conformation may result in the changes in catalytic activity, stereoselectivity, regioselectivity as well as stability (Gumel et al., 2011). Thus, solvent selection is very important in lipase catalysed reactions (Klibanov, 1990; Liu et al., 2009).

The term log P (the partition coefficient between water and octanol) is a tool of forecasting the denaturing effect of a solvent on an enzyme. Laane et al. (1987) reported that solvent with log P less than 2 is not suitable for enzyme-catalysed reactions since they distort the essential water from the enzyme, thereby inactivating them. Solvents with log P in the range of two to four are weak water distorters and their effect on the enzyme activity is not predictable. Solvents with log P larger than four do not distort the essential water layer, thereby being the ideal reaction media (Zhao et al., 2006).

However, a strongly hydrophobic solvent is not always the best selection for lipase catalyzed synthesis. For example, *N*α-acetyl amino acids, which were used as reactants in the reactions of this thesis, are not soluble in organic solvents and this

can adversely affect the product yield of the reaction. Thus, two or more solvents may change the polarity and ionization capacity of a nonaqueous reaction medium (Gumel et al., 2011).

2.4.3.2.4 *Nα*-Acetyl Amino Acids as Reactants

In this thesis, *N*-acetyl-L-leucine and *N*-acetyl-L-valine were incorporated into the 2-monoacylglycerol produced by alcoholysis of refined olive pomace oil. There were three basic reasons for selecting these amino acid derivatives:

1. Leucine and valine are essential amino acids
2. Leucine and valine are the most hydrophobic amino acids
3. Caloric value of leucine and valine is lower than that of fatty acids present in olive pomace oil.

Leucine and valine are essential amino acids which means that you have to get them from your diet. Leucine, isoleucine, and valine are branched-chain amino acids (BCAA) which consist of about one third of skeletal muscle tissue within the human body. These amino acids decrease the fat mass of the body while increasing the muscle mass (Sharp and Pearson, 2010). The benefits of leucine and valine can be ordered as: (i) regulate glucose level in blood, (ii) increase muscle development, (iii) decrease the nicotin level in liver, (iv) are effective on the therapy of brain and liver diseases, (v) help to decrease fat mass of the body, (vi) prevent the muscle mass loss related with getting older, (vii) give an appetite to the cancer patient (Belitz et al., 2009; Foster, 2011; Wagenmakers, 2004).

It is necessary to use appropriate protecting groups are required to prevent the formation of undesired bonds and side reactions in the systems in which amino acids are used as reactant (Llobet et al., 2009). *tert*-butoxycarbonyl (Boc) and acetyl groups may be used in these reaction systems. Boc protecting group is easily separated when subjected to acidic environment. On the other hand, acetyl exists in natural structures and provides the permanent protection of amine functions. It prevents the interaction of amine function with the acidic side functions and conserves the non-ionic character of the neutral amino acids (Morán et al., 2001).

2.5 Olive Pomace Oil

Olive oil production acquired an enormous socio-economic importance over the centuries by a continuous upward trend. Worldwide olive oil production is about 3.0 million tons per year, 75% (about 2.3 million tons) of which produced in the European Union (main producers: Spain, Greece, and Italy) in 2013-2014 (IOOC, 2014). Olive pomace (Figure 2.6) is a byproduct of olive oil production and is a very abundant agricultural waste in the Mediterranean countries (Redel-Macías et al., 2012).

Figure 2.6 Olive pomace after olive fruit processing

The most important product obtained from olive pomace is the olive pomace oil. This oil is useful in cosmetic and soap industries and also it can be used for the production of edible oil after refined at suitable conditions (Göğüş and Bozkurt, 2009). In olive oil industry, about 4.5 kg of pomace is obtained per liter of olive oil produced. Olive pomace contains 4–12% oil depending on the extraction technology (Meziane and Kadi, 2008).

The residual oil in the olive pomace is economically regained in oil extraction plants after decreasing the moisture content of olive pomace to 5–8%. The extracted oil is called as crude pomace oil. Refined olive pomace oil (ROPO) is that acquired from crude pomace oil by a refining process (Göğüş and Maskan, 2006).

The quality of the oil recovered from the olive pomace oil is mainly depends on process conditions. Drying applications with long storage periods and high temperature makes the oil inferior grade. After elimination of impurities, its

triacylglycerol composition is same with that of olive oil since olive pomace oil is actually olive oil but extracted using solvent (Çiftçi et al., 2009).

2.5.1 Extraction of Olive Pomace Oil

Olive oil production process is achieved by an order of operation consisting of: acceptance of olives, separating of impurities, washing with water, crushing of olives, malaxation of paste, olive oil extraction, final centrifugation, storage, filtration and bottling (Figure 2.7) (Valta et al., 2015).

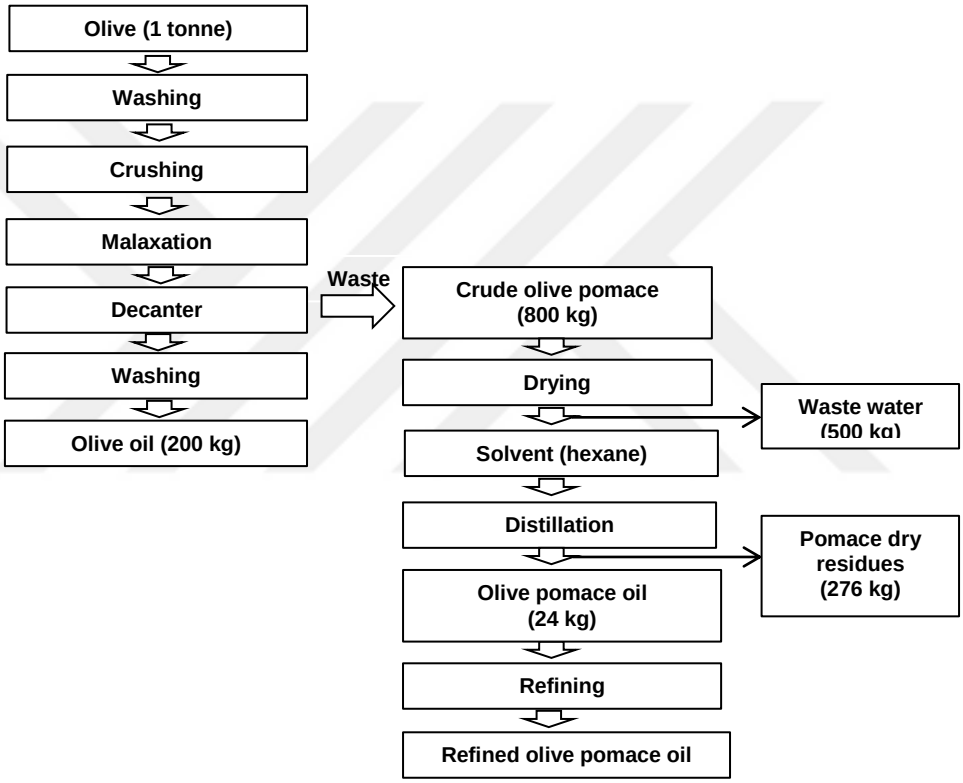


Figure 2.7 Flowchart of olive oil and refined olive pomace oil production

Mechanical production of olive paste lasts with two remaining products: pomace and water. Olive pomace existed from conventional pressing and three phase decanter centrifugation is the origin of income for the oil mills since they sell it to industrial plants where the residual oil is extracted by hexane. Olive pomace must be transferred directly to the plant for drying. By this way, fermentation of olive pomace may be stopped and hydrolysis of the oil may be prevented (Petrakis, 2006). The amount of residual oil in the pomace changes due to the extraction type. The amount of extracted oil is generally 6–8% in pressure systems and 3–5% in the three phase

extraction systems. The recovery of the oil from the pomace is consisted of two steps,

(1) Drying of the pomace until approximately 7-8% humidity to obtain optimum extraction yield,
(2) The dried pomace is crunched and extracted using hexane. Hexane is removed by distillation from the extracted crude olive pomace oil (Figure 2.7) (Boskou, 2011; Petrakis, 2006).

2.5.2 Refining of Olive Pomace Oil

The extracted oil from the pomace is dark green (high chlorophyll content), high acidity (>3% as oleic acid) and has a bad odor and taste with a high waxes content. Because of the solvent extraction, olive-residue oil has also minor components such as waxes, sterols, erythrodiol and uvaol at higher levels than those of olive oils. (Boskou, 2011). Therefore, it is not suitable for human consumption and it is necessary for olive pomace oil to be subjected to refining to become edible (Petrakis, 2006).

Refining of olive pomace involves the same methods operated for olive oil refining. However, ROPO may not be used for human consumption in many countries and European Union countries because of flavor considerations. A blend of refined olive pomace oil and virgin olive oil is called as olive pomace oil and it is fit for human consumption (Vossen, 2007).

2.5.2.1 Degumming

Degumming is processed to remove phospholipids in the olive pomace oil using phosphoric or citric acid. Phosporic or citric acid form hydrated phospholipids at 60-80°C. These hydrated phospholipids are removed from the oil phase using decantation in batch system or continuous centrifugation (Boskou, 2011; IOOC, 2006; Petrakis, 2006).

2.5.2.2 Neutralization

Nearly all free fatty acids are removed from olive pomace oil using alkali neutralization by convertion of free fatty acids into oil soaps using NaOH. The oil-

insoluble soaps are decanted using batch neutralizers to be separated. In continuous systems, degumming and alkali neutralization present in series and separation takes place with centrifugation (Petrakis, 2006).

2.5.2.3 Bleaching

Bleaching removes the undesirable colours of the neutralized pomace oil. The pigments in olive oil are carotenoids and chlorophylls, and also the components obtained from these pigments because of the acidity. The seperation of chlorophylls from olive pomace oil is principally consequential since chloropylls can not be removed during deodorization unlike carotenoid. Primary and secondary oxidation compounds are also adsorbed during bleaching, beside metals, detergents, phospholipids, pesticides, and polyaromatic substances (Gandul et al., 1999; Ouyan et al., 1980; Ruiz-Méndez et al., 2011; 2005). 2-3% acid-activated earth is stirred with the preheated oil (90-100°C) for 30 min under 30-40 mmHg in bleaching step. Filtration is used to remove activated earth and charcoal from the bleached oil (Petrakis, 2006). Vacuum should be used during bleaching to reduce oxidation (Ruiz-Méndez et al., 2013).

2.5.2.4 Deodorization

Other components such as fatty acid ethyl esters and phenols may be developed in the time of olive-pomace oil storage. Substantial amounts of fatty acid ethyl esters, developed by their reaction with short-chain alcohols ending with the fermentation of organic materials in the presence of water, can be present with a percentage of 1 to 5 (Ruiz-Méndez and Ramos-Hinojosa, 2003). These compounds can be removed during deodorization. Deodorization is a steam distillation at reduced pressures (4-8 mmHg) and elevated temperatures (180-200°C) (Petrakis, 2006).

2.5.2.5 Winterization

Winterization is employed for pomace oil which is rich in waxes to avoid precipitation or cloudiness during storage (Boskou, 2011; Petrakis, 2006). The aim is to remove any compound that causes the cloudy final product (de Greyt and Kellens 2000). The process consists of freezing (5-8°C), ‘maturing’ to increase the crystal size and the addition of 5% water. The aqueous phase with the wax is separated by

centrifugation from the dewaxed oil. Solids formed are separated by filtration. (Boskou, 2011; Petrakis, 2006).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Refined olive pomace oil (ROPO) was obtained from Bilginoğlu Marbil Yağ San. ve Tic. AŞ., Izmir, Turkey. Lipases from *Mucor miehei* (immobilized on macroporous ion-exchange resin), *Candida antarctica* (immobilized on a macroporous acrylic resin), *Rhizopus oryzae* (immobilized on immobead 150), *Pseudomonas fluorescences* (immobilized in Sol-Gel-AK), *Aspergillus niger* (immobilized in Sol-Gel-AK), *lipase B Candida antarctica* (immobilized on immobead 150), proteases from *Rhizopus* spp., *Aspergillus oryzea*, *Bacillus* spp., *Bacillus licheniformis* were purchased from Sigma-Aldrich. Thin layer chromatography (TLC) plates (Kieselgel G) were obtained from Merck (Darmstadt, Germany). *N*-acetyl-L-leucine and *N*-acetyl-L-valine were obtained from Ark Pharm Inc. (Libertyville, IL, USA). Chromarods (Chromarod S-IV) were purchased from NTS (Germany). Ethanol and *n*-hexane were purchased at HPLC grade from Sigma–Aldrich. All other reagents and solvents were of analytical or chromatographic grade.

3.2 Methods

3.2.1 Free Fatty Acid Content and Peroxide Value of ROPO

Free fatty acid content and peroxide value of refined olive pomace oil were analyzed according to official methods AOCS Official Method Ca 5a-40 (AOCS, Ca 5a-40) and AOCS Official Method Cd 8-53 (AOCS, Cd 8-53), respectively.

3.2.2 Triacylglycerol (TAG), Diacylglycerol (DAG) and Monoacylglycerol (MAG) Composition

The composition of ROPO including triacylglycerol, diacylglycerol and monoacylglycerol was determined using the method proposed by the AOCS Official

Method Ce 5b-89 by HPLC (Shimadzu, Japan). 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 injected into the column (Sphereclone 5 μ ODS (2), 250 x 4.6 mm; Phenomenex, USA) with an accompanying guard column (40 x 3-mm id) of the same phase and eluted at a flow rate of 1.0 mL/min. The column temperature was set at 30°C and elution was monitored with an RID-10A (Shimadzu, Japan). All triacylglycerol contents were expressed in area percentages. All analyses were performed in duplicate and average values were reported.

3.2.3 Fatty Acid Composition

Fatty acid composition at *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 TAG. The products of lipolysis were separated by TLC plates that were developed with petroleum ether:diethyl ether:acetic acid (70:30:1, v:v:v). The *sn*-2 MAG band was scraped off and was extracted with diethyl ether. *sn*-2 MAG of ROPO was converted to fatty acid methyl esters (FAMEs) prior to GC analysis. The method suggested by international olive oil council (IOOC, 2001) was used for methylation. 100 mg of the oil sample was mixed with 2 mL heptane. 0.2 N methanolic potassium hydroxide solution was added to the mixture and vortexed vigorously for 30 sec. After the upper layer became clear, upper solution was decanted and analyzed by GC as described below. The fatty acid composition at *sn*-1,3 position was also calculated. The mean composition of each fatty acid in positions *sn*-1 and *sn*-3 can be calculated from its concentration in the intact triacylglycerol and in position *sn*-2 by means of the relationship (Equation 3.1).

$$[\text{position 1 or 3}] = (3 \times [\text{FA\% in TAG}] - [\text{FA\% in position 2}])/2 \quad (3.1)$$

3.2.4 GC Analysis

After methylation, the fatty acid composition was determined with an Agilent 7890A gas chromatograph (Agilent Technologies, USA) equipped with a flame ionization detector, a split/splitless injector and a HP-88 capillary column (88 % Cianopropylaryl 100 m x 0.250 mm ID x 0.20 μm) following the method suggested

by Çiftçi et al. (2009). The temperature of the injector was 250ºC and the split ratio was 50:1. The oven was kept at 120ºC for 1 min, then the temperature was increased from 120ºC to 175ºC at a rate of 10ºC/min, held for 10 min, and then increased from 175 to 210ºC at a rate of 5ºC/min, held for 5 min, followed by increase at a rate of 5ºC/min to 230ºC and held at this temperature for 5 min. The detector was set at 260ºC. Helium was used as the carrier gas, flowing at a rate of 1.5 mL/min. Fatty acid methyl esters were identified by comparison with the relative retention times of standard mixtures.

3.2.5 Production of 2-Monoacylglycerol (2-MAG) from Refined Olive Pomace Oil

2-MAG was synthesized from ROPO using *sn*-1,3 specific enzyme with enzymatic hydrolysis and ethanolysis.

3.2.5.1 Enzymatic Hydrolysis Reaction

3.2.5.1.1 Lipase Screening for Hydrolysis Reaction

Effects of lipases from different sources on 2-MAG synthesis using enzymatic hydrolysis were investigated. For this aim, lipase from *Porcine pancrease and* immobilized lipases from *Mucor miehei, Candida antarctica* and lipase B from *Candida antarctica* were tested. The reaction conditions were enzyme load 15 (wt.)%, temperature 50°C, time 12 h, oil:water ratio (1:5, v:v) in the reactions which were occurred using lipases from *Mucor miehei* and *Candida antarctica* and lipase B from *Candida antarctica*. The reaction conditions were enzyme load 15 (wt.)%, temperature 25°C, time 12 h, oil:buffer ratio (1:5, v:v), buffer concentration 0.025 M (pH 8-8.5) in the reactions which were occurred using lipase from porcine pancrease. Different reaction conditions were studied for lipase from porcine pancrease and other lipases depending on their optimum reaction conditions. 2.37 g ROPO (874.91 g/mol) was used in the reactions.

3.2.5.1.2 Synthesis of 2-MAG using Lipase from Porcine Pancrease

ROPO was hydrolyzed using *sn*-1,3 specific lipases from *Porcine pancreas*. The range of reaction parameters were time (2-24 h), enzyme load (5-20 wt%), buffer concentration (0.01-0.5) for the reaction. Oil:buffer solution ratio was 1:5 (v:v) in

these reactions. The reactions were carried out in a rotary incubator (New Brunswick Scientific, Nova 40, USA) at 250 rpm. The reaction with porcine pancreatic lipase were ended using 20 mL ethanol:acetone (1:1, v:v) mixture. The efficiency of the hydrolysis reaction was expressed as the the conversion% (Equation 3.2).

$$\text{conversion } (\%) = \frac{\text{weight of free fatty acids after hydrolysis reaction}}{\text{theoretical weight of fatty acids at } sn\text{-}1,3 \text{ positions}} \quad (3.2)$$

$$weight\ of\ free\ fatt\ acids\ after\ hydrolysis\ reaction\ (oleic\ acid) = N * 28.2 * V$$

In hydrolysis reaction, the weight of free fatty acids were determined using AOCS method Ca 5a-40 (AOCS, 5a-40). Here, N is the normality of NaOH used in neutralisation; V is the used volume of NaOH in the neutralization. Theoretical weight of fatty acids at *sn*-1,3 position was determined using the results of GC analysis.

3.2.5.1.3 Neutralization of Free Fatty Acids

Neutralization was done in a similar manner as Lee and Akoh (1998) with some modification. Reaction mixture (5-6 g) was mixed with 150 mL hexane, 1 mL phenolphthalein solution and required amount of 0.5 N KOH in 20% (vol) ethanol (B mL) in a separatory funnel. The upper phase was collected. Lower phase was washed again with 50 mL hexane and the 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 was added to the collected upper phase. After shaken, the upper phase was collected and centrifuged for 10 min at 6000 rpm to remove the soaps. After centrifugation, the upper phase collected and the hexane was evaporated to obtain the neutralized product containing TAG, DAG and MAG.

3.2.5.1.4 Analysis of Hydrolysis Reaction Products

The products of lipolysis were determined by reversed phase HPLC using the method proposed by Çiftçi et al., (2009). Oil was diluted in acetone, filtered and injected into an HPLC system consisting of a quadratic pump (model LC-10ADVP; Shimadzu, Japan) equipped with a column (Sphereclone 5 µm ODS (2), 250 x 4.6

mm; Phenomenex, USA) with an accompanying guard column (40 x 3 mm id) of the same phase and an ultraviolet (UV) detector (Schimadzu, SPD 20A). Elution was monitored by UV absorbance at 215 nm. The mobile phase consisted of acetone and acetonitrile (50:50, v:v) with a flow rate of 1.0 mL/min. The column temperature was set at 50ºC with a column heater. Compositions of the compounds were given in percentage area.

3.2.5.2 Enzymatic Ethanolysis Reaction

3.2.5.2.1 Lipase Screening for Ethanolysis Reaction

Ethanolysis of ROPO (2.29 mmol) was performed in a 50-mL tightly closed screw-capped flask. ROPO was combined with 8 mL ethanol. The reactions were catalyzed by immobilized lipase from *Mucor miehei*, *Candida antarctica*, *Rhizopus oryzae*, *Pseudomonas fluorescences*, *Aspergillus niger* or Lipase B from *Candida antarctica* added in the amount of 10% (w:w based on the weight of ROPO). The reactions were carried out in a rotary incubator (New Brunswick Scientific, Nova 40, USA) at 55°C, 6 h and 250 rpm. At the end of the reactions, the product was separated from the enzyme by decanting. The excess ethanol was evaporated in a rotary vacuum evaporator (Heidolph Instrument GmbH & Co. KG. Schwabach, Germany) at 40°C and the solvent free mixtures were stored at -35°C for subsequent analysis. All reactions were performed in duplicate and the mean values were reported.

3.2.5.2.2 Preliminary Studies for Experimental Design of Ethanolysis Reaction

Pre-experiments were done to determine the ranges of the parameters to be studied prior to experimental design generated by Design Expert 7.0 using response surface methodology. Temperature (40-70°C), time (2-24 h), enzyme load (5-25 wt%), ethanol:oil molar ratio (3:1-70:1) were studied as reaction parameters since they are the most important parameters which effect the ethanolysis reaction. Ethanolysis of ROPO (2.29 mmol) was performed in a 50-mL tightly closed screw-capped flask. ROPO was combined with required amount of ethanol and lipase. Time and temperature were set at specificied values. The reactions were carried out in a rotary incubator (New Brunswick Scientific, Nova 40, USA). At the end of the reactions, the product was separated from the enzyme by decanting. The excess ethanol was evaporated in a rotary vacuum evaporator (Heidolph Instrument GmbH & Co .KG.

Schwabach, Germany) at 40°C and the solvent free mixtures were stored at -35°C for subsequent analysis. All reactions were performed in duplicate and the mean values were reported.

3.2.5.2.3 Synthesis of 2-MAG using Lipase from *Candida antarctica*

As a result of lipase screening, the most effective lipase was found as lipase from *C. antarctica.* In further experiments, immobilized lipase from *C. antartica* was used in the synthesis of 2-MAG. ROPO (2.29 mmol) and the corresponding amount of ethanol and enzyme were mixed as given in Table 3.1. The values of time and temperature were set according to experimental central composite design (Table 3.1). The reactions were performed in a rotary incubator (New Brunswick Scientific, Nova 40, USA) at 250 rpm. At the end of the reactions, the product was separated from the enzyme by decanting. The excess ethanol was evaporated in a rotary vacuum evaporator (Heidolph Instrument GmbH & Co. KG. Schwabach, Germany) at 40°C and the solvent free mixtures were stored at -35°C for subsequent analysis. All reactions were performed in duplicate and the mean values were reported.

Table 3.1 The ranges of parameters studied in the experimental design of ethanolysis reaction

Parameter	- α	-1	0	+1	+α
Time (h)	1.00	4.00	7.00	10.00	13.00
Enzyme load (wt%)	6.00	10.00	14.00	18.00	22.00
Temperature (ºC)	37.50	45.00	52.50	60.00	67.50
Ethanol:oil molar ratio	15.00	30.00	45.00	60.00	75.00

3.2.5.2.4 Determination of Lipase Reusability

The reusability of the selected lipase after lipase screening in the ethanolysis of ROPO was studied under the optimum reaction conditions determined by response surface methodology (RSM). After completion of each reaction, the enzyme was washed with ethanol, dried and reused in the next reaction. 2-MAG yield (%) was determined for each reaction.

3.2.5.2.5 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 to obtain maximum yield of 2-MAG. CCRD was composed of 30 experimental points consisting of 16 axial points, eight star points and six center points (Table 3.2). All 30 runs were performed in a totally random order to avoid bias. The independent variables and their levels were as follows: reaction time (4–10 h), reaction temperature (45–60ºC), enzyme load (10–18 wt%) and ethanol:oil molar ratio (30:1-60:1).

The data obtained were analyzed using RSM (Stat-Ease, Design-Expert software, version 7). Analysis of variance (ANOVA), regression analysis and model generation were used to evaluate the effects of factors and to optimize reaction conditions. The level of significance for all tests was set at 95% confidence level. The goodness of the models established was determined using the coefficient of determination, R^2, together with the absolute average deviation values and ANOVA (Arifin et al., 2010). The first- or second-order coefficients were generated by regression analysis with backward elimination. The quadratic response surface model was fitted to the equation (3.3): where Y_i (i=1) is the response for the 2-MAG yield (%). β_0, β_i, β_{ii}, β_{ij} are constant coefficients of intercept, linear, quadratic and interaction terms, respectively, x_i and x_j are independent variables and ε is the random error.

$$Y_i = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} X_i^{\,2} + \sum_{i=1}^{3} \sum_{j=i+1}^{4} \beta_{ij} X_i X_j + \varepsilon \qquad (3.3)$$

3.2.5.2.6 Purification of 2-MAG

Separation of the 2-MAG by solvent extraction was carried out using a modification of the method described by Rodríguez et al. (2012). The ethanol free reaction product was dissolved in ethanol:water mixture (90:10, v:v) in the proportion 1:9 (v:v) at 50°C. For extracting esters, triacylglycerols, diacylglcyerols and fatty acids, this mixture was washed two times with an equal volume of hexane. The ethanolic phases were mixed and extracted with hexane to remove the residual ethyl esters

Table 3.2 A five-level, four factorial central composite rotatable design generated for ethanolysis reaction of ROPO to synthesize 2-MAG

Run	t[a] (h)	En[b] (wt%)	T[c] (°C)	Ethanol:Oil Molar Ratio	2-MAG (%)[d]		2-MAG Yield (%)[e]	
					Experimental	Predicted	Experimental	Predicted
1	4	10	45	30	32.16±0.50[f]	30.46	79.02±0.50	74.81
2	10	10	45	30	23.38±0.75	23.35	57.44±0.75	57.34
3	4	18	45	30	22.15±1.20	21.46	54.42±1.20	52.69
4	10	18	45	30	4.06±0.35	1.47	9.98±0.35	3.45
5	4	10	60	30	18.93±0.27	19.70	46.51±0.27	48.36
6	10	10	60	30	30.78±0.56	27.18	75.63±0.56	66.74
7	4	18	60	30	24.85±0.37	21.53	61.06±0.37	52.85
8	10	18	60	30	12.96±0.45	16.08	31.84±0.45	39.47
9	4	10	45	60	32.09±1.25	31.23	78.85±1.25	76.69
10	10	10	45	60	32.62±0.98	32.84	80.15±0.98	80.64
11	4	18	45	60	34.09±0.75	34.58	83.76±0.75	84.93
12	10	18	45	60	21.77±0.25	23.26	53.49±0.25	57.11
13	4	10	60	60	13.64±0.26	13.17	33.51±0.26	32.32
14	10	10	60	60	26.42±0.48	29.37	64.91±0.48	72.13
15	4	18	60	60	25.07±1.15	27.36	61.60±1.15	61.59
16	10	18	60	60	32.04±0.58	30.63	78.72±0.58	74.81
17	1	14	52,5	45	17.63±0.49	18.94	43.32±0.49	46.62
18	13	14	52,5	45	15.59±0.56	15.10	38.30±0.56	37.19
19	7	6	52,5	45	24.35±0.82	25.27	59.83±0.82	62.18
20	7	22	52,5	45	17.60±0.62	17.53	43.32±0.62	43.15
21	7	14	37,5	45	32.85±0.84	34.27	80.71±0.84	83.77
22	7	14	67,5	45	31.47±0.65	30.87	77.32±0.65	75.42
23	7	14	52,5	15	13.66±1.25	17.53	33.56±1.25	42.51
24	7	14	52,5	75	35.37±0.56	34.27	86.90±0.56	80.14
25	7	14	52,5	45	29.19±0.64	32.32	71.72±0.64	79.59
26	7	14	52,5	45	32.06±0.56	32.32	78.77±0.56	79.59
27	7	14	52,5	45	32.23±0.58	32.32	79.19±0.58	79.59
28	7	14	52,5	45	35.18±0.87	32.32	86.44±0.87	79.59
29	7	14	52,5	45	33.07±0.45	32.32	81.25±0.45	79.59
30	7	14	52,5	45	32.24±0.72	32.32	79.21±0.72	79.59

[a] time; [b] enzyme load; [c] temperature; [d] 2-MAG (%) in final reaction mixture; [e] (2-MAG (wt%) in final reaction mixture/theoretical yield)*100; [f] Mean (SD) (n= 3).

from the ethanolic phase. The ethanol remained in the ethanolic phase was evaporated using rotary vacuum evaporator at 40°C. The residual water was evaporated at vacuum oven set at 40°C for 4 h. Figure 3.1 summarizes the experimental procedure used for the purification. The purified 2-MAG was stored at -35°C until analysis.

For low temperature crystallization, the resultant reaction product (2 g) was dissolved in 20 mL n-hexane at room temperature. The solution was cooled to -18^{o}C and left at this temperature for 1 h. White crystals formed were separated with centrifugation at -10ºC for 15 min at 8000 rpm. The purified 2-MAG were stored at -35°C until analysis.

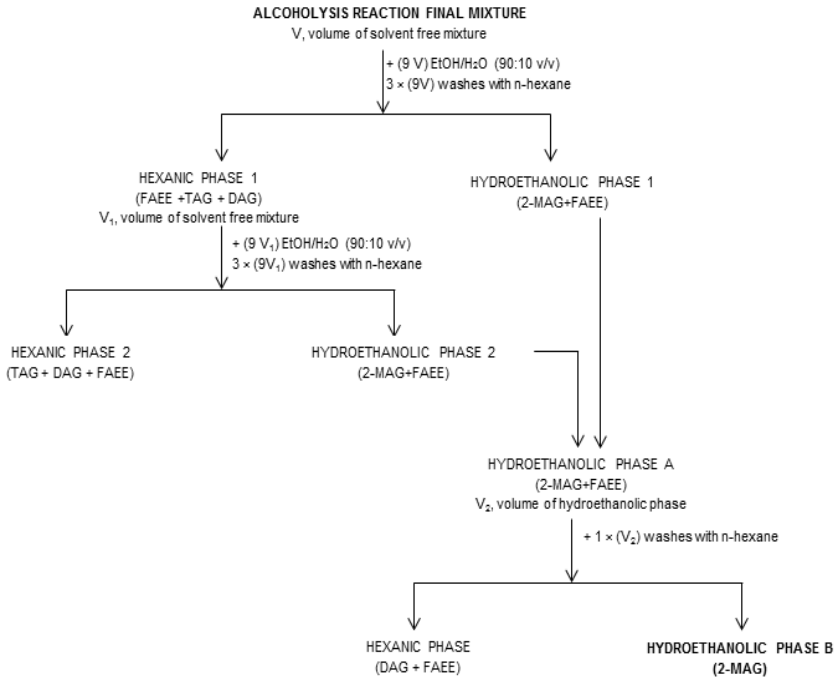


Figure 3.1 Experimental procedure for the recovery of 2-MAG from the alcoholysis final mixture by solvent extraction. FAEE, fatty acid ethyl ester; TAG, triacylglycerol; DAG, diacylglycerol; 2-MAG, 2-Monoacylglycerol.

3.2.5.2.7 Determination of the Ethanolysis Reaction Mixture Composition by Thin Layer Chromatography- Flame Ionization Detector (TLC-FID)

The procedure was performed according to the method described by Adamczak and Bornscheuer (2013). Boric acid-treated chromarods (Chromarod S-IV) were used for the analysis with the Iatroscan device (Iatroscan MK-5; Iatron Laboratories, Tokyo,

Japan) to separate 2-MAG and 1(3)-MAG. The preparation of boric acid treated chromarods was performed by diping the rods into the 3% boric acid solution and then drying for 5 min at 120ºC. The rods loaded with the samples were eluted for 8 cm with 100% chloroform. After drying the developed sample at room temperature for 5 min, the Chromarods were again developed for 10 cm with chloroform:(methanol: NH_4OH, 8: 2, v:v) (70:0.05, v:v). The developed sample was then dried in an oven at 120ºC for 5 min. Scanning was performed under the following conditions: gas flow rate 160 mL/min, air flow rate 2.0 L/min, scanning speed 30 s/scan. The yield of 2-MAG synthesis was expressed as a percentage of mass of 2-MAG synthesized (% by weight) to the theoretical mass of 2- MAG (40.70% by weight). The theoretical mass was calculated based on the *sn*-2 fatty acid composition of ROPO.

3.2.6 Model Reaction Between Glycerol and *N*α-acetyl-L-amino acids

Model reactions between 1 mmol glycerol and 4 mmol *N*-acetyl-L-leucine or *N*-acetyl-L-valine were performed using 500 µL DMF in 50-mL tightly closed screw-capped flask. The reactions were catalyzed by lipases or proteases from different sources. Enzymes were added in the amount of 20 (wt%, based on the weight of *N*α-acetyl-L-amino acid) in the reactions. Immobilized lipases from *Mucor miehei, Candida antarctica, Thermomyces lanuginosus* and proteases from *Rhizopus spp., Aspergillus oryzea, Bacillus spp., Bacillus licheniformis* were studied as catalysts. The reactions were carried out in a rotary incubator (New Brunswick Scientific, Nova 40, USA) at 55°C, 24 h and 225 rpm. At the end of the reactions, the product was separated from the enzyme by decanting. The solvent was evaporated under N_2 and the solvent free mixtures were stored at -35°C for subsequent analysis. All reactions were performed in duplicate and the mean values were reported.

3.2.7 Incorporation of Nα-acetyl-L-amino acids into Purified 2-MAG

3.2.7.1 Solvent Screening for Solubility of Reactants

The solubilites of *N*-acetyl-L-leucine, *N-acetyl*-L-valine and 2-MAG at different solvent systems were determined at 25°C. DMF, ethanol, methanol, 0.1% TFA (in water), 0.085% TFA (in water:acetonitrile), acetone, acetonitrile, cyclohexane, dichloromethane, hexane, methyl ethyl ketone were tested as solvents.

3.2.7.2 Solvent Screening for Reaction Medium

The enzymatic reactions were performed using 1 mmol *N*-acetyl-L-leucine and 0.25 mmol 2-MAG in 6 mL of the relevant solvent for 72 h. Lipases from different sources and different solvent combinations were tested in the reactions. Enzyme load was used as 20 (wt%, based on the weight of *N*-acetyl-L-leucine) in the reactions. The reactions were carried out in a rotary incubator (New Brunswick Scientific, Nova 40, USA) at 55°C and 225 rpm. At the end of the reactions, DMF was removed using nitrogen and the other solvents were evaporated in a rotary vacuum evaporator (Heidolph Instrument GmbH & Co.KG. Schwabach, Germany) at 40°C and the solvent free mixtures were stored at -35°C for subsequent analysis. All reactions were performed in duplicate and the mean values were reported.

3.2.7.3 Preliminary Studies for Experimental Design

As a result of lipase screening, the most effective enzyme was found as lipase from *Thermomyces lanuginosus.* In further experiments, immobilized lipase from *T. lanuginosus* was used for the incorporation of *N*-acetyl-L-leucine (or *N*-acetyl-L-valine) into 2-MAG. The effects of independent parameters (temperature, time, enzyme load and 2-MAG: *N*-acetyl-L-leucine (or *N*-acetyl-L-valine) molar ratio on the reaction were studied. The ranges were selected as 10-40 (wt%) for enzyme load, 2-72 h for time, 40-65°C for temperature, 1:2-1:6 for 2-MAG: *N*-acetyl-L-leucine (or *N*-acetyl-L-valine) molar ratio. Reactions were performed using lipase from *Thermomyces lanuginosus* in 5 mL ethanol:hexane (4:1, v:v) solvent mixture. The reactions were carried out in a rotary incubator (New Brunswick Scientific, Nova 40, USA) at 225 rpm. Solvents were evaporated in a rotary vacuum evaporator (Heidolph Instrument GmbH & Co.KG. Schwabach, Germany) at 40°C and the solvent free mixtures were stored at -35°C for subsequent analysis. All reactions were performed in duplicate and the mean values were reported.

3.2.7.4 Incorporation of *N*-acetyl-L-leucine (or *N*-acetyl-L-valine) into 2-MAG

0.5 mmol 2-MAG and 2 mmol of *N*-acetyl-L-leucine (or *N*-acetyl-L-valine) were reacted in 5 mL ethanol:hexane (4:1, v:v) mixture. The ranges of the parameters were given in Table 3.3. The corresponding amount of enzyme was added into the reaction as given in Table 3.4. The values of time and temperature were set according to an

experimental central composite design (Table 3.4). The reactions were performed in a rotary incubator (New Brunswick Scientific, Nova 40, USA) at 225 rpm. At the end of the reactions, the product was separated from the enzyme by decanting. The excess ethanol and hexane were evaporated in a rotary vacuum evaporator at 40°C and the solvent free mixtures were stored at -35°C for subsequent analysis. All reactions were performed in triplicate and the mean values were reported.

Table 3.3 Independent variables and their levels for central composite design in the optimization of lipase catalyzed incorporation of *N*-acetyl-L-leucine and *N*-acetyl-L-valine into the 2-MAG

Parametre	- α	-1	0	+1	+α
Time (h)	2.50	8.00	16.00	24.00	29.45
Enzyme load (wt%)	9.88	15.00	22.50	30.00	35.11
Temperature (ºC)	34.88	40.00	47.50	55.00	60.11

3.2.7.5 Experimental Design and Optimization by Response Surface Methodology

A five-level, three-factorial central composite rotatable design was employed to study the response patterns and to determine the optimum combination of variables to obtain maximum incorporation yield. CCRD was composed of 20 experimental points consisting of six center points (Table 3.4). All 20 runs were performed in a totally random order to avoid bias. The independent variables and their levels were as follows: reaction time (8–24 h), reaction temperature (40–55ºC), enzyme load (15–30 wt%).

The data obtained 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 all tests was set at 95% confidence level. The goodness of the models established was determined using the coefficient of determination, R^2, together with the absolute average deviation values and ANOVA (Arifin et al., 2010).

The first- or second-order coefficients were generated by regression analysis with backward elimination. The quadratic response surface model was fitted to the equation (3.4): where Y_i (i=1) is the response for the total reaction product (%). 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.

$$Y_i = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^{\,2} + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j + \varepsilon \qquad (3.4)$$

Table 3.4 A five-level, three factorial central composite rotatable design generated for the incorporation of the *N*-acetyl-L-leucine and *N*-acetyl-L-valine into the 2-MAG

Run	t[a] (h)	En[b] (wt.) %	T[c] (°C)	Total reaction product[d] (%)		Total reaction product[e] (%)	
				Experimental	Predicted	Experimental	Predicted
1	8.00	15.00	40.00	18.63±0.50[f]	19.81	8.06±0.16	8.31
2	24.00	15.00	40.00	22.18±0.35	21.95	8.05±0.22	8.64
3	8.00	15.00	55.00	29.14±0.75	29.69	8.83±0.18	9.55
4	24.00	15.00	55.00	24.07±0.22	23.44	10.39±0.21	9.88
5	8.00	30.00	40.00	20.63±0.36	21.15	14.98±0.72	14.75
6	24.00	30.00	40.00	23.10±0.45	23.29	13.59±0.23	15.08
7	8.00	30.00	55.00	25.04±0.23	25.16	15.08±0.09	16.00
8	24.00	30.00	55.00	19.35±0.21	18.91	16.25±0.12	16.33
9	2.50	22.50	47.50	28.66±0.52	27.39	13.10±0.91	12.04
10	29.45	22.50	47.50	23.13±0.63	23.93	13.65±0.36	12.60
11	16.00	22.50	34.88	18.65±0.12	17.81	11.61±0.22	11.27
12	16.00	22.50	60.11	22.06±0.05	22.44	13.19±0.25	13.37
13	16.00	9.88	47.50	24.55±0.27	24.34	6.57±0.93	6.90
14	16.00	35.11	47.50	21.58±0.02	21.66	18.13±0.27	17.74
15	16.00	22.50	47.50	22.46±0.13	23.00	12.26±0.33	12.32
16	16.00	22.50	47.50	23.47±0.15	23.00	11.05±0.06	12.32
17	16.00	22.50	47.50	23.56±0.22	23.00	13.86±0.17	12.32
18	16.00	22.50	47.50	22.75±0.32	23.00	11.65±0.19	12.32
19	16.00	22.50	47.50	23.22±0.11	23.00	12.42±0.05	12.32
20	16.00	22.50	47.50	22.84±0.21	23.00	13.72±0.01	12.32

[a] time; [b] enzyme load; [c] temperature; [d] The reaction was performed between 2-MAG and *N*-acetyl-L-leucine; [e] The reaction was performed between 2-MAG and *N*-acetyl-L-valine; [f] standart deviation. Each run were performed three times.

3.2.7.6 Purification of the *N*-acetyl-L-leucine (or *N*-acetyl-L-valine) Incorporated 2-MAG

For the separation of *N*-acetyl-L-leucine (or *N*-acetyl-L-valine) from the final reaction mixture, the suggested method of Morán et al. (2001) were applied. For this aim, a mixture of celite and activated charcoal 1:1 (150–160 g of each) was packed in a Buchner fritted funnel (10×13 cm, with a fine-porosity fritted-glass disk) to a final

bed volume of 150 mL. The eluent was passed through the stationary phase by vacuum. Celite and activated charcoal were used as absorbants for amino acids. The chromatographic system was treated initially with an aqueous solution of acetic acid (10% v/v; 150 mL). Then, aliquots of the residue containing 3.75 g of crude reaction products were loaded. Elution of the products was achieved isocratically by methanol:water (13:7, v:v).

3.2.7.7 Model Reaction Between 2-MAG and *N*-acetyl-L-leucine for Verification of Reaction

Reactions were performed between 0.014 mmol 2-MAG (purity, >99%) and 0.056 mmol *N*-acetyl-L-leucine were performed in 50-mL tightly closed screw-capped flask. The reactions were catalyzed by immobilized lipase *Candida antarctica* and *Thermomyces lanuginosus* supported by acrylic resin in 2 mL DMF:hexane (5:95, v:v) and ethanol:hexane (80:20, v:v). Enzymes were added in the amount of 20% (w:w, based on the substrate). The reactions were carried out in a rotary incubator (New Brunswick Scientific, Nova 40, USA) at 55°C, 72 h and 225 rpm. At the end of the reactions, the product was separated from the enzyme by decanting. DMF was evaporated under N_2 and hexane was evaporated using rotary vacuum evaporator. The solvent free mixtures were stored at -35°C for subsequent analysis.

3.2.7.8 HPLC Analysis

The final reaction mixtures were analyzed by HPLC (Shimadzu, Kyoto, Japan) equipped with UV detector (Shimadzu, Japan) using the triacylglycerol analysis method by HPLC (Kocak et al., 2011). A mobile phase consisting of 50:50 (v:v, acetone:acetonitrile) was used isocratically in the analysis. The products were diluted in acetone, filtered and injected into the column (Sphereclone 5 μ ODS (2), 250x4.6 mm; Phenomenex, CA, USA) with an accompanying guard column (40x3-mm id) of the same phase and eluted at a flow rate of 1.0 mL/min. The column temperature was 50 °C. Composition of the products was given in percentage area.

3.2.7.9 TLC/FID Analysis

The procedure was performed according to the method described by Adamczak and Bornscheuer (2013). The samples were diluted in methanol. The rods loaded with the

samples were eluted for 8 cm with 100% chloroform. After drying the developed sample at room temperature for 5 min, the Chromarods were again developed for 10 cm with chloroform:(methanol: NH_4OH, 8: 2, v:v) (70:0.05, v:v). The developed sample was then dried in an oven at 120ºC for 5 min. Scanning was performed under the following conditions: gas flow rate 160 mL/min, air flow rate 2.0 L/min, scanning speed 30 s/scan. The total product composition was defined as mass percentages.

3.2.7.10 LC-MS-MS Analysis

The products were purified using HPLC column (Sphereclone 5 µ ODS (2), 250x4.6 mm; Phenomenex, CA, USA). By direct injection in Ab-SciEx 3200 Q-Trap MSMS detector (Darmstadt, Germany) with electrospray ionization probe (Framingham, MA,USA), mass spectra of the products were recorded using the method of Tasdemir et al. (2015).

3.2.8 Physicochemical Properties of *N*-acetyl-L-leucine (or *N*-acetyl-L-valine) Incorporated 2-MAG

3.2.8.1 Density (g/mL)

The densities of the products and ROPO were determined using the AOCS method Cc 10a-25 (AOCS Cc 10a-25) at 25°C.

3.2.8.2 Melting Profile and Solid Fat Content (SFC)

The melting profile and SFC of the final products were analyzed by DSC (Perkin Elmer DSC-6, Norwalk, CN, USA) with AOCS Official Method Cj 1-94 (AOCS Cj 1-94). The DSC instrument was calibrated with indium (m.p. 156.6°C, ΔH_f =28.45 J/g). Nitrogen was used as the purge gas and flowed at 40 mL/min. The sample was completely melted at 80°C before being weighed (5-10 mg) into an aluminium pan which was then sealed. An empty, hermetically sealed aluminum pan was be 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 5°C/min and held at -60°C for 10 min. At the end of the cooling, the sample was heated at 5°C/min to 80°C. The onset (T_{onset}) and peak temperatures (T_{peak}) were determined according to the melting profile. The SFC was

calculated at various temperatures from the DSC heating thermogram data by partial integration according to Tieko Nassu and Guaraldo Gonçalves (1995). DSC analysis results were the mean of two experiments.

3.2.8.3 Oxidative Stabilities

The oxidative stabilities of the products and ROPO were determined by a Perkin Elmer differential scanning calorimeter (DSC-6, Norwalk, Conn., U.S.A.) with the method of Tan et al. (2002). Instrument was calibrated with indium standard. Samples of 5±0.5 mg were weighed into open aluminum pans. An empty pan was used as reference. The isothermal temperature program was studied at four different temperatures (110, 120, 130 and 140°C). Oxygen was passed through the sample chamber at 50 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.

3.2.8.4 Viscosity (mPa·s)

Viscosities of the products and ROPO were measured using Brookfield RV DV3T digital viscometer. The temperature was setted at 25°C and RV5 was used as spindle. The spindle speed was 250 rpm during the analysis.

3.2.8.5 Specific Heat Capacity, Thermal Expansion Coefficient and Thermal Conductivity

The specific heat capacities of the samples and ROPO were determined using DSC. The melting curve which was obtained using DSC (Section 3.2.8.2) was used in the calculation of specific heat capacity. Specific heat capacity was calculated using the absorbed energy during the melting time. Thermal conductivity was determined using HACH Conductivity HQ 40d. Thermal expansion coefficients were determined using the suggested method of Coupland and McClements (1997).

3.2.8.6 Vapour Absorption Capacity

Products and ROPO were spread on a stainless steel laboratory dish and the samples were hold at 20°C at relative humidities of 65% for 1 week. At the end of the holding

time, the moisture contents were determined using Karl Fischer coulometric moisture titrator (KEM MKC-501, Kyoto, Japan).

3.2.8.7 Microstructure

Microstructure of ROPO and the products were investigated using a polarized light microscope (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°C for 15 min in order to erase the crystal memory and 20 μL of sample was 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 (24°C). Images were captured under polarized light with 20X magnification on the gray-scale.

3.2.8.8 Solubility Properties of the Products

3.2.8.8.1 Solubility of the Products in Water (25ºC)

The products and ROPO (2 mL) were dissolved in alcohol (4 mL). Then, the mixtures were titrated with distilled water until the mixtures became cloudy. The used water in the titration was multiplied with 100. The result gave the ‘solubility value’ for the samples.

3.2.8.8.2 Crismer Value

Crismer value determines the miscibility of the oil in standart solvent mixture (t-amyl alcohol, ethyl alcohol and water (5:5:0.27, v:v:v). Crismer values of the samples were measured using AOCS method Cb 4-35 (AOCS Cb 4-35). The samples were hold at 95°C water bath for 5 min. Then, the samples were poured into a test tube, which was assigned at 2 mL and 4 mL previously, up to 2 mL. The mixture of t-amyl alcohol, ethyl alcohol and water (5:5:0.27, v:v:v) was added to the sample up to 4 mL. The mixture in the test tube was continued to be heated until the temperature was about 5°C above the point of clarification. After that, the mixture was allowed to be cool down. The temperature at which cloudiness was observed in the solution was recorded. Free fatty acidity (Acidity%) was measured using the

AOCS method Ca 5a-40 (AOCS Ca 5a-40). Correction factor was assumed as 2.07 which was given in the standart for olive oil. Crismer value was determined using Equation 3.5.

Crismer value, corrected= Turbidity observed (°C) + (%Acidity x correction factor) (3.5)

3.2.8.9 Caloric Value Determination

The caloric values of the samples and ROPO was determined by Deng Çevre Analiz Lab. San. ve Tic. Ltd. Şti., (Gaziantep, Turkey). For caloric value determination, the method suggested in TS 1740:2006 was used. The pressure was set at 420-450 psi. 0.1 mg sample was weighed in the bomb and the bomb was placed into the bomb calorimeter (AC-350, LECO Corporation, St Joseph, MO, USA). 1 mL water was added into the added to the bomb and O_2 was given to the system. The relative humidity was 35±5% and temperature was 25±5^{o}C during the analysis.

3.2.8.10 Statatistical Analysis

One way analysis of variance, Duncan multiple range test was conducted to determine the differences in the means of the samples using SPSS Statics 22.0. Each measurement was triplicated.

CHAPTER 4
RESULTS and DISCUSSION

4.1 Acidity and Peroxide Value of Refined Olive Pomace Oil (ROPO)

The acidity and peroxide value of refined olive pomace oil was found to be 0.21% (oleic acid) and 2.5 meq/kg, respectively.

4.2 Triacylglycerol (TAG), Diacylglycerol (DAG) and Monoacylglycerol (MAG) Composition of ROPO

The TAG, DAG and MAG composition of refined olive pomace oil was determined using HPLC equipped with refractive index detector (Figure 4.1). Refined olive pomace oil composed of 95% triacylglycerol and 5% DAG, MAG and trace amounts of free fatty acids. Each individiual TAG percentage in the oil was given Table 4.1. The average molecular weight of refined olive pomace oil was calculated using the triacylglycerol composition and found to be as 874.91 g/mol. The major triacylglycerol in ROPO was found as triolein (37.21%). TAG composition is one of the most important factors that affects the chemical characteristics of an oil (Çiftçi et al., 2009).

4.3 Fatty Acid Composition

The total and *sn*-2 fatty acid compositions of refined olive pomace oil were analyzed using GC (Table 4.2). The major fatty acids of ROPO were oleic, palmitic, linoleic and stearic acid with a content of 71.02, 12.23, 9.51 and 3.61%, respectively (Table 4.2). Palmitoleic, margaric, heptadecenoic, α-linolenic, arachidic, eicosenoic, eicosapentenoic, arachidonic and lignoceric acid were found in minor amounts (<1%). Refined olive pomace oil had high unsaturated fatty acid content (83.30%). The major abundant fatty acids at *sn*-2 position were oleic and linoleic acids with the percentages of 87.40 and 11.30, respectively. Palmitoleic (16:1) and linolenic acids (18:3) were observed in minor amounts at *sn*-2 position (<%1). The major abundant

fatty acids at *sn*-1,3 positions were oleic and palmitic acids with the percentages of 62.83 and 18.34%, respectively.

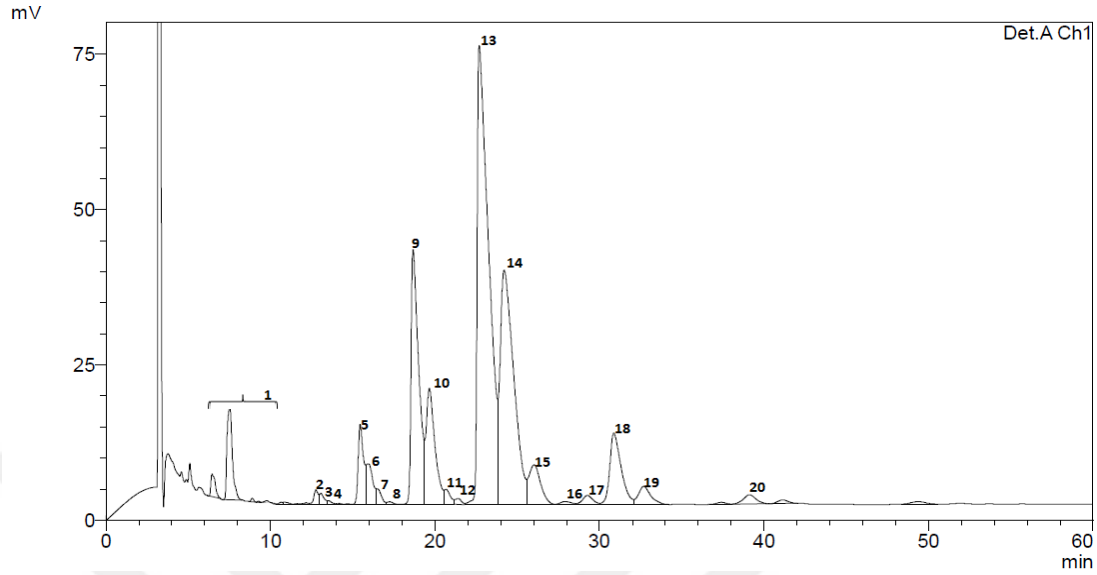


Figure 4.1 HPLC chromatogram of refined olive pomace oil. 1, MAG+DAG; 2, LLL; 3, OLLn+PoLL; 4, LnLP; 5, LLO; 6, LnOO+PoOL; 7, PoPoO; 8, POLn; 9, OOL+LnPP; 10, POL+SLL; 11, PLL; 12, PLP; 13, OOO; 14, POO; 15, POP; 16, PPP; 17, ALO; 18, SOO; 19, POS+SLS, 20, SPS. L, linoleic acid; O, oleic acid; Ln, linolenic acid; P, palmitic acid; Po, palmitoleic acid; S, stearic acid; A, arachidic acid.

Table 4.1 Triacylglycerol composition of refined olive pomace oil

TAG	(%)	TAG	(%)
LLL	0.42	**PLP**	0.26
OLLn+PoLL	0.38	**OOO**	37.21
LnLP	0.10	**POO**	22.40
LLO	2.89	**POP**	3.29
OOLn+PoOL	1.95	**PPP**	0.22
PoPoO	0.57	**ALO**	0.64
POLn	0.10	**SOO**	5.68
OOL+LnPP	13.60	**SLS+POS**	1.57
POL+SLL	7.26	**SPS**	0.81
PLL	0.65		
		TOTAL	**100**

L, linoleic acid; O, oleic acid; Po, palmitoleic acid; P, palmitic acid; S, stearic acid, A, arachidic acid.

Table 4.2 Fatty acid composition of refined olive pomace oil

The fatty acid composition			
Fatty Acid	Total FA% in TAG	Total FA% in *sn*-2	Total FA% in *sn*-1,3
C16:0 (Palmitic acid)	12.23	-	18.34
C16:1 (Palmticoleic acid)	0.60	0.62	0.59
C17:0 (Margaric acid)	0.12	-	0.18
C17:1 (Heptadecanoic acid)	0.14	-	0.21
C18:0 (Stearic acid)	3.61	-	5.41
C18:1 (Oleic acid)	71.02	87.40	62.83
C18:2 (Linoleic acid)	9.51	11.30	8.62
C18:3 (α-Linolenic acid)	0.59	0.68	0.55
C20:0 (Arachidic acid)	0.62	-	0.93
C20:1 (Eicosenoic acid)	0.38	-	0.57
C20:3 (Eicosatrienoic acid)	0.26	-	0.39
C20:4 (Arachidonic acid)	0.80	-	1.2
C24:0 (Lignoceric acid)	0.12	-	0.18

4.4 Production of 2-Monoacylglycerol from Refined Olive Pomace Oil

4.4.1 Enzymatic Hydrolysis

4.4.1.1 Lipase Screening

Four lipases from different sources were analyzed for their ability to catalyze the hydrolysis reaction of ROPO to produce 2-monoacylglycerol. Immobilized lipases from *Mucor miehei, Candida antarctica,* porcine pancreas and immobilized lipase B from *Candida antarctica* were tested for their catalytic activity. The lipases from *Mucor miehei* and porcine pancreas are *sn*-1,3 specific lipases. The *sn*-1,3 position specificity of the lipase from *C. antarctica* and lipase B from *C. antarctica* depends on the reaction conditions. Tris-HCl buffer was used in the hydrolysis reaction which was carried out using porcine pancreatic lipase to adjust the pH 8. Because, the optimum pH for porcine pancreatic lipase is 8-8.5. For the other enzymes, the pH of the reaction medium is already at their optimum value (pH=~7). So, it was not necessary to use any buffer to adjust the pH for a specific value in these reactions. Figure 4.2 showed the conversion percentages obtained in the hydrolysis reactions carried out using different lipases. The highest conversion rates were observed when lipases from *C. antarctica* and porcine pancrease were used as biocatalyst. Lipase

from *M. miehei* showed the lowest conversion rate. Although some studies were carried out using *C. antarctica*, lipase from porcine pancreas was used in the further experiments. Because, it was thought that lipase from *C. antarctica* might loose its *sn*-1,3 specificity during the hydrolysis reaction.

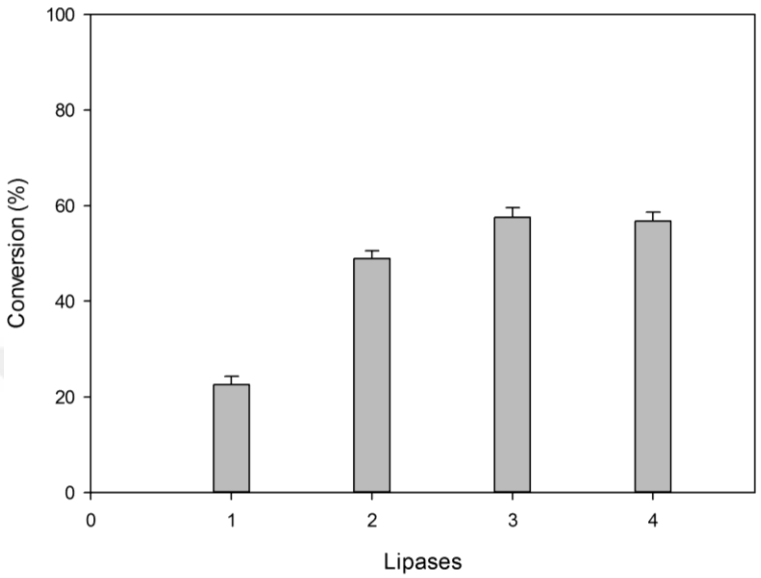


Figure 4.2 Effect of lipases on the hydrolysis reaction of olive pomace oil. 1, Lipase from *Mucor miehei*; 2, Lipase B from *Candida antarctica*; 3, Lipase from *Candida antarctica*. Reaction conditions: Enzyme load 15% (wt.), temperature 50°C, time 12 h, oil:water ratio (100:60, v:v). 4, Porcine pancreatic lipase. Reaction conditions: Enzyme load 15% (wt.), temperature 25°C, oil:buffer ratio 1:5 (v:v), buffer concentration 0.025 M

4.4.1.2 Effect of Different Parameters on the Hydrolysis Reaction Carried Out Using Porcine Pancreatic Lipase

Some pre-experiments should have been done to determine the ranges of the parameters to be studied prior to experimental design generated by Design Expert 7.0 using response surface methodology (RSM). To understand the main effect of one parameter, this parameter should be variable while the other parameters are constant.

Effect of enzyme load

The effect of enzyme concentration on the hydrolysis reaction of ROPO carried out with porcine pancreatic lipase was given in Figure 4.3. Increase in the enzyme concentration resulted in the increase in conversion rate. The maximum conversion rate was seen when the 15 % (wt.) enzyme load was used in the specified reaction

conditions. Enzyme loads higher than 20% (wt.) was not studied since the enzyme could not be dissolved in the specified buffer ratio (1:5, oil:buffer, v:v),

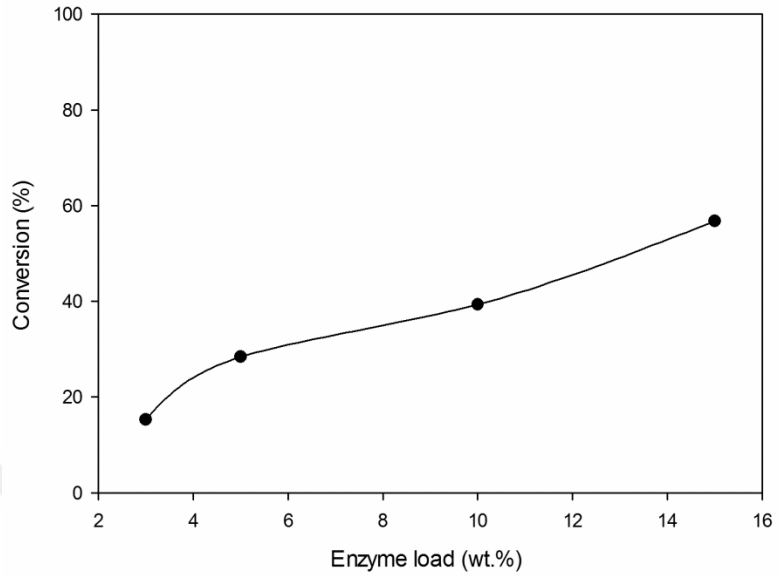


Figure 4.3 The effect of enzyme load on the hydrolysis reaction of refined olive pomace oil with porcine pancreatic lipase. The reaction conditions were temperature 25°C, time 12 h, oil:buffer ratio (1:5, v:v), buffer concentration 0.025 M

Effect of buffer concentration

The effect of buffer concentration on the hydrolysis reaction performed using porcine pancreatic lipase was studied in the range of 0.01 and 0.5 M buffer concentration (Figure 4.4). The conversion rate decreased from 74% to 55% when the buffer concentration increased from 0.01 M to 0.02 M. The conversion rate decreased to 49% when the buffer concentration was 0.1 M. The highest conversion was observed when the buffer concentration was 0.01 M in the studied range.

Effect of time

The effect of time was studied to understand the change in the rate of hydrolysis carried out using porcine pancreatic lipase in the time range of 5-24 h. There was a considerable increase in the conversion rate in the earlier 12 h (Figure 4.5). The conversion increased from 39.31 to 84.25% when the reaction time was increased from 4 to 12 h. The reaction reached to equilibrium at 15 h and there was not a remarkable increase after 15 h.

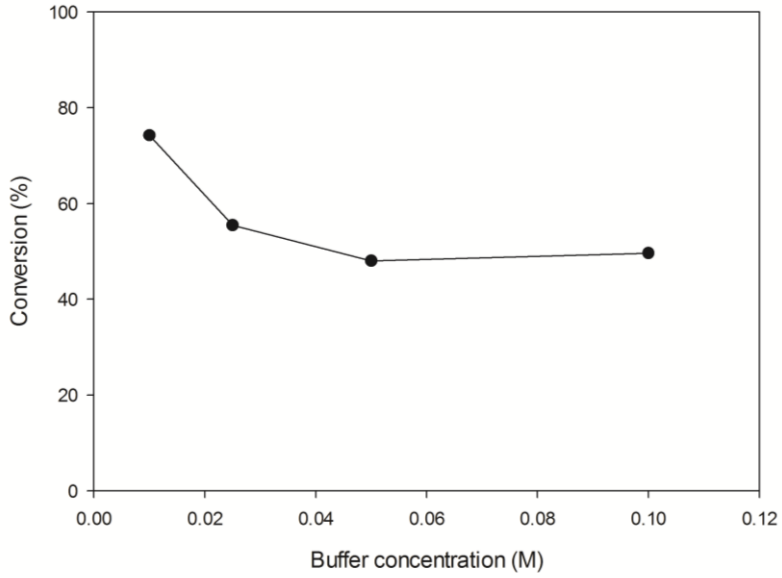


Figure 4.4 The effect of buffer concentration on the hydrolysis reaction of refined olive pomace oil with porcine pancreatic lipase. Reaction conditions: time 12 h, temperature 25°C, oil:buffer ratio (1:5, v:v), enzyme load 15% (wt.)

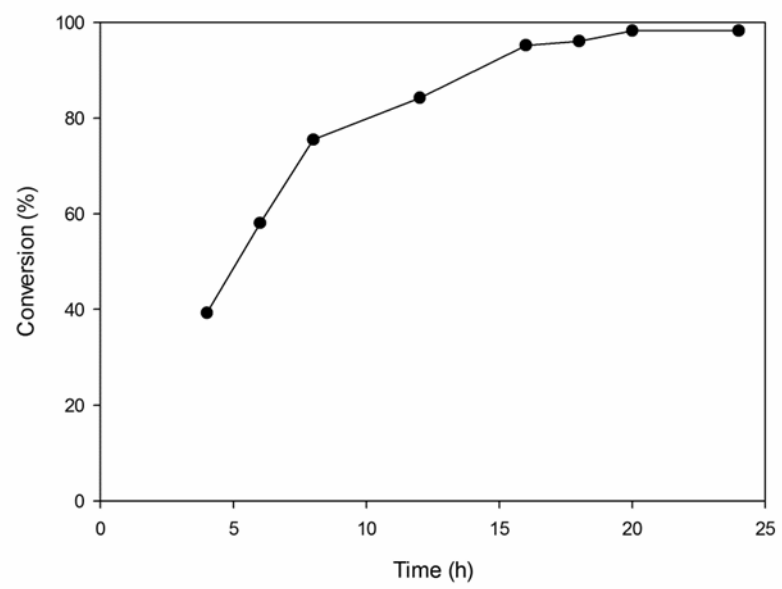


Figure 4.5 The effect of time on the hydrolysis reaction of refined olive pomace oil with porcine pancreatic lipase. The reaction conditions were enzyme load 15 (wt.)%, temperature 25°C, oil:buffer ratio (1:5, v:v), buffer concentration 0.01 M

4.4.1.3 Recovery and Purification of 2-MAG from Hydrolysis Reaction

The hydrolysis reaction product mixture contains free fatty acids (FFA), MAG, DAG and TAG. Firstly, neutralization of the free fatty acids were carried out for the further purification of 2-MAG. It was expected that acylgylcerols were collected in hexane phase during neutralization due to the study of Lee and Akoh (1998). In this manner,

the hexane phase was analyzed using HPLC to determine its acylglycerol mixture and to ensure that the monoacylglcyerols were collected in hexane phase (Figure 4.6). However, it was found that the collected sample in the hexane phase contained higher amount of DAG and TAG (84.40%) than that of 2-MAG (15.60%). The reason of this situation could be explained with the water-like structure of monoacylglycerols. Monoacylglycerols might have more affinity to polar phase (water) than nonpolar phase (hexane). Since the monoacylglycerol composition of the collected phase was lower, the recovery efficiency of 2-monoacyglycerol decreased considerably in further purification applications. The prelimenary trial experiments which were done in our laboratory showed that ethanolysis reaction was more efficient in the synthesis and recovery of 2-MAG with high purity. In Figure 4.7, HPLC chromatogram of the ethanolysis reaction product which would be subjected to purification procedure might be observed. Ethanolysis reaction product contained 10.50% of DAG and 89.50% of 2-MAG and fatty acid ethyl ester (FAEE) mixture. As a result, it was concluded that synthesis of 2-MAG using enzymatic ethanolysis would have been more suitable than enzymatic hydrolysis. Although enzymatic hydrolysis was a suitable method for the production of 2-MAG, recovery and purification of 2-MAG from the ethanolysis reaction mixture was easier and required less experimental step.

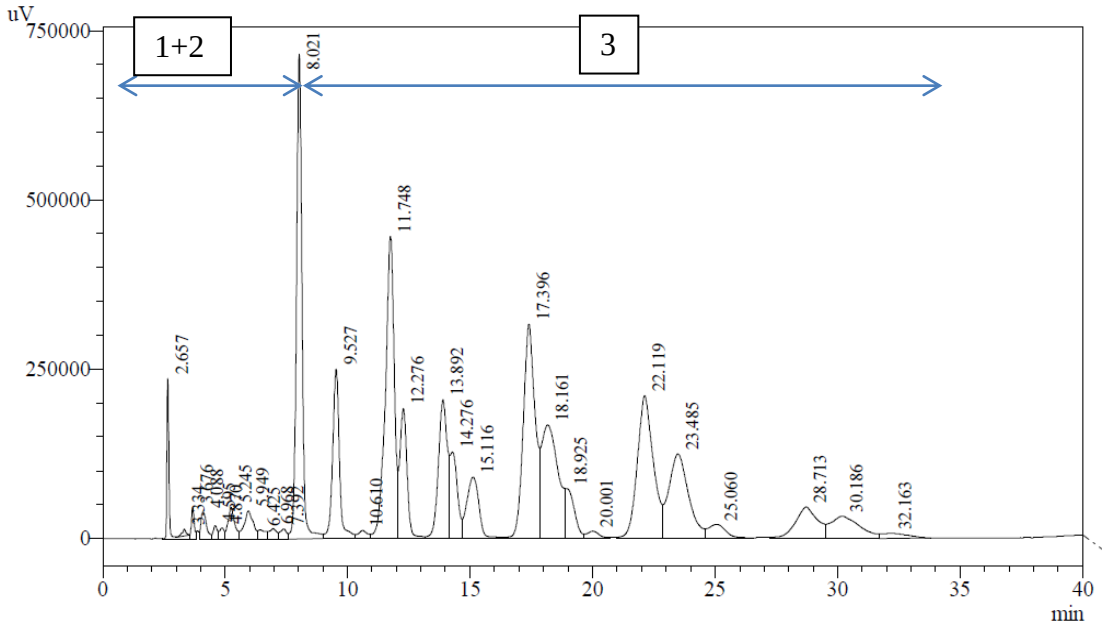


Figure 4.6 HPLC chromatogram of hexane phase after removal of fatty acids by neutralization of hydroysis reaction mixture. 1, Monoacylglycerol; 2, Diacylglycerol; 3, Triacylglycerol

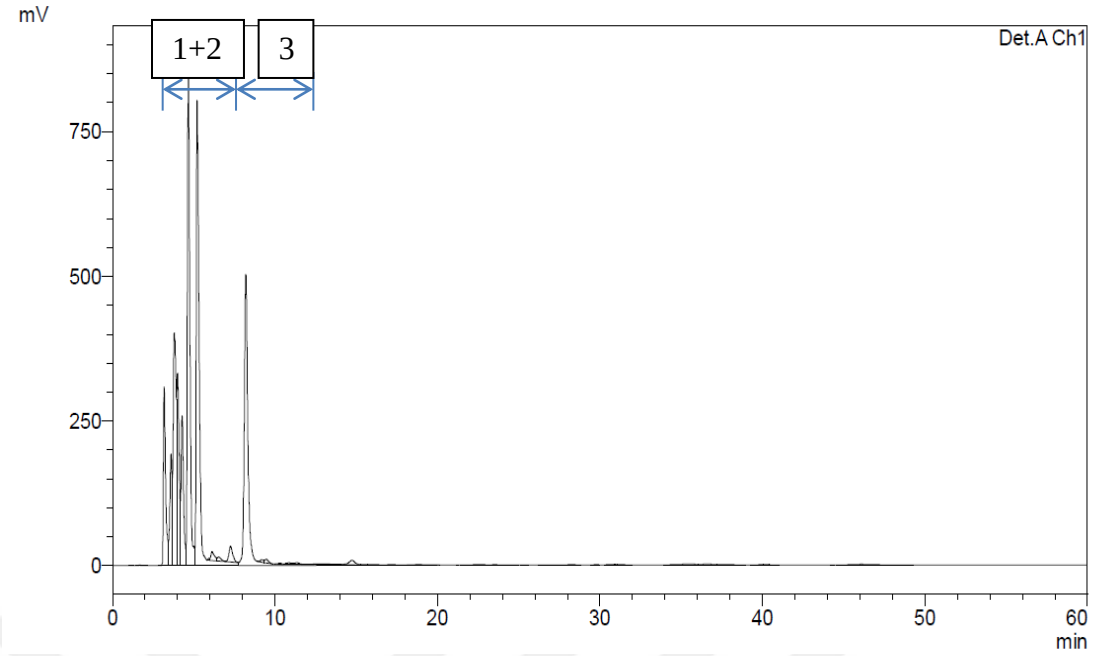


Figure 4.7 HPLC chromatogram of the ethanolysis product mixture of refined olive pomace oil. 1, Fatty acid ethyl esters; 2, Monoacylglycerol; 3, Diacylglycerol

4.4.2 Enzymatic Ethanolysis

4.4.2.1 Lipase Screening

Common and commercially available, *sn*-1,3 specific or non-specific, six lipases were screened for their ability to synthesize 2-monoacylglycerols in the ethanolysis reaction of ROPO (Table 4.3). Weber and Mukherjee (2008) and Parkin (2007) reported that immobilized lipases from *Mucor miehei*, *Rhizopus oryzae*, *Aspergillus niger* and *Candida antarctica* lipase B were *sn*-1,3 specific lipases. Moreover, the lipases from *Pseudomonas fluorescences* and *Candida antarctica* were identified as nonspecific in the literature (Weber and Mukherjee 2008; Heldt-Hansen et al., 1989). Beside *sn*-1,3 specific lipases, the nonspecific lipases were also screened since nonspecific lipases were also found effective in the alcoholysis reactions in literature (Wongsakul et al., 2003; Irimescu et al., 2001a). In this study, lipase from *A. niger* showed the lowest catalytic effect on the synthesis of 2-MAG whereas lipase from *C. antarctica* showed the highest catalytic impact. Esteban et al. (2009) also reported that lipase from *C. antarctica* showed high activity and stability in the synthesis of 2-MAG from fish oil. Irimescu et al. (2001b) and Esteban et al. (2009) noticed that *C. antarctica*, a positionally nonspecific lipase, showed strict regiospecificity in an

excess amount of ethanol. Figure 4.8 showed TLC/FID chromatogram of ethanolysis reaction product.

Table 4.3 Effect of lipase source on the synthesis of 2-MAG from refined olive pomace oil

Lipase sources	Content (wt%)[a]				2-MAG yield (%)[b]
	FAEE	TAG	DAG	2-MAG	
Candida antarctica	51.90	2.31	14.09	31.69	77.86
Lipase B *Candida antarctica*	33.62	31.43	25.85	9.10	22.35
Mucor miehei	1.42	86.57	7.84	4.17	10.24
Pseudomonas fluorescences	3.48	81.80	10.64	4.08	10.02
Rhizopus oryzae	0.35	89.94	6.20	3.51	8.62
Aspergillus niger	0.47	91.38	5.09	3.06	7.51

[a] Content of reaction product; [b] (2-MAG (wt%) in final reaction mixture/theoretical yield)*100.

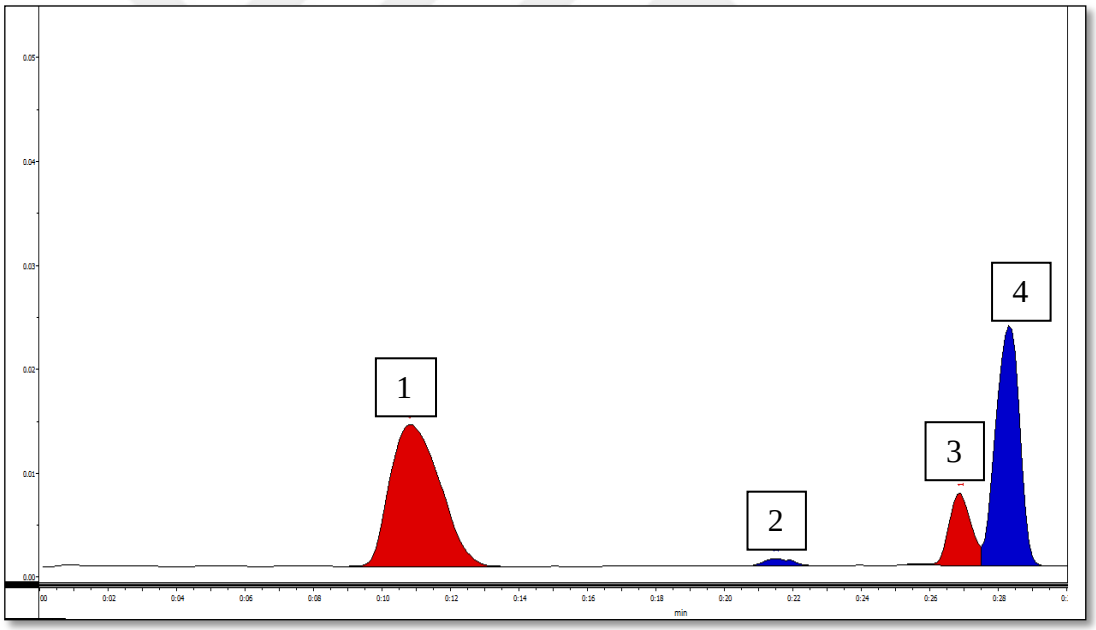


Figure 4.8 TLC/FID chromatogram of ethanolysis reaction product. 1, Fatty acid ethyl esters; 2, free fatty acids; 3, diacylglycerols; 4, monoacylglycerol

4.4.2.2 Preliminary Studies for Experimental Design of Ethanolysis Reaction

Some pre-experiments should have been done to determine the ranges of the parameters to be studied prior to experimental design generated by Design Expert 7.0 using response surface methodology. To understand the effect of one parameter, this parameter should be variable while the other parameters are constant. In this respect, the studies for enzyme load (5-25 %wt.), time (2-24 h), temperature (40-70°C) and

ethanol:oil molar ratio (3:1-70:1) were carried out. In Figure 4.9-4.12, the effects of the studied parameters on the 2-MAG (%) might be observed. The detailed investigation of the effect of reaction parameters by RSM was given in Section 4.4.2.3.

Effect of Ethanol:Oil Molar Ratio

2-MAG yield was increased with increasing ethanol:oil molar ratio (Figure 4.9). At lower ethanol:oil molar ratios (1:1-10:1), 2-MAG yield was about 5%. The lower 2-MAG yield (~5 %) was a result of production of glycerol at lower ethanol:oil molar ratios. This result showed that the *sn*-1,3 specificity of lipase from *C.antarctica* increased at higher ethanol:oil molar ratios.

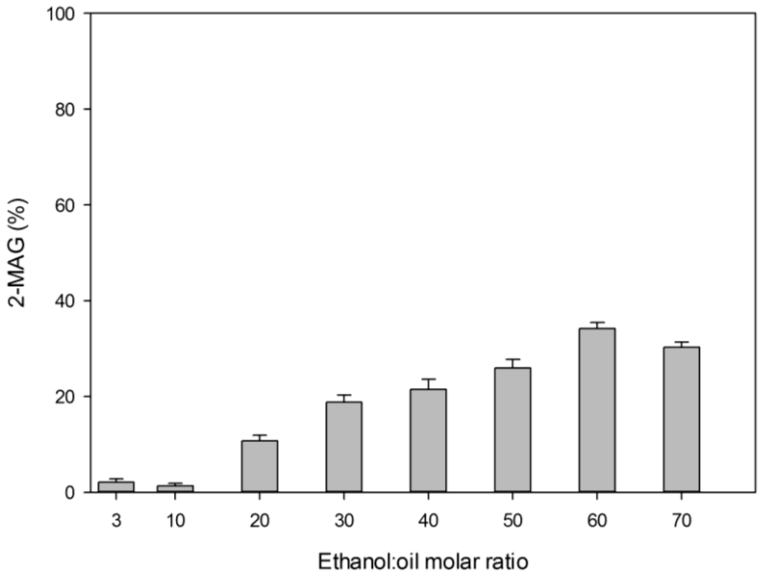


Figure 4.9 The effect of ethanol:oil molar ratio on the ethanolysis of refined olive pomace oil carried out using lipase from *C. antarctica*. The reaction conditions were enzyme load 15 (wt%), temperature 50°C, time 6 h

Effect of temperature

The preliminary studies to determine the effect of temperature were carried out at 40, 50, 60 and 70ºC. The 2-MAG yields were close to each other when the reactions were occured at 40, 50 and 60ºC. 2-MAG yield (%) was 31.09, 30.22, 32.10 at the temperatures of 40, 50 and 60°C, respectively. However, the 2-MAG yield decreased to 27.54% when the reaction was occured at 70°C (Figure 4.10). It may be a result of low activity of the enzyme at high temperatures. The optimum temperature range for

the lipase from *C. antarctica* is between 40 and 70°C (Rodriguez et al., 2012). The determined results were similar to the suggested optimum temperature range.

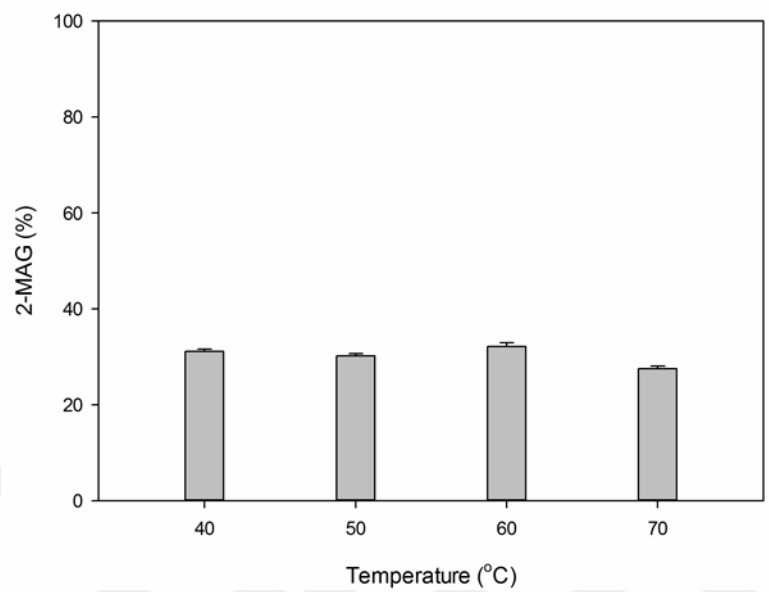


Figure 4.10 The effect of temperature on the ethanolysis of refined olive pomace oil carried out using lipase from *C. antarctica*. The reaction conditions were enzyme load 15% (wt.), ethanol:oil molar ratio 60:1, time 6 h

Effect of time

2-MAG yield increased up to 34.17% in the first six hours of the reaction (Figure 4.11). However, 2-MAG synthesis decreased by increasing time after 6 h. This result was similar to the literature in which they studied the ethanolysis reaction carried out using lipase from *C. antarctica* (del Mar Munio et al., 2008; Irimescu et al., 2002; Köse et al., 2002).

Effect of enzyme load

Enzyme load is also an important parameter which affects the ethanolysis reaction. 2-MAG yield was found to be lower at the reactions occured using high and low enzyme loads (Figure 4.12). For instance, when the enzyme load was used as 5 ve 25 (wt.) %, 2-MAG yields were 19 and 23%, respectively. However, when the enzyme load was 15 (wt%), 2-MAG yield increased to 34%. The lower yields at higher enzyme loads might be related with poor contact of substrate and enzyme due to the weak mixing when the enzyme load was higher.

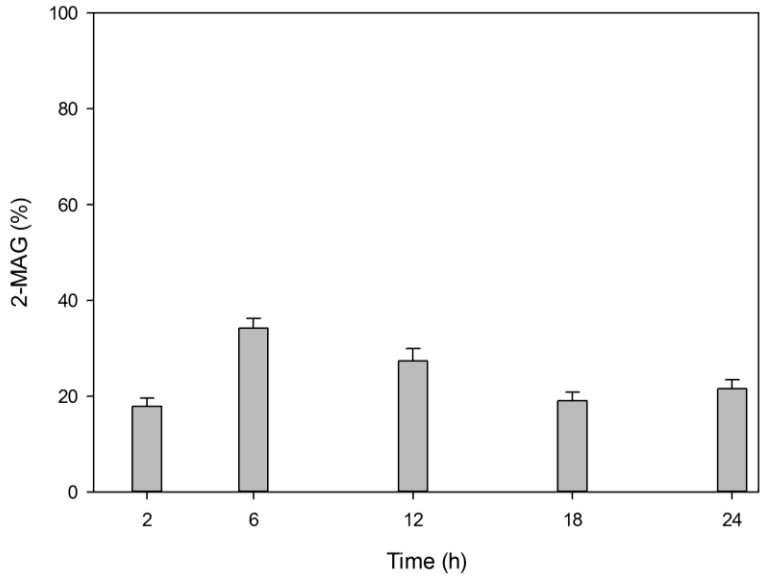


Figure 4.11 The effect of time on the ethanolysis of refined olive pomace oil carried out using lipase from *C. antarctica*. The reaction conditions were enzyme load 15 (wt%), ethanol:oil molar ratio 60:1, temperature 50°C

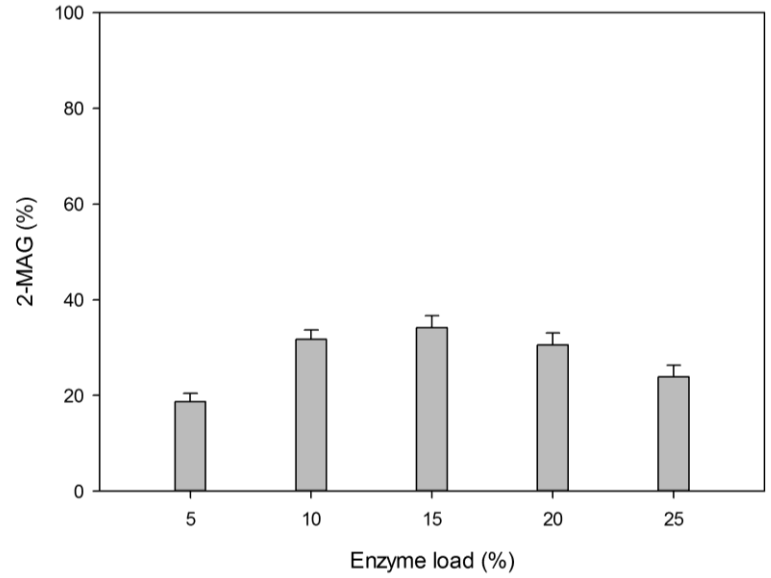


Figure 4.12 The effect of enzyme load on the ethanolysis of refined olive pomace oil with lipase from *C. antarctica*. The reaction conditions were temperature 50°C, ethanol:oil molar ratio 60:1, time 6 h

4.4.2.3 Investigation of the Main Effects of Different Parameters and Their Interactions on Ethanolysis Reaction Using Response Surface Methodology

The effects of the reaction parameters on the ethanolysis reaction for the synthesis of 2-MAG were investigated. Table 3.2 shows the experimental and predicted values for the weight percent of 2-MAG (%) in final reaction product and 2-MAG yield (%)

at experimental points generated by RSM. The predicted values were obtained from the model fitting technique using Design Expert 7.0 and were seen to be closely correlated to the experimental values.

Table 4.4 shows the regression coefficients and P values of independent variables as linear, quadratic and interactive for 2-MAG yield (%). The significant P value (<0.05) for model and non-significant lack of fit (P<0.05) with a high coefficient of determination (0.94) indicated that quadratic model was a good fit. The best fitting quadratic model was determined by regression and backward elimination by means of elimination of insignificant factors and interactions in the models.

Table 4.4 ANOVA and model equation for response surface quadratic model of 2-MAG yield after ethanolysis reaction

Source	Coefficients	F Value	p-value Prob > F
Model[a]		20.98	< 0.0001
Intercept	79.60	-	-
Linear			
Time	-2.36	3.25	0.0901[b]
Enzyme load	-4.76	13.23	0.0022[c]
Temperature	-2.09	2.55	0.1299[b]
Ethanol:oil molar ratio	9.41	51.74	< 0.0001[c]
Interactive			
Time × Enzyme load	-7.94	24.58	0.0001[c]
Time × Temperature	8.96	31.32	< 0.0001[c]
Time × Ethanol:Oil molar ratio	5.36	11.18	0.0041[c]
Enzyme load × Temperature	6.65	17.26	0.0007[c]
Enzyme load × Ethanol:Oil molar ratio	7.59	22.46	0.0002[c]
Temperature × Ethanol:Oil molar ratio	-4.48	7.83	0.0129[c]
Quadratic			
Time × Time	-9.42	60.57	< 0.0001[c]
Enzyme load × Enzyme load	-6.73	30.91	< 0.0001[c]
Ethanol:Oil molar ratio × Ethanol:Oil molar ratio	-4.57	14.23	0.0017[c]
Lack of Fit		2.20	0.1981

[a] The coefficient of determination (R^2) of the model was 0.94; [b] Not significant at 'Prob>F'>0.05, [c] Significant at 'Prob>F' <0.05

Effect of ethanol:oil molar ratio

The coefficient obtained for ethanol:oil molar ratio showed that linear term of ethanol:oil molar ratio had the most significant effect on 2-MAG yield (Table 4.4). The synthesis of 2-MAG was improved with increasing ethanol:oil molar ratio. The preliminary studies of ethanolysis reaction (Section 4.4.2.2) showed that at lower

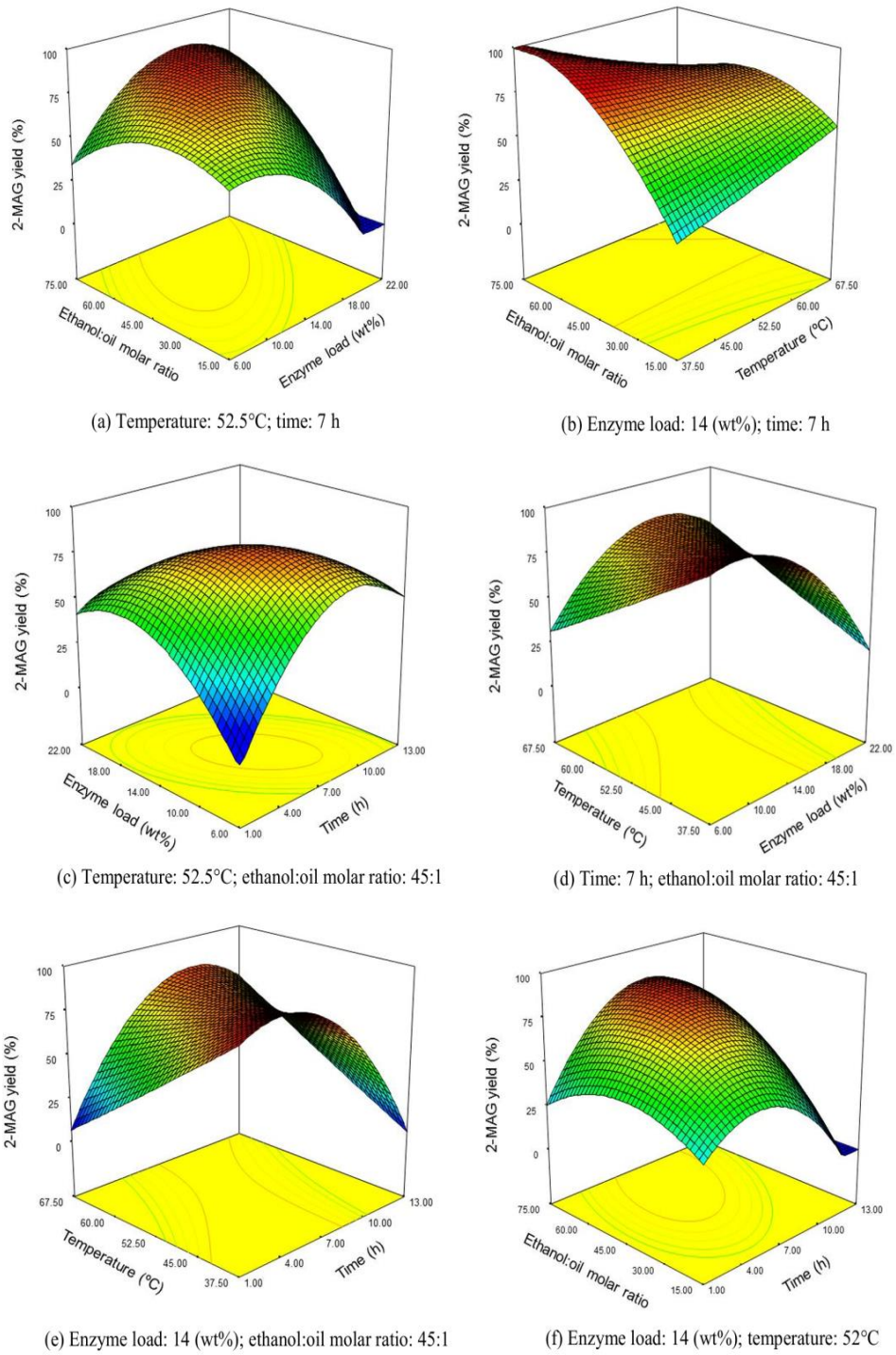
ethanol:oil ratios (1:1-10:1), yields of 2-MAG were low since glycerol was released at the end of the reactions. It might be concluded that specificity of lipase from *C. antarctica* on *sn*-1,3 positions of triacylglycerols increases at higher ethanol:oil molar ratios. This result was similar with the study of Irimescu et al. (2002) in which this selective specificity of enzyme was explained with the ethanol effect. Ethanol, a polar solvent, may fix the tertiary structure of the enzyme by hindering the substrate from accessing the catalytic pocket with the acyl group at the second position of substrate.

The interaction of ethanol:oil molar ratio with time, temperature and enzyme load were found to be significant at 95% confidence level for the 2-MAG yield (%) (Table 4.4). The 2-MAG yield (%) decreased after the center point (45:1, ethanol:oil molar ratio) at lower enzyme loads (wt%) while it increased at higher enzyme loads (Figure 4.13a). Although the effect of ethanol:oil molar ratio was positive, the interaction of ethanol:oil molar ratio and temperature had a negative effect on 2-MAG yield (Table 4.4). This relationship was also confirmed by the response plot (Figure 4.13b). At lower temperatures, increase in ethanol:oil molar ratio caused increase in the 2-MAG yield until 60:1 (ethanol:oil molar ratio) and further increase did not change the 2-MAG yield (%) (Figure 4.13b). However, at higher temperatures, 2-MAG yield (%) decreased slowly after 60:1 (ethanol:oil molar ratio) (Figure 4.13b). The decrease at high ethanol:oil molar ratio by increasing temperature could be explained by the deactivation of lipase by the excess of ethanol at higher temperatures or deacylation of 2-MAG to glycerol because of the high temperature effect as mentioned also in the study of Irimescu et al., (2002).

Effect of enzyme load

Enzyme load was the other significantly effective parameter on the ethanolysis reaction (Table 4.4). Although the enzyme load showed negative effect individually on 2-MAG yield (%), the interactions of enzyme load with temperature or ethanol:oil molar ratio showed positive effects. The effect of enzyme load on 2-MAG yield (%) was improved when the positive moderating effect of increasing temperature or ethanol:oil molar ratio was considered.

The interaction of enzyme load and time had statistically significant negative effect on the 2-MAG yield (Table 4.4). Valverde et al. (2013) found also a similar



(a) Temperature: 52.5°C; time: 7 h

(b) Enzyme load: 14 (wt%); time: 7 h

(c) Temperature: 52.5°C; ethanol:oil molar ratio: 45:1

(d) Time: 7 h; ethanol:oil molar ratio: 45:1

(e) Enzyme load: 14 (wt%); ethanol:oil molar ratio: 45:1

(f) Enzyme load: 14 (wt%); temperature: 52°C

Figure 4.13 Response surface plots for 2-MAG yield (%) as a function of (a) Ethanol:oil molar ratio and enzyme load (wt%), (b) Ethanol:oil molar ratio and temperature, (c) Enzyme load (wt%) and time, (d) Temperature and enzyme load (wt%), (e) Temperature and time, (f) Ethanol:oil molar ratio and time. 2-MAG 2-Monoacylglycerol

interactive relationship between time and enzyme load in the alcoholysis reaction. In Figure 4.13c, it was seen that at longer reaction times, higher 2-MAG yield could be obtained using lower enzyme load. At short reaction times, increase in enzyme load resulted in increase in 2-MAG yield (%) and the reaction reached to equilibrium at the enzyme load of 14 (wt%) (Figure 4.13c). However, the results showed that at longer reaction times, enzyme load more than 10 (wt.)%. decreased the 2-MAG yield significantly. The decrease in 2-MAG yield at longer reaction times may be explained by acyl migration. Similarly, Wang et al. (2014) also reported that 2-MAG was converted to 1-MAG by acyl migration at longer reaction times since 2-MAG is extremely unstable. As seen in Figure 4.13d, it was seen that 2-MAG yield (%) increased slightly up to 80% with increasing enzyme load and then decreased exponentially with further increase at lower temperatures. However, at higher temperatures 2-MAG yield (%) was increased considerably with increasing enzyme load up to 16 (wt.)%.

Effect of time

Although the effect of linear term of time was found to be insignificant, the quadratic term and the interactions of time with each independent parameter had significantly important effect on the yield of 2-MAG (%) (Table 4.4). There was a strong interaction between reaction time and temperature ($P<0.05$). The increase in 2-MAG yield with increasing time was slower at high reaction temperatures than low reaction temperatures (Figure 4.13e). 2-MAG yield reached to 70% even if the reaction time was 1 h at 37.5ºC. This value was 10% when the reaction temperature was 67.5ºC at 1 h. For all the levels of ethanol:oil molar ratio, 2-MAG yield (%) decreased considerably after 7 h (Figure 4.13f). This trend was similar to the findings of del Mar Munío et al. (2008), Irimescu et al. (2002), and Shimada et al. (2003). Shimada et al. (2003) explained this decrease with two phenomena: (1) the velocity of the reaction slowed because the reaction approached equilibrium after 7 h and then acyl migration occurred, (2) the lipase behaved as nonspecific after 7 h and 1(3)-MAG underwent ethanolysis after acyl migration occurred. Adamczak and Bornscheuer (2013) reported that the ethanolysis reaction reached to equilibrium at 12-14 h. Schmid et al. (1998) indicated that the reaction of alcoholysis was very fast and 8 h was required to convert tripalmitin to 2-MAG completely. However, in this study the

reaction reached to equilibrium at less than 5 h according to the response plot analysis, if the other reaction parameters are set to optimum values.

Effect of temperature

The reaction temperature was varied from 37.5 to 67.5°C in the experimental points. The linear term of temperature was not significantly effective on the synthesis of 2-MAG (Table 4.4). However, interaction terms of reaction temperature with other reaction parameters had a significant impact on the synthesis of 2-MAG (Table 4.4). This means that the effect of temperature on the reaction yield depends on the other reaction parameters. Temperature was not removed by backward elimination to maintain the hierarchy of the model. In Figure 4.13d, it was observed that the reaction was developed faster at low temperatures. At low temperatures, higher yields of 2-MAG were obtained at low enzyme loads (Figure 4.13e). However, high enzyme loads were required at high temperature levels. The interactive effect of enzyme load and temperature on the ethanolysis reaction was negative (Table 4.4). After the center point of ethanol:oil molar ratio, increase in temperature reduced the 2-MAG yield (%) significantly.

4.4.2.4 Optimization and Verification of the Model

The reaction conditions were optimized for the maximum 2-MAG yield (%) using optimization function of Design Expert Software 7.0. The enzyme load was targeted at 10 (wt%) because of the economic considerations while the other variables were set due to the ranges given in Table 3.1. At selected ranges the optimum conditions were found as being 45ºC temperature, 5 h time, 10% enzyme load, 50:1 ethanol:oil molar ratio with a high desirability (0.98). Under these conditions, the predicted 2-MAG yield was 84.83%. To verify the predicted results of this model, three experiments were conducted at the optimized reaction conditions and the mean value of 2-MAG yield was 82.54%, in a good agreement with the predicted value. The lipase from *C. antarctica* remained stable for at least 11 uses in the ethanolysis of ROPO performed at the optimum conditions and the 2-MAG yield (%) remained approximately constant over the 11 reaction cycle catalyzed by the lipase from *C. antarctica*.

4.4.2.5 Purification of 2-Monoacylglycerols

The majority of ethanolysis reaction mixture was consisted of 2-MAG and fatty acid ethyl esters. The reaction mixture might also contain minor amounts of DAG and residual TAG in ethanol. The ethanolysis reaction product was purified to separate the 2-MAG by two methods: solvent extraction and low temperature crystallization. In literature, the purification of 2-MAG was carried out combining the three ethanolic phases obtained after washing the ethanolic phase with hexane (Rodríguez et al., 2012; Zhou et al., 2001; del Mar Munío et al., 2008). However, in our study it was observed that recovery of 2-MAG decreases and FAEE content increases in the ethanolic phase if three ethanolic phases were combined. Thus, it was concluded that combination of two ethanolic phases was more feasible. The combined ethanolic phases might be further washed with hexane to succeed higher purity (Table 4.5). The pirinciple of purification with low temperature crystallization based on the crystallization temperature differences of reaction products. Finally, 96% purity was obtained with both methods (Table 4.5-4.6). However, 2-MAG recovery yield (g 2-MAG extracted/100 g of total product) obtained by solvent extraction was considerably higher than that of the low temperature crystallization. 2-MAG recovery yield obtained was 80.10% for solvent extraction while it was 69.00% for low temperature crystallization. The purified 2-MAG was pale yellowish and sticky at room temperature. Figure 4.14 showed the TLC/FID chromatogram of the purified 2-monoacyglycerols.

Table 4.5 Composition of the final reaction mixture and hydroalcoholic phases after each extraction with hexane

	Content (wt%)[a]					
	FAEE	TAG	FA	DAG	2-MAG (purity)	2-MAG recovery yield[b]
Reaction final mixture	44.16	-	2.69	24.82	34.33	
Hydroethanolic phase 1	5.85	-	-	11.21	82.94	63.78
Hydroethanolic phase 2	9.25	-	-	12.71	78.04	82.85
Hydroethanolic phase 3	13.54	-	-	14.17	65.32	78.41
Hydroethanolic phase 4	34.82	-	-	25.32	39.86	81.68
Hydroethanolic phase A	47.27	-	-	-	52.73	
Hydroethanolic phase B	3.39	-	-	-	96.51	80.10

[a] Weight percentage of each lipidic species; [b] g 2-MAG extracted/100 g of total product

Table 4.6 Composition of the reaction mixture after crystallization

	Content (wt%)[a]					
	FAEE	TAG	FA	DAG	2-MAG (purity)	2-MAG recovery yield[b]
After crystallization	-	3.09	-	-	96.91	69.00

[a] Weight percentage of each lipidic species; [b] g 2-MAG extracted/100 g of total product

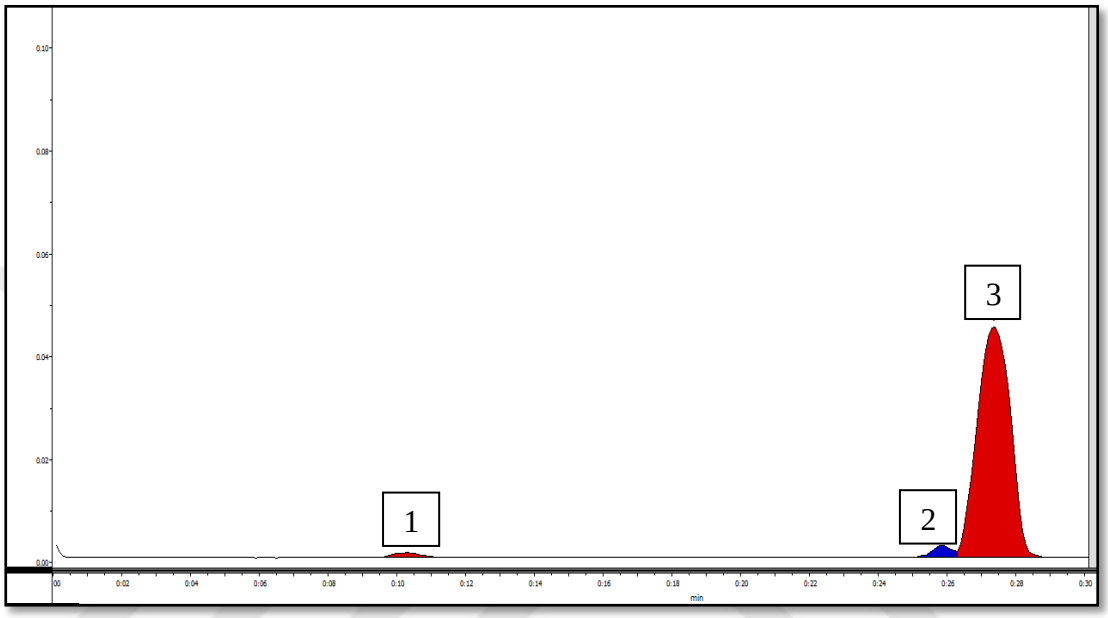


Figure 4.14 TLC/FID chromatogram of purified 2-monoacylglycerol. 1, fatty acid ethyl ester; 2, diacylglycerol; 3, monoacylglycerol.

4.4.2.6 *Sn*-2 Position Analysis of the Purified 2-Monoacylglycerol

The major *sn*-2 fatty acids of purified 2-MAG were oleic acid and linoleic acid with a content of 83.23 and 15.01%, respectively (Table 4.7). It can be concluded that the fatty acid profile of the purified 2-MAG was not significantly different from the initial *sn*-2 fatty acid composition of the ROPO. This revealed that lipase from *C. antarctica* behaved as an *sn*-1,3 specific lipase in the ethanolysis reaction. The result was in good aggreement with the study of Shimada et al. (2003) in which they concluded that ethanolysis of oil with *C. antarctica* lipase can be applied to analysis of *sn*-2 fatty acid composition in TAG. Small differences in the *sn*-2 composition may be related to the different affinities of the fatty acids to ethanol used as a polar solvent during solvent extraction. The linoleic and linolenic acids may show higher affinity to ethanol when compared to oleic acid.

Table 4.7 Fatty acid composition of the purified 2-monoacylglycerol

Fatty acids	Fatty acid (*sn*-2)[a] (%)	Fatty acid (*sn*-2)[b] (%)
C16:0 (Palmitic acid)	-	-
C16:1 (Palmiticoleic acid)	0.62	0.62
C17:0 (Margaric acid)	-	-
C17:1 (Heptadecanoic acid)	-	-
C18:0 (Stearic acid)	-	-
C18:1 (Oleic acid)	87.40	83.23
C18:2 (Linoleic acid)	11.30	15.01
C18:3 (α-Linolenic acid)	0.68	1.14
C20:0 (Arachidic acid)	-	-
C20:1 (Eicosenoic acid)	-	-
C20:3 (Eicosatrienoic acid)	-	-
C20:4 (Arachidonic acid)	-	-
C24:0 (Lignoceric acid)	-	-

[a] The fatty acid composition of ROPO at *sn*-2 position; [b] The fatty acid composition of the purified 2-monoacylglycerol after ethanolysis reaction. 4.5 Incorporation of *N*-acetyl-L-leucine (or *N*-acetyl-L-valine) to the Purified 2-MAG

4.5 Enzymatic Esterification of 2-MAG with *N*-acetyl-L-leucine (or *N*-acetyl-L-valine)

4.5.1 Model Reaction Between Glycerol and *N*-acetyl-L-leucine (or *N*-acetyl-L-valine)

Some of the selected lipases and proteases were screened for their selectivity to amino acid derivatives as substrate before the enzymatic incorporation of *N*α-acetyl-L-amino acids into the purified 2-monoacylglycerol (Table 4.8-9). The incorporation of amino acid derivatives into the glycerol was performed using the method of Morán et al. (2001) with some modifications. In the study of Moran et al. (2001), amino acids were dissolved in glycerol and the reactions were performed in solvent free medium. However, *N*-acetyl-L-leucine and *N*-acetyl-L-valine were slightly soluble in glycerol. In this respect, DMF was added into this reaction to dissolve the *N*-acetyl-L-leucine and *N*-acetyl-L-valine. The reaction conditions were determined by performing pre-experiments. The percentages of the amino acid incorporated products in the final reaction mixture were given in Table 4.8-4.9. The results showed that the lipases and proteases accepted the amino acid derivatives as substrate in the incorporation reaction. However, lipases were selected for further analysis. There were four reasons for this selection: (1) the proteases which were

studied in this study had lower enzymatic activity than lipases, (2) proteases had the disadvantage of being in free form, (3) proteases were significantly more expensive than the lipases, (4) proteases were non-specific enzymes whereas lipases were *sn*-1,3 specific which were studied in this thesis.

Table 4.8 Effect of different enzymes on the incorporation of *N*-acetyl-L-leucine into glycerol

Enzyme	Source	2-acyl-(1)3-acetylleucine-rac-glycerol (%)	2-acyl-1,3-di-acetylleucine-rac-glycerol (%)
Lipase[a]	*Mucor miehei*	7.80	8.92
Lipase[a]	*Candida antarctica*	29.32	59.92
Lipase[a]	*Thermomyces lanuginosus*	30.77	58.30
Protease[b]	*Rhizopus* spp.	18.32	29.73
Protease[b]	*Aspergillus oryzea*	15.56	16.67
Protease[b]	*Bacillus* spp.	5.54	6.78
Protease[b]	*Bacillus licheniformis*	20.84	14.56

[a] immobilized enzyme; [b] free enzyme. 1 mmol glycerol and 4 mmol *N*-acetyl-L-leucine was reacted using 500 µL DMF for 24 h. 20 % (wt., on the basis of *N*-acetyl-L-leucine) enyzme load was used in the reactions.

Table 4.9 Effect of different enzymes on the incorporation of *N*-acetyl-L-valine into glycerol

Enzyme	Source	2-acyl-(1)3-acetylvaline-rac-glycerol (%)	2-acyl-1,3-di-acetylvaline-rac-glycerol (%)
Lipase[a]	*Mucor miehei*	8.62	9.83
Lipase[a]	*Candida antarctica*	25.64	49.67
Lipase[a]	*Thermomyces lanuginosus*	29.87	59.60
Protease[b]	*Rhizopus* spp.	16.42	25.37
Protease[b]	*Aspergillus oryzea*	15.66	16.63
Protease[b]	*Bacillus* spp.	5.58	6.89
Protease[b]	*Bacillus licheniformis*	20.97	15.01

[a] immobilized enzyme; [b] free enzyme. 1 mmol glycerol and 4 mmol *N*-acetyl-L-valine was reacted using 500 µL DMF for 24 h. 20 % (wt., on the basis of *N*-acetyl-L-valine) enyzme load was used in the reactions.

4.5.2 Solvent Screening for Substrate Solubility

Selection of solvent system is very critical for enzymatic reactions in which enzymes have to be active and stable (Plou et al., 2002). A suitable solvent system should be

selected in the incorporation of *N*-acetyl-L-leucine and *N*-acetyl-L-valine into the purified 2-MAG. The solvent system should be suitable both for enzyme to be active and stable and for substrates to be dissolved. Lipases are active and stable in nonpolar solvents while amino acids require polar solvents to be dissolved. In this respect, firstly different solvent and/or solvent systems were studied to test the solubility of *N*-acetyl-L-leucine, *N*-acetyl-L-valine and 2-MAG (Table 4.10). The acetylated leucine and valine were not dissolved in 0.1% TFA (in water) and 0.085% TFA (in water-acetonitrile). These solvents formed emulsions with 2-MAG since they contained water. Because of that reason, these solvents were not suitable for the enzymatic reaction. *N*-acetyl-L-leucine and *N*-acetyl-L-valine were slightly soluble in acetone, acetonitrile, ethanol and methanol. Amino acids were not soluble in nonpolar solvents. DMF was found as the most suitable solvent since amino acid derivatives and 2-MAG were dissolved in this solvent (Table 4.10).

Table 4.10 The solubilities of *N*-acetyl-L-leucine, *N*-acetyl-L-valine and 2-MAG in different solvents (25ºC)

	***N*-acetyl-L-leucine**	***N*-acetyl-L-valine**	**2-MAG**
Dimethylformamide	++	++	++
Ethanol	+	+	++
Methanol	+	+	++
0.1% TFA (in water)	++	++	-
0.085%TFA (in water-acetonitrile)	++	++	++
Acetone	+	+	++
Acetonitrile	+	+	++
Chloroform	-	-	+
Cyclohexane	-	-	+
Dichloramethane	-	-	+
Hexane	-	-	+
Methyl ethyl ketone	-	-	-

++, soluble; +, slightly soluble; -, not soluble.

4.5.3 Solvent System Selection for Reaction Medium

Enzymatic reactions were performed in consideration of the solubilities of the substrates and activities of the enzymes (Table 4.11). For that purpose, the solvents

and/or solvent mixtures in which the substrates were soluble and the enzymes had higher activity were tested.

When DMF was used as reaction medium, 2-acyl-(1)3-acetylleucine-rac-glycerol and 2-acyl-1,3-di-acetylleucine-rac-glycerol were determined as 7.80% and 0.09%, respectively. The polarity of the solvent system was decreased to increase the activity of the enzyme. The products were determined as <1% in the reactions which were performed in hexane:DMF (80:20, v:v) and acetone:DMF (80:20, v:v)). It was observed that the product percentages increased if the DMF ratio was decreased. The highest incorporation was observed in the reaction which was performed in hexane:DMF (95:5, v:v). In that reaction, 2-acyl-(1)3-acetylleucine-rac-glycerol and 2-acyl-1,3-di-acetylleucine-rac-glycerol were determined as 10.30% and 11.81%, respectively. The HPLC chromatograms of 2-MAG and final reaction mixtures were given in Figure 4.15.

The effect of hexane:DMF (95:5, v:v) solvent mixture on reusebility of lipase from *T. lanuginosus* was studied. After the first reaction was performed with *T. lanuginosus,* second reaction was carried out using the same enzyme. It was determined that the the activity of enzyme decreased significantly.

Because of the decrease in enzyme activity, the effect of different ratios of ethanol:hexane mixture on lipase activity was studied. Reactions were performed in ethanol and ethanol:hexane (50:50, v:v) using lipases from *M. miehei, C. antarctica* and *T. lanuginosus.* In those reactions, any product were not dedected in HPLC analysis. In the reactions which were performed using *T. lanuginosus* in acetone, the products were detected (<1%). However, *M. miehei* did not show activity in acetone and no product was observed. The optimum ethanol:hexane mixture ratio was found to be 80:20 (v:v). The lipase activity did not change in its repeatable usages in the solvent reaction medium for 6 reactions. In this system, ethanol provides the solubility of Nα-protected amino acids while hexane allows lipase to show higher activity.

Table 4.11 The effect of solvent systems and lipase sources for the enzymatic incorporation of ***N*-acetyl-L-leucine** in to 2-MAG

Solvent	Lipases	2-acyl-(1)3-acetylleucine-rac-glycerol (%)	2-acyl-1,3-di-acetylleucine-rac-glycerol (%)
Ethanol	*M. miehei*	ND*	ND
Ethanol	*C. antarctica*	ND	ND
Ethanol	*T. lanuginosus*	ND	ND
Acetone	*M. miehei*	ND	ND
Acetone	*T. lanuginosus*	0.41	0.36
DMF	*T. lanuginosus*	7.80	0.09
(Ethanol:hexane)[a]	*M. miehei*	ND	ND
(Ethanol:hexane)[a]	*C. antarctica*	ND	ND
(Ethanol:hexane)[a]	*T. lanuginosus*	ND	ND
(Ethanol:hexane)[b]	*T. lanuginosus*	11.52	13.20
(Ethanol:hexane)[c]	*T. lanuginosus*	2.25	3.80
(Hexane:DMF)[b]	*T. lanuginosus*	ND	0.01
(Acetone:DMF)[b]	*T. lanuginosus*	0.20	0.17
(Hexane:DMF)[c]	*T. lanuginosus*	10.30	11.81
(Cyclohekzan:DMF)[c]	*T. lanuginosus*	11.67	9.85
(Isooctane:DMF)[c]	*T. lanuginosus*	5.12	5.93
(Acetone:DMF)[c]	*T. lanuginosus*	1.80	1.23

*ND Not detected; [a] (50:50, v:v); [b] (80:20, v:v); [c] (95:5, v:v). 0.25 mmol 2-MAG and 1 mmol *N*-acetyl-L-leucine was reacted using 5 mL solvent for 72 h. 20 % enzyme load (wt., on the basis of *N*-acetyl-L-leucine) was used in the reactions

4.5.4 Model Reaction Between 2-MAG and *N*-acetyl-L-leucine

Verification for the reaction of purified 2-MAG which was obtained by ethanolysis reaction and *N*-acetyl-L-leucine was performed studying the reaction between commercial pure 2-MAG (purity >98%) and *N*-acetyl-L-leucine in hexane:DMF (95:5, v:v) and ethanol:hexane (80:20, v:v). The retention times of the products of both reactions were the same in HPLC analysis. The HPLC chromatograms of final reaction mixture and pure 2-MAG were given in Figure 4.16.

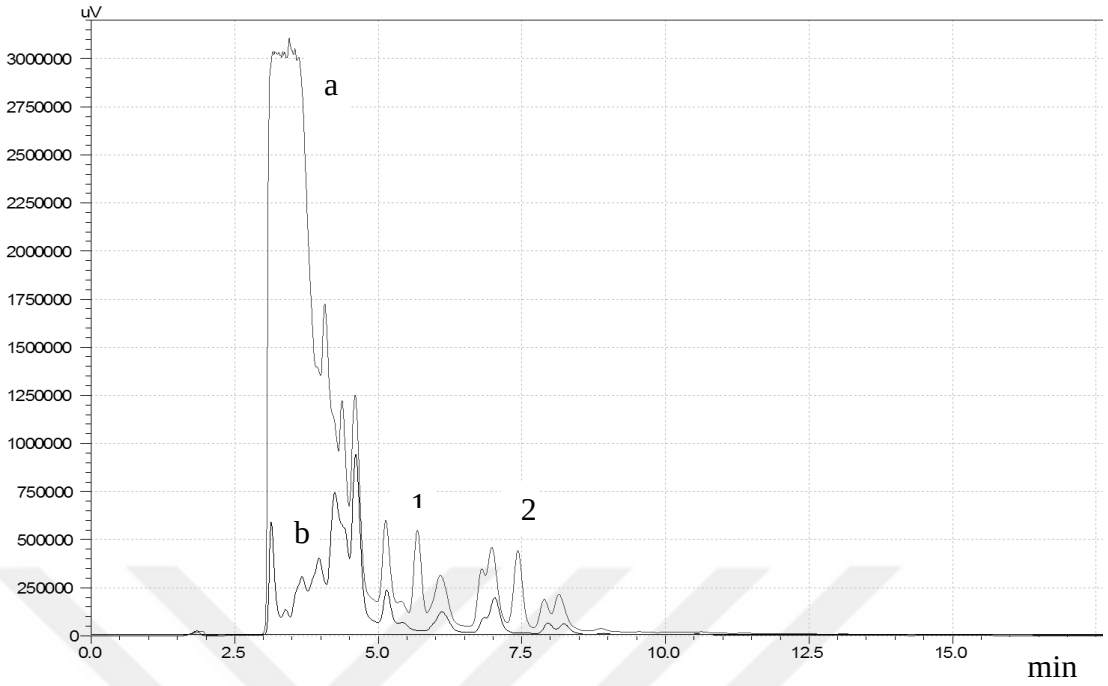


Figure 4.15 The HPLC chromatogram of final reaction mixture performed between purified 2-MAG and *N*-acetyl-L-leucine. 1, 2-acyl-(1)3-acetylleucine-rac-glycerol; 2, 2-acyl-1,3-di-acetylleucine-rac-glycerol. (a) final reaction mixture, (b) purified 2-monoacylglycerol.

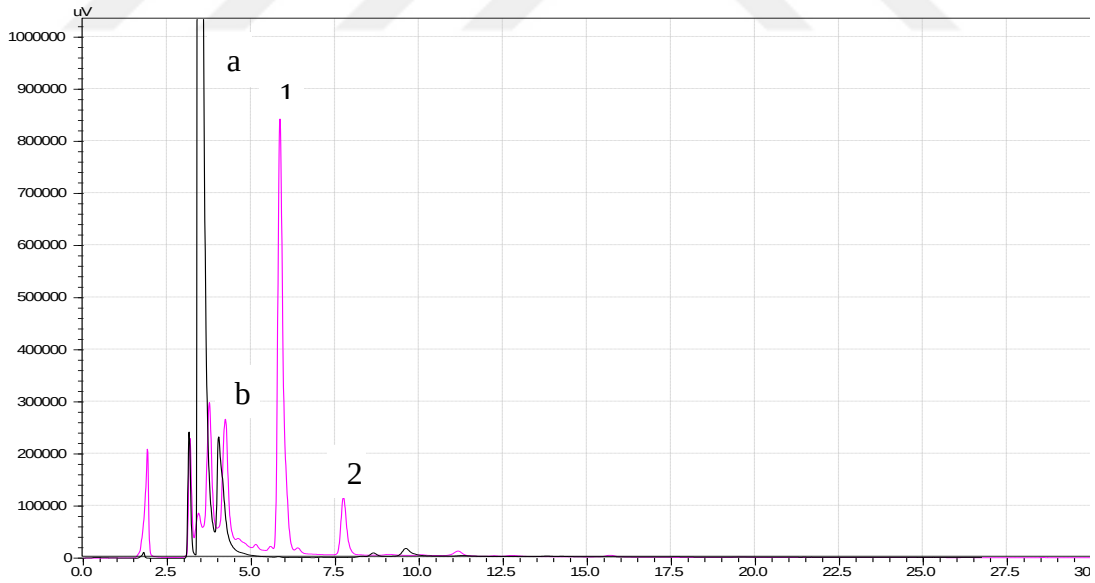


Figure 4.16 The HPLC chromatogram of final reaction product mixture performed between pure 2-MAG and *N*-acetyl-L-leucine. 1, 2-acyl-(1)3-acetylleucine-rac-glycerol; 2, 2-acyl-1,3-di-acetylleucine-rac-glycerol. (a) pure 2-monoacylglycerol, (b) final reaction mixture

4.5.5 Identification of the Reaction Products

Theoretical molecular mass of possible products are: 2-oleic-1,3-di-acetylleucine-rac-glycerol, 666.93; 2-linoleic-1,3-di-acetylleucine-rac-glycerol, 664.91; 2-olein-(1)3-acetylleucine-rac-glycerol, 511.73 and 2-linolein-(1)3-acetylleucine-rac-glycerol, 509.72. At the end of the esterification reaction of 2-MAG with *N*-acetyl-L-leucine, two new different peaks were generated at HPLC chromatogram compared to that of 2-MAG (Figure 4.15-4.16). The peak with late retention time in HPLC belonges to both 2-oleic-1,3-di-acetylleucine-rac-glycerol and 2-linoleic-1,3-di-acetylleucine-rac-glycerol according to LC-MS-MS analysis. The peak with earlier retention time gave the spectrums for both 2-olein-(1)3-acetylleucine-rac-glycerol and 2-linolein-(1)3-acetylleucine-rac-glycerol. LC-MS-MS spectras (Figure 4.17-4.20) confirmed the proposed molecular structures of 2-oleic-1,3-di-acetylleucine-rac-glycerol and 2-linoleic-1,3-di-acetylleucine-rac-glycerol by showing a peak at *m*/*z* 668.9 and 663.9, respectively corresponding to the deprotonated molecular ion $[M-H]^-$. Structures of 2-olein-(1)3-acetylleucine-rac-glycerol and 2-linolein-(1)3-acetylleucine-rac-glycerol gave a peak at *m*/*z* 509.1 and 508.8, respectively corresponding to the deprotonated molecular ion $[M-H]^-$. Main fragmentations are from oleic acid ($C_{18}H_{33}O_2$) showed at a peak *m*/*z* 280.8, from linoleic acid ($C_{18}H_{34}O_2$) at *m*/*z* 283.0 and ($C_{18}H_{34}O$) at m/z 264.6, from glycerol (C_3H_5) at *m*/*z* 39.0, from *N*-acetyl-L-leucine ($C_8H_{15}NO_3$) at *m*/*z* 174.6, branch side of *N*-acetyl-L-leucine (C_4H_9) at *m*/*z* 57.1.

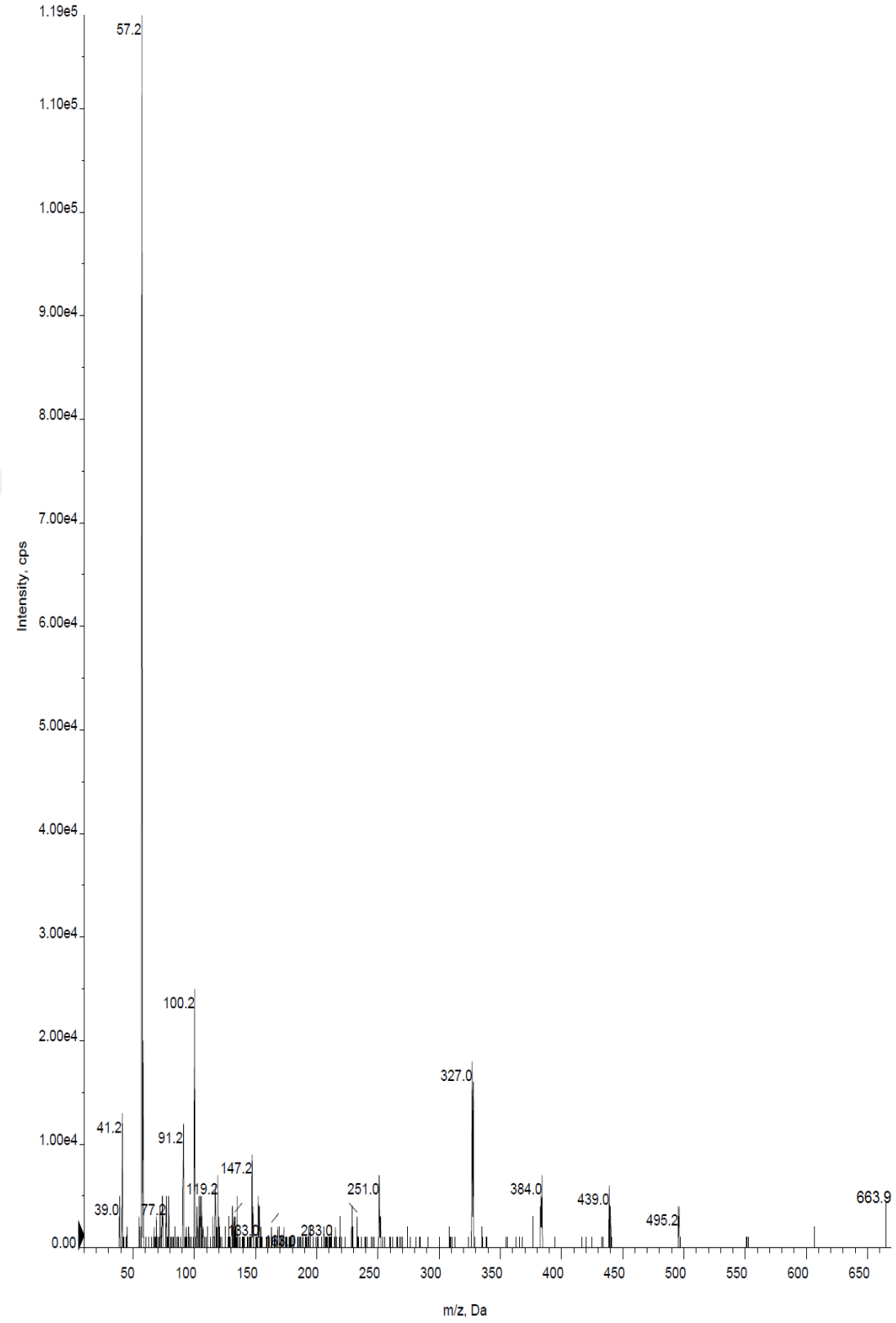


Figure 4.17 Mass spectrum of 2-linolein-1,3-acetylleucine-rac-glycerol

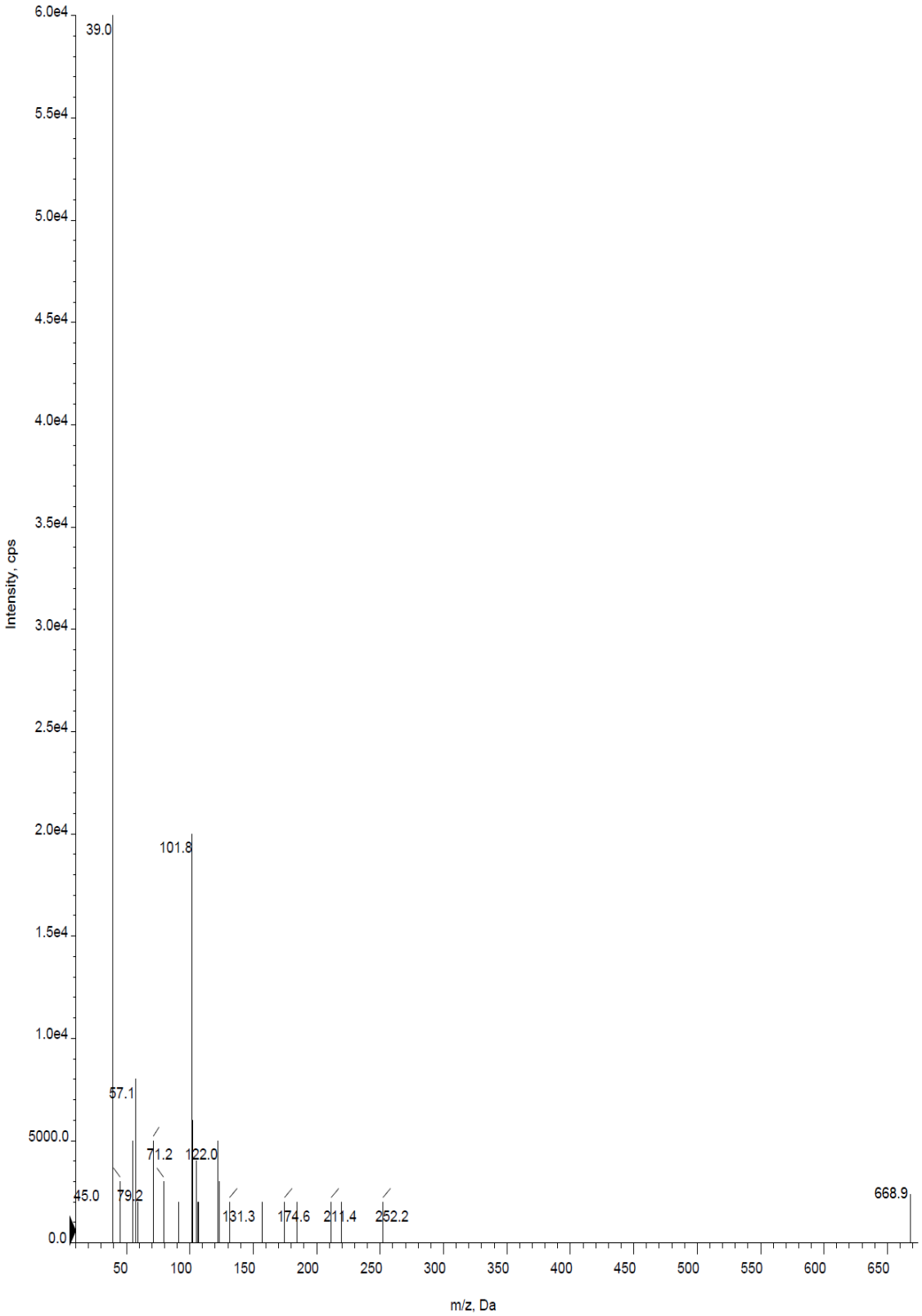


Figure 4.18 Mass spectrum of 2-olein-1,3-acetylleucine-rac-glycerol

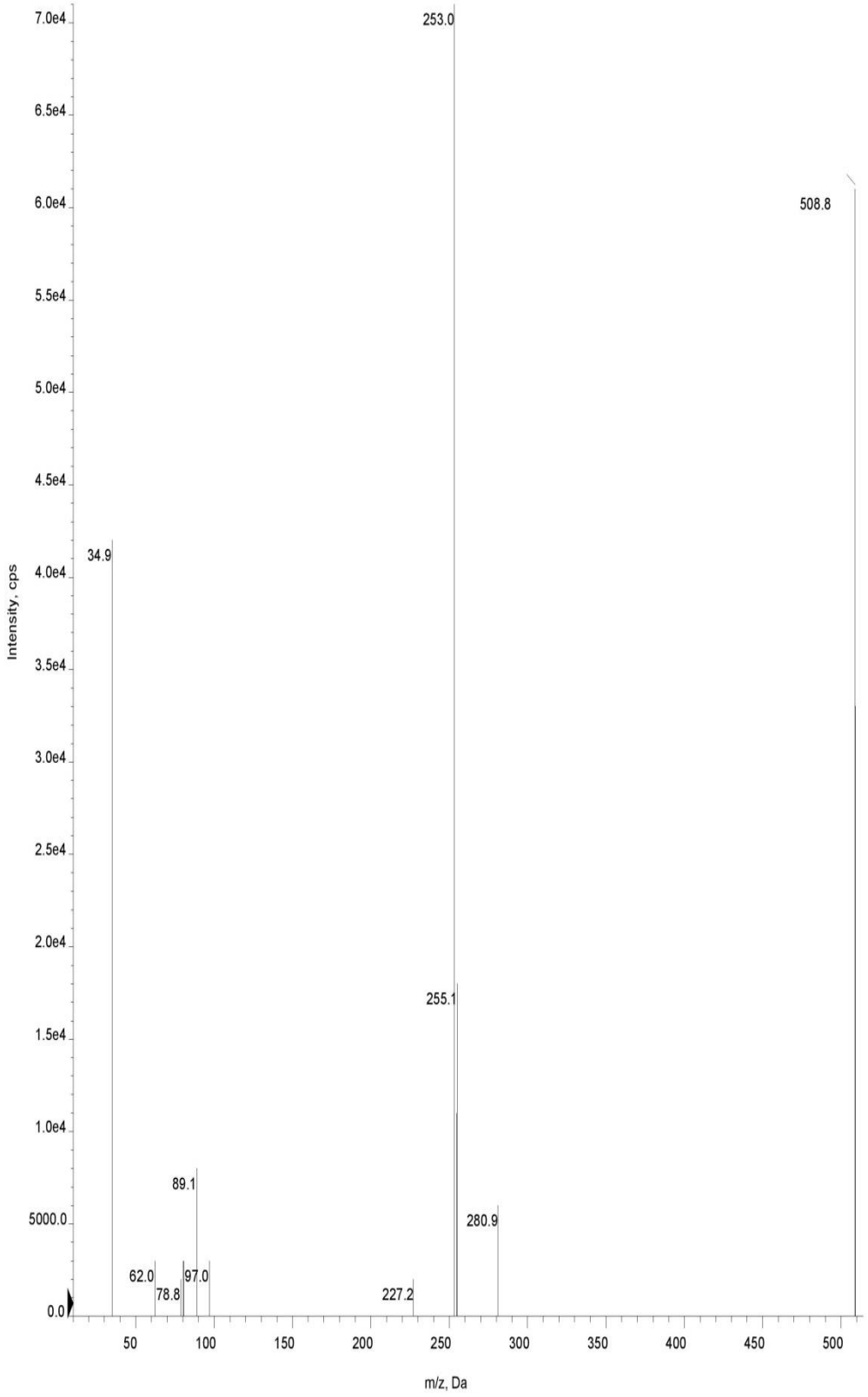


Figure 4.19 Mass spectrum of 2-linolein-1(3)-acetylleucine-rac-glycerol

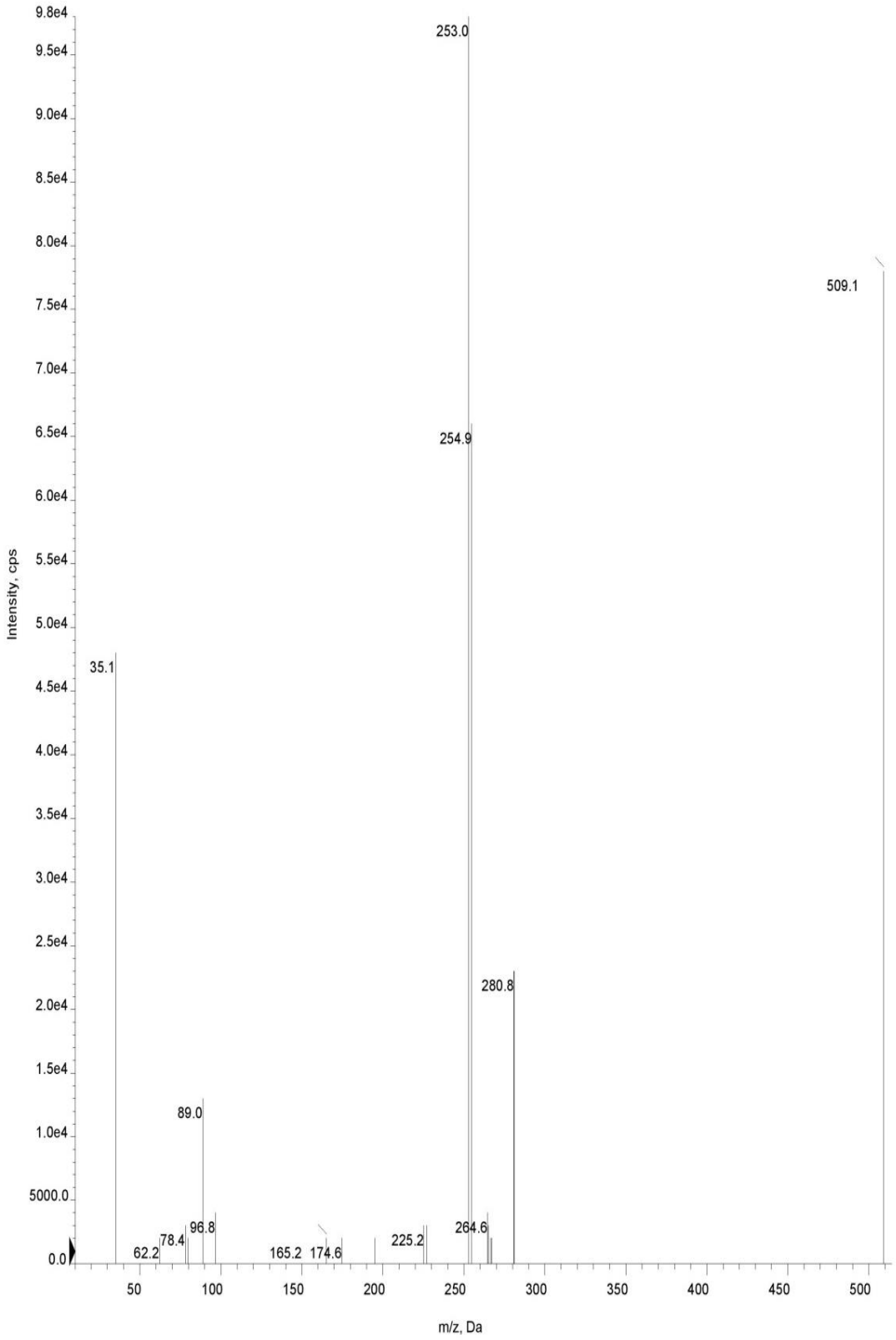


Figure 4.20 Mass spectrum of 2-olein-(1)3-acetylleucine-rac-glycerol

4.5.6 Preliminary Studies for Experimental Design of the Enzymatic Reaction

The lipase from *T. lanuginosus* showed the highest efficiency for the incorporation of Nα-protected amino acids into the 2-MAG. *N*-acetyl-L-leucine and *N*-acetyl-L-valine were used in the reactions as Nα-protected amino acids. Some pre-experiments should have been done to determine the ranges of the parameters to be studied prior to experimental design generated by Design Expert 7.0 using response surface methodology. The most important parameters which affect the enyzmatic reaction were temperature, time, enzyme load and substrate molar ratio. The percentages of *N*-acetyl-L-leucine and *N*-acetyl-L-valine incorporated 2-MAG in the final reaction product mixture were followed as total reaction product (%).

Effect of 2-MAG: *N*-acetyl-L-leucine (or *N*-acetyl-L-valine) molar ratio

The effect of substrate mole ratio was studied by changing the 2-MAG: *N*-acetyl-L-leucine (or *N*-acetyl-L-valine) mole ratio between 1:2 and 1:6 (2-MAG: *N*-acetyl-L-leucine (or *N*-acetyl-L-valine), mol:mol. The highest incorporation was observed when the 2-MAG:*N*α-acetyl-L-amino acid mole ratio was 1:4 (Figure 4.21-4.22). It was concluded that 2-MAG:*N*α-acetyl-L-amino acid mole ratio should not have been included in the experimental design because of the solubility reasons. If the amount of *N*-acetyl-L-leucine (or *N*-acetyl-L-valine) increased, the amount of solvent had to be increased also due to the solubility problems. However, the changes in the amount of solvent would prevent the comparision of the experimental results of each run in experimental design. The increase or decrease in the amount of the solvent affects the contact of substrate and enzyme. Thus, the percentage of the total raction product is affected. Because of those findings, 2-MAG: *N*-acetyl-L-leucine (or *N*-acetyl-L-valine), mole ratio was fixed at 1:4 in the experimental design.

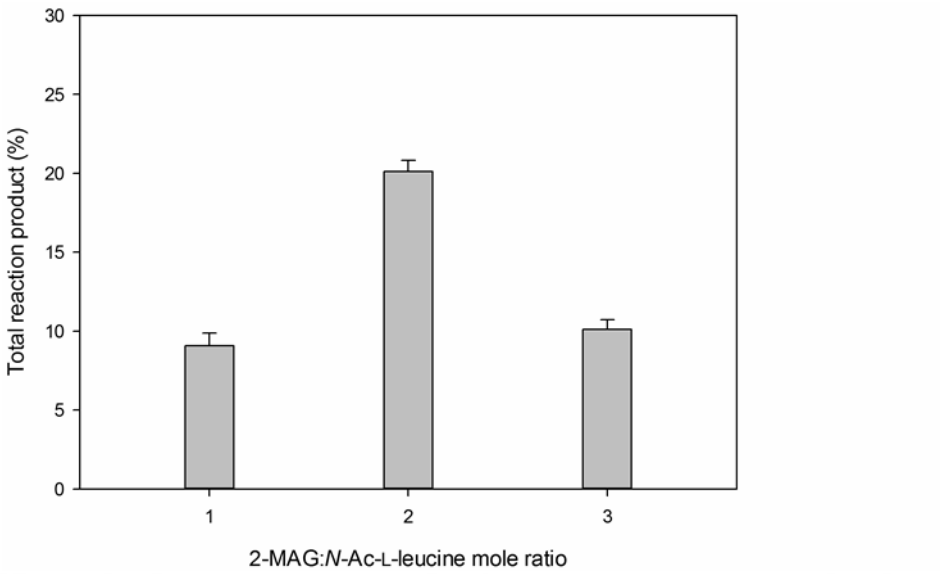


Figure 4.21 Effect of 2-MAG:*N*-acetyl-L-leucine mole ratio on the enzymatic reaction carried out between 2-MAG and *N*-acetyl-L-leucine using lipase from *T. lanuginosus*. Reaction conditions: Enzyme load 20 (wt.)%, time 24 h, temperature 50°C. (1) 1:2, (2) 1:4, (3) 1:6

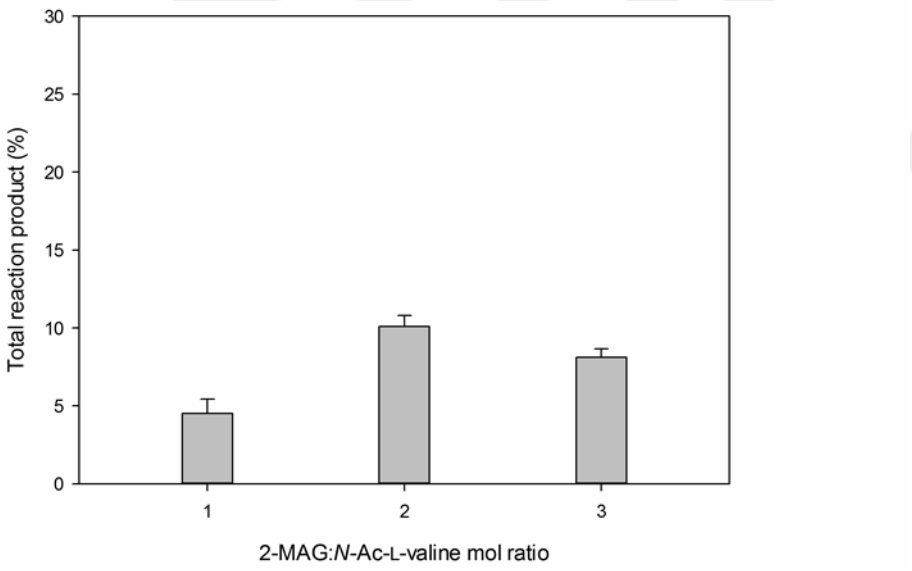


Figure 4.22 Effect of 2-MAG:*N*-acetyl-L-leucine mole ratio on the enzymatic reaction carried out between 2-MAG and *N*-acetyl-L-valine using lipase from *T. lanuginosus*. Reaction conditions: Enzyme load 20 (wt.)%, time 24 h, temperature 50°C. (1) 1:2, (2) 1:4, (3) 1:6

Effect of enzyme load

Enzyme load is one of the most important parameters that affect enzymatic reactions. The total reaction product (%) decreased when lower or higher enzyme loads were used (Figure 4.23-4.24). In Figure 4.23, it was seen that total reaction products (%)

were found to be 1.00 and 16.53 when 10 and 40 (wt.)% enzyme loads were used respectively. When 30 (wt.)% enzyme load was used, the total reaction product was increased to 25.93%. The same trend was seen for the reactions carried out with *N*-acetyl-L-valine. The lower yields at higher enzyme loads (40%) might be related with poor contact of substrate and enzyme due to the weak mixing when the enzyme load was higher.

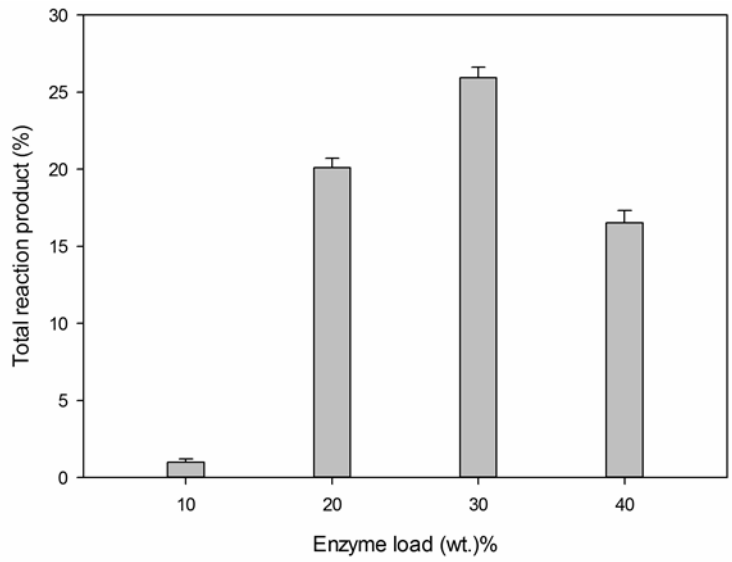


Figure 4.23 Effect of enzyme load on the enzymatic reaction carried out between 2-MAG and *N*-acetyl-L-leucine using lipase from *T. lanuginosus.* Reaction conditions: time 24 h, 2-MAG:*N*-acetyl-L-leucine molar ratio 1:4, temperature 50°C

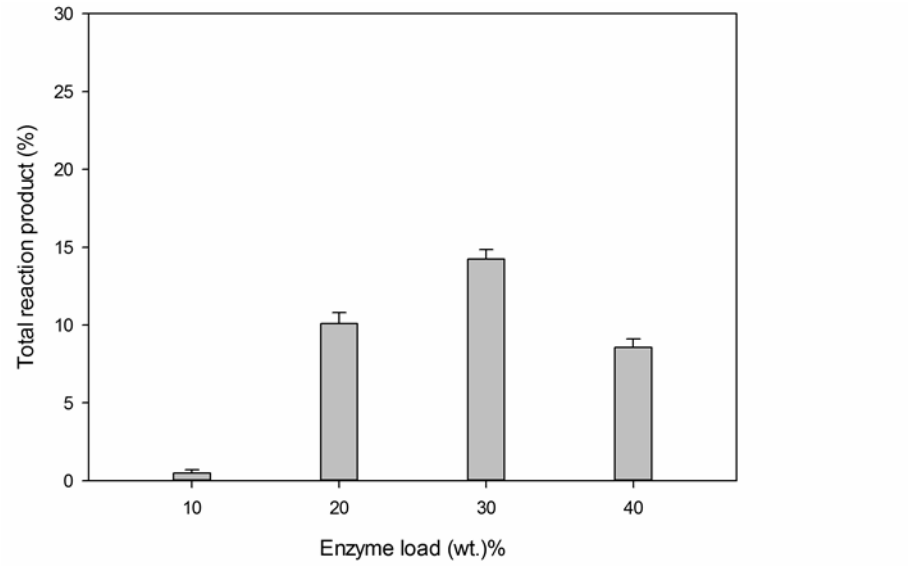


Figure 4.24 Effect of enzyme load on the enzymatic reaction carried out between 2-MAG and *N*-acetyl-L-valine using lipase from *T. lanuginosus.* Reaction conditions: time 24 h, 2-MAG:*N*-acetyl-L-valine molar ratio 1:4, temperature 50°C

Effect of time

The reactions were carried out for 2-72 h with the purpose of observing the effect of time on the reaction. The total reaction product increased to 21.5% in the first 12 h and reached to equilibrium at 12 h at the reaction occured between 2-MAG and *N*-acetyl-L-leucine. The total reaction product decreased after 48 h (Figure 4.25). In the case of reaction carried out between 2-MAG and *N*-acetyl-L-valine, the total reaction product continued to increase in 24 h and decreased after 24 h (Figure 4.26).

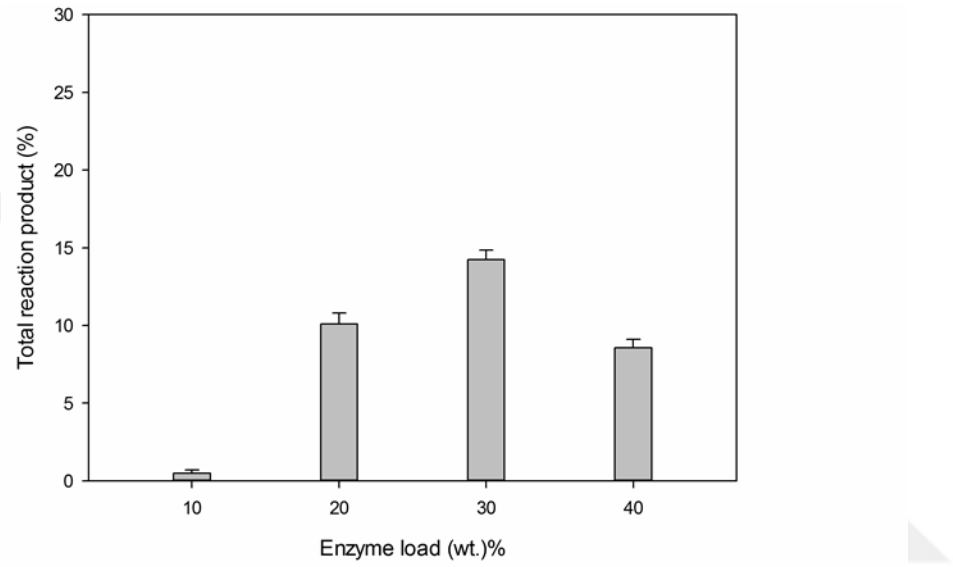


Figure 4.25 Effect of time on the enzymatic reaction between 2-MAG and *N*-acetyl-L-Leucine. Reaction conditions: Enzyme load 20 (wt.) %, temperature 50°C, 2-MAG: *N*-acetyl-L-leucine mole ratio 1:4.

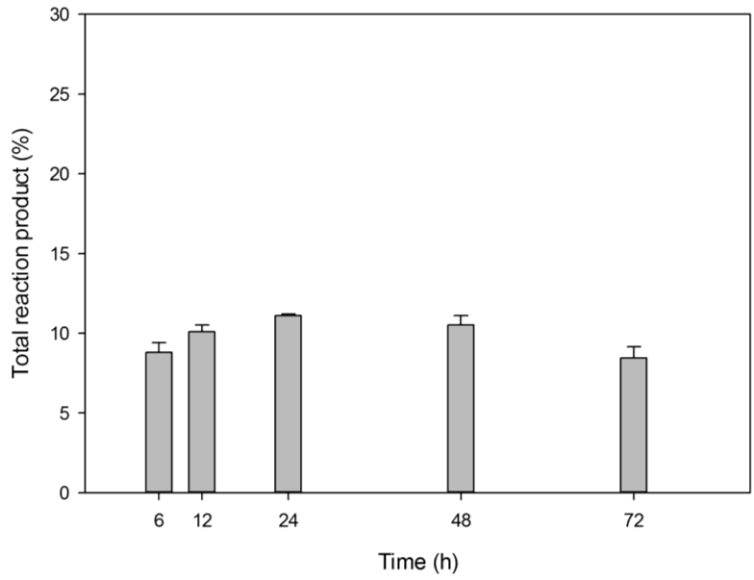


Figure 4.26 Effect of time on the enzymatic reaction between 2-MAG and *N*-acetyl-L-Valine. Reaction conditions: Enzyme load 20 (wt.) %, temperature 50°C, 2-MAG: *N*-acetyl-L-valine mole ratio 1:4.

4.5.7 Investigation of the Main Effects of Different Parameters and Their Interactions on the Enzymatic Reaction using the Response Surface Methodology

The main and interactive effects of the parameters for the incorporation of *N*-acetyl-L-leucine and *N*-acetyl-L-valine into the 2-MAG with enzymatic method were investigated using response surface methodology. The range of the parameters studied at experimental design was given at Table 3.3. Table 3.4 shows the experimental and predicted values for the total reaction product (%) at each of the 20 experimental points generated by RSM for two different amino acids: *N*-acetyl-L-leucine and *N*-acetyl-L-valine. The predicted values were obtained from the model fitting technique using Design Expert 7.0 and were seen to be closely correlated to the experimental value. Table 4.12-13 shows the regression coefficients and P values of independent variables as linear, quadratic and interactive for total reaction product (%). The significant P value (<0.0001) for model and non-significant lack of fit (P<0.05) indicated that quadratic model was a good fit.

4.5.7.1 Enzymatic Reaction Between 2-MAG and *N*-acetyl-L-leucine

4.5.7.1.1 Investigation of the Main Effects of Different Parameters and Their Interactions on the Enzymatic Reaction between 2-MAG and *N*-acetyl-L-leucine using the RSM

RSM was implemented to model total reaction products for the enzymatic reaction between 2-MAG and *N*-acetyl-L-leucine. The data of response obtained from reaction under different reaction conditions were shown in Table 3.4. The predicted model was significant at the 99% confidence level and the lack of fit was not significant ($p<0.05$) with high coefficient of determinations (R^2) 0.95. The high values of coefficient of determination, significance of model and non-significant lack of fit indicate that the model was a good fit. The best fitting quadratic model was determined by regression and backward elimination by means of elimination of insignificant factors and interactions in the models. The coefficients and P values were given in Table 4.12.

Table 4.12 ANOVA and model equation coefficients for response surface quadratic model of total reaction product after enzymatic reaction carried out between 2-MAG and *N*-acetyl-L-leucine

Sources	Coefficient	F Value	P Value Prob > F
Model[a]		33.44	< 0.0001
Intercept	-72.78		
Linear			
Time	1.06	25.57	0.0003[b]
Temperature	3.04	45.82	< 0.0001[b]
Enzyme load	1.13	15.39	0.0020[b]
Interactive			
Time × Temperature	-0.03	62.35	< 0.0001[b]
Temperature × Enzyme load	-0.02	30.52	0.0001[b]
Quadratic			
Time × Time	0.01	22.81	0.0005[b]
Temperature × Temperature	-0.01	26.73	0.0002[b]
Lack of fit		4.39	0.0613[c]

[a] Determination coefficient of the model (R^2); 0.95. [b]'Significant at 'Prob>F' <0.05, [c] not significant at 'Prob>F'>0.05.

Effect of temperature

Temperature was found as the most effective parameter on the reaction ($p<0.0001$) (Table 4.12). The positive coefficient of temperature (+3) showed that increasing temperature raised the percentage of total reaction product. However, significant quadratic term of temperature ($p<0.001$) with negative coefficient (-0.02) assigned that temperature had a weaker effect on the total reaction product (%) as the temperature increased. The reason of this trend may be the decreasing stability of the 2-MAG at high temperatures (Compton et al., 2007).

Although the main effect of temperature was positive, its interactive effect with time negative. The interactive effect reflected to the response surface plots (Figure 4.27a). For instance, the total reaction product (%) increased from 20.30 to 27.50% at 8 h (Figure 4.27a) while decreased slightly from 22.10 to 21.20% at 24 h by increasing temperature from 40 to 55 °C. The reasons of the decrease at long reaction time and high temperature might be (1) the hydrolysis of ester bond between *N*-acetyl-L-leucine and hydroxy groups of 2-MAG (2) the unstability of 2-MAG.

Effect of enzyme load

Enzyme load had a statistically significant ($p<0.01$) and positive effect (+1.13) on the total reaction product (%). Interaction between enzyme load and temperature was found significant ($p<0.0001$). The negative coefficient of this interaction (-0.02) indicated that there is an inverse relationship between enzyme load and temperature on the percentage of total reaction product. The trend of the relationship was confirmed by Figure 4.27b. The total reaction product increased from 19.90 to 21.35% at 40°C while decreased from 25.70 to 21.35% at 55 °C with increasing enzyme load in the given range (Figure 4.27b). This means that increasing temperature decreased the positive effect of enzyme load on total reaction product. Fomuso and Akoh (1997) reported that temperature changes can also affect parameters, such as enzyme stability, affinity of enzyme for substrate, and preponderance of competing reactions.

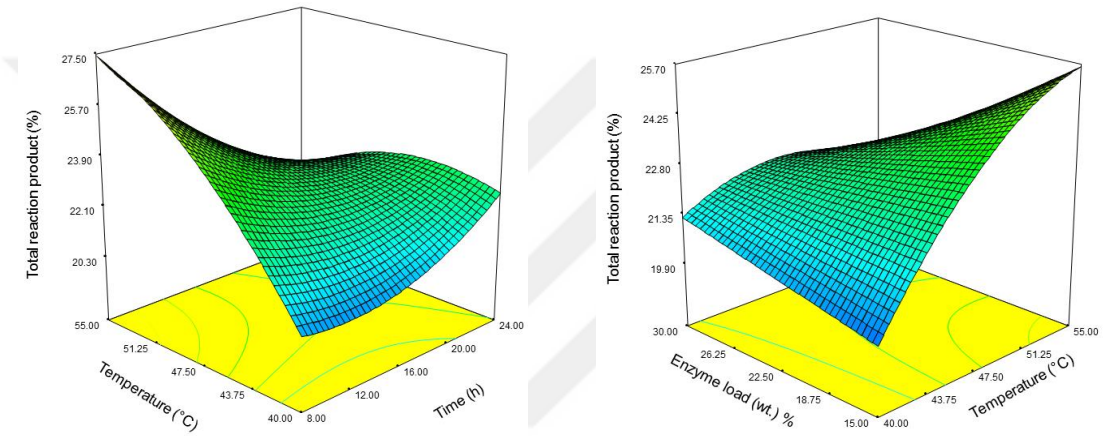
Effect of time

The main effect of reaction time on the enzymatic reaction was significantly ($p<0.001$) positive (+1.06) (Table 4.12). This means that total reaction product (%) increased with increasing reaction time. The positive coefficient (+0.01) of quadratic term of time showed that the effect of time becomes more strong on the percentage of total reaction product as time increased ($p<0.0001$). Although the interaction terms of time and temperature, enzyme load and temperature was statistically significant, the interaction of time and enzyme load was not statistically significant ($p>0.05$). Figure 4.27c shows that increasing enzyme load causes a slight, nonsignificant decrease in the total reaction product (%) at the same reaction time. At high enzyme load and long reaction times the reaction may shift towards hydrolysis of *N*-acetyl-L-leucine rather than esterification.

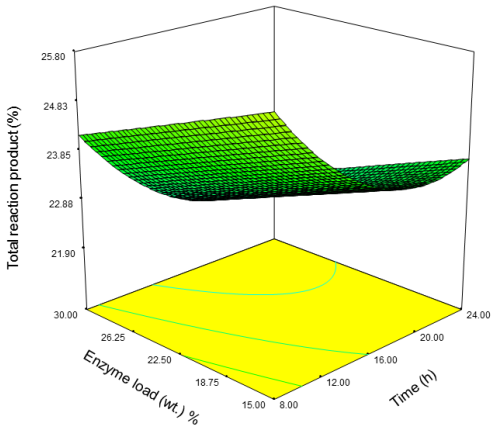
4.5.7.1.2 Optimization and Verification

The reaction conditions were optimized for the maximum total reaction product (%) using Design Expert Software 7.0. In the reactions, substrate mole ratio was fixed at 1:4 (2-MAG:*N*-acetyl-L-leucine, mol:mol). The optimum conditions for the highest incorporation of *N*-acetyl-L-leucine was found to be as temperature 54ºC, time 8 h, enzyme load 15 (wt.)%. At these reaction conditions, the total reaction product was

29.3%. For the verification of the model, experiments were performed at optimum conditions. The mean of total reaction product percentage of three experiments were 28.5%. This implied that the model was successfully applied. The lipase from *T. lanuginosus* was stable for at least 6 uses in the reaction of enzymatic incorporation of *N*-acetyl-L-leucine at the optimum conditions and the total reaction product (%) remained approximately constant over the 6 reaction cycle catalyzed by the lipase from *T. lanuginosus*.



(a) Enzyme load: 22.50 (wt.) % (b) Time: 16.00 h



(c) Temperature: 47.50 °C

Figure 4.27 Response surface plots for the total reaction product after the reaction of 2-MAG and *N*-acetyl-L-leucine. Total reaction product contains 2-acyl-(1)3-acetylleucine-rac-glycerol and 2-acyl-1,3-di-acetylleucine-rac-glycerol (a) temperature-time, (b) enzyme load-temperature, (c) enzyme load-time

4.5.7.2 Enzymatic Reaction Between 2-MAG and *N*-acetyl-L-valine

4.5.7.2.1 Investigation of the Main Effects of Different Parameters and Their Interactions on the Enzymatic Reaction Between 2-MAG and *N*-acetyl-L-valine Using RSM

RSM was implemented to model total reaction products (%) for the enzymatic reaction between 2-MAG and *N*-acetyl-L-valine. The data of response obtained from reaction under different reaction conditions were shown in Table 3.4. The predicted model was significant at the 99% confidence level and the lack of fits were not significant ($p<0.05$) with high coefficients of determinations (R^2) 0.91. The high values of coefficient of determination, significance of model and non-significant lack of fit indicate that the model was a good fit. The best fitting linear model was determined by regression and backward elimination by means of elimination of insignificant factors and interactions in the models. The coefficients and p values were given in Table 4.13.

Table 4.13 ANOVA and model equation for response surface linear model of total reaction product after enzymatic reaction between 2-MAG and ***N***-acetyl-L-valine

Sources	Coefficient	F Value	P Value Prob > F
Model[a]		58.85	< 0.0001
Intercept	-1.63		
Linear			
Time	0.02	0.44	0.5139[b]
Temperature	0.08	6.37	0.0225[c]
Enzyme load	0.42	169.74	< 0.0001[c]
Lack of fit		0.52	0.8291[b]

[a] Determination coefficient of the model (R^2), 0.91; [b] not significant at Prob>F'>0.05; [c] Significant at 'Prob>F' <0.05

Effect of enzyme load

Enzyme load was found to be the most significantly important parameter that affects the enzymatic incorporation of *N*-acetyl-L-valine into the 2-MAG ($p<0.0001$) (Table 4.13). The main effect of enzyme load had a positive effect on the percentages of total reaction product according to its positive coefficient (+0.42). This indicated that increasing enzyme load increased the total reaction product. Also, confirming this

result, in Figure 4.28a,c, it was seen that the increase in enzyme load increased the total reaction product linearly.

Effect of temperature

The temperature had a significant ($p<0.05$) and positive effect on the reaction carried out between 2-MAG and *N*-acetyl-L-valine (Table 4.13). The increase in temperature caused increase in total reaction product according to its positive coefficient (+0.08). The response surface plots and ANOVA results were in good agreement. The interaction of temperature with time and enzyme load were seen on Figure 4.28a,b. In these Figures, it could be seen that the changes in enzyme load and time did not change the effect of temperature on the reaction (Figure 4.28a,b). These results confirmed that there was no interactive effect between temperature and time or enzyme load.

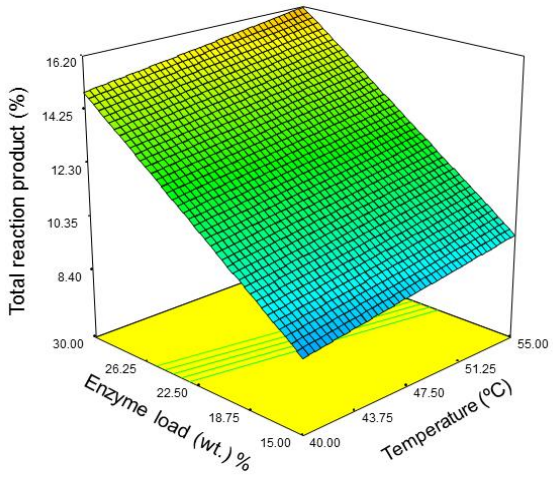
Effect of time

Due to the ANOVA results, reaction time did not have a significant effect on the reaction ($p>0.05$) (Table 4.13). In Figure 28a,b, it was seen that the changes in reaction time did not change the total reaction product with any change in enzyme load or temperature. The results indicated that the incorporation of *N*-acetyl-L-valine into the 2-MAG was completed in 8 h and after that the incorporation yield did not changed.

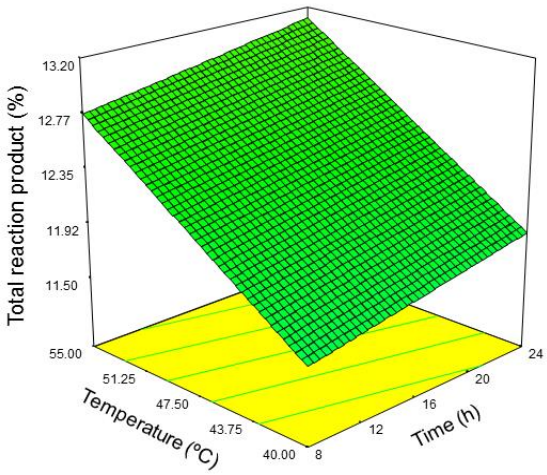
4.5.7.2.2 Optimization and Verification

The reaction conditions were optimized for the maximum total reaction product (%) using optimized function of Design Expert Software 7.0. In the reactions, substrate mole ratio was fixed at 1:4 (2-MAG: *N*-acetyl-L-valine, mol:mol). At these reaction conditions, the total reaction product percentage was 16.33. For the verification of the model, experiments were performed at optimum conditions. The average total reaction product percentage of three experiments was 18.22. This implied that the model was successfully applied. The lipase from *T. lanuginosus* remained stable for at least 6 uses in the reaction of enzymatic incorporation of *N*-acetyl-L-valine at

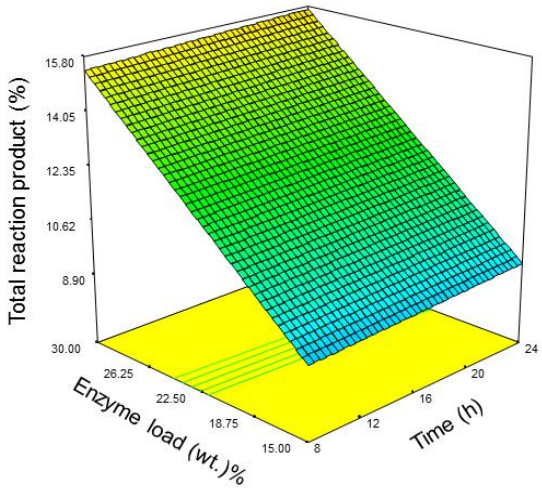
optimum conditions and the total reaction product (%) remained approximately constant over the 6 reaction cycle catalyzed by the lipase from *T. lanuginosus*.



(a) Enzyme load: 22.50 (wt.) %



(b) Time: 16.00 h



(c) Temperature: 47.50 °C

Figure 4.28 Response surface plots for the total reaction product after the reaction of 2-MAG and *N*-acetyl-L-valine. Total reaction product contains 2-acyl-(1)3-acetylvaline-rac-glycerol ve 2-acyl-1,3-di-acetylvaline-rac-glycerol (A) enzyme load-temperature, (B) temperature-time, (C) enzyme load (wt%)-time.

4.5.8 Removal of *N*-acetyl-L-leucine and *N*-acetyl-L-valine from the Final Reaction Mixture

The final reaction mixture contains 2-acyl-(1)3-acetylleucine(valine)-rac-glycerol and 2-acyl-1,3-di-acetylleucine(valine)-rac-glycerol and unreacted 2-MAG, *N*-acetyl-L-leucine or *N*-acetyl-L-valine. Firstly, bulk production was carried out for the collection of the reaction products at optimum conditions which were determined

using response surface methodology for purification. The purity of the purified products of the reactions carried out by *N*-acetyl-L-leucine and *N*-acetyl-L-valine was 70.10 and 65.70%, respectively.

4.6 Investigation of Some Physical, Chemical and Physicochemical Properties of the Purified Products

Some of the physical, chemical and physicochemical properties of the ROPO and purified products were given in Table 4.14.

4.6.1 Density

The densities of ROPO and purified products were given in Table 4.14. *N*-acetyl-L-leucine incorporated product had a density of 0.952 g/mL while *N*-acetyl-L-valine incorporated product had a density of 0.944 g/mL. The densities of the products were found to be significantly higher than ROPO (0.916 g/mL). The reason of the difference may be related with the fact that the density of *N*-acetyl-L-leucine (1.06 g/mL) and *N*-acetyl-L-valine (1.094 g/mL) were higher than the major fatty acids of the ROPO (i.e. oleic acid, 0.89 g/mL). Liquid oils have a density between 0.91 and 0.92 g/mL. Oil density generally increases with low molecular weight fatty acids and hydrogenation (Hidalgo and Zamora, 2005). Thus, the low molecular weight of the amino acid derivative may be the reason of high density of modified lipid.

Table 4.14 Some of the physical, chemical and physicochemical properties of the ROPO and purified products at 25°C

	ROPO	Purified product[1]	Purified product[2]
Density (g/mL)	0.916±0.00[3,A]	0.952±0.00[B]	0.944±0.00[C]
Viscosity (cp)	78.40±0.32[A]	87.75±0.15[B]	87.54±0.22[C]
Specific heat capacity (cal/g°C)	0.46±0.05[A]	0.38±0.01[A]	0.37±0.01[B]
Thermal expansion coefficient	3.52±0.00[A]	3.65±0.00[B]	3.58±0.00[C]
Thermal conductivity (W/m*K)	0.17±0.00[A]	0.15±0.00[A]	0.15±0.00[A]
Vapour absorption capacity (ppm)	995.00±5.10[A]	4800.00±5.80[B]	4775.00±6.10[B]
Solubility in water (mL)	1.50±0.10[A]	2.70±0.10[B]	2.50±0.00[C]
Crismer value	80.12±0.25[A]	78.30±0.16[B]	77.15±0.25[C]
Calorie (kcal/g)	9.49±0.00[A]	7.72±0.05[B]	6.20±0.07[C]

[1] Obtained from the reaction of 2-MAG and *N*-acetyl-L-leucine; [2] Obtained from the reaction of 2-MAG and *N*-acetyl-L-valine; [3] Standart deviation (n=3). [A-C] Means within each rows with different letters are significantly (P<0.05) different.

4.6.2 Melting profile and solid fat content (SFC)

Onset and melting peak temperatures for ROPO and purified products were given in Table 4.15. The onset and melting peak temperatures of ROPO were -9.33 ve 6.88°C, respectively. The onset and melting peak temperatures of the products obtained from the reaction of 2-MAG and *N*-acetyl-L-leucine were -7.37 and 4.86°C. The onset and melting peak temperatures of the products obtained from the reaction of 2-MAG and *N*-acetyl-L-valine were -7.12 and 4.73°C. The reason of the lower melting point of the products might be explained by the presence of amino acid derivatives which have lower carbon chain than fatty acids of ROPO. The solid fat contents of ROPO and the products were given in Table 4.16. Solid fat content of the products were determined from the area under their melting curves. 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 ease of spreading and oil exudation. 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. Solid fat contents of the purified products indicated that the products had the similar values with ROPO and behaved as liquid oil at room temperature.

Table 4.15 Melting profiles of ROPO and purified products

Samples	T_{onset} (°C)	T_{peak} (°C)
ROPO	-9.33	6.88
Purified product[1]	-7.37	4.86
Purified product[2]	-7.12	4.73

[1] Obtained from the reaction of 2-MAG and *N*-acetyl-L-leucine; [2] Obtained from the reaction of 2-MAG and *N*-acetyl-L-valine.

Table 4.16 Solid fat content of ROPO and purified products

Samples	SFC(%) at (°C)				
	-20	**-10**	**0**	**4**	**10**
ROPO	100.00	97.02	32.23	8.96	0.00
Purified product[1]	100.00	99.87	45.60	9.40	0.00
Purified product[2]	100.00	99.97	45.40	9.43	0.00

[1] Obtained from the reaction of 2-MAG and *N*-acetyl-L-leucine; [2] Obtained from the reaction of 2-MAG and *N*-acetyl-L-valine.

4.6.3 Oxidative stability

Differential scanning calorimeter (DSC) technique was applied successfully to obtain the oxidative stability of the purified products and ROPO. The thermal changes which were occured during oxidation of the oil were recorded by DSC. A rapid increase in the amount of evolved heat is observed during initiation of the oxidation reaction and it is recorded as induction time. The induction times of the products and ROPO were given in Table 4.17. Both of the products showed lower induction time than refined olive pomace oil. The reason of lower induction time might be the presence of phenolics and tocopherols in refined olive pomace oil which increases the oxidative stability.

Table 4.17 Induction times of refined olive pomace oil and purified products

Samples	**Temperature (°C)**			
	110	**120**	**130**	**140**
ROPO	252.00	117.00	62.19	40.89
Purified product[1]	150.00	78.60	45.72	42.51
Purified product[2]	148.00	76.40	44.80	41.57

[1] Obtained from the reaction of 2-MAG and *N*-acetyl-L-leucine; [2] Obtained from the reaction of 2-MAG and *N*-acetyl-L-valine.

4.6.4 Viscosity

Liquid viscosity is important in the design of process equipment for the fatty acid industry. For example, it is an important parameter in estimating efficiency of distillation columns for separation of fatty acids. Liquid viscosity data are required in the design of heat transfer equipment. Process piping design and pressure drop determination also require viscosity data (Noureddini et al., 1992). The viscosities of the *N*-acetyl-L-leucine and *N*-acetyl-L-valine incorporated products were found to be 87.75 and 87.54 cp, respectively (Table 4.14). The products had statistically higher viscosities than that of ROPO (78.40).

4.6.5 Specific Heat Capacity, Thermal Expansion Coefficient and Thermal Conductivity

The specific heat capacity of ROPO (0.46 kal/g°C) was statistically higher than those of the amino acid derivative incorporated products (Table 4.14). The specific heat capacity of *N*-acetyl-L-leucine incorporated product was 0.38 cal/g°C and *N*-acetyl-L-

valine incorporated product was 0.37 cal/g°C. These results suggested that more energy is required to heat refined olive pomace oil compared to the same amount of *N*-acetyl-L-leucine (or *N*-acetyl-L-valine) incorporated product. Thermal coefficients of the products and ROPO were significantly different from each other. ROPO had the lowest thermal expansion coefficient. The thermal conductivities of ROPO and the products were statistically similar to each other (Table 4.14). The thermal conductivities of ROPO and *N*-acetyl-L-leucine (or *N*-acetyl-L-valine) incorporated products were 0.17 W/mK and 0.15 W/mK, respectively. Thermal expansion coefficients were determined using the density values of the compounds at different temperatures (Table 4.14). It can be concluded that incorporation of *N*-acetyl-L-leucine (or *N*-acetyl-L-valine) did not change the thermal expansion coefficient.

4.6.6 Vapour Absorption Capacity

ROPO and the purified products were hold at an environment at which relative humidity was 65% for one week period. At the end of this period, the water content of *N*-acetyl-L-leucine and *N*-acetyl-L-valine incorporated products were found as 4800 and 4775 ppm, respectively. This value was 995 ppm for refined olive pomace oil. The statistical analysis showed that vapour absorption capacity of refined olive pomace oil were statistically lower that the purified products. In literature, it was reported that the maximum vapour absorption capacity of triacylglycerols might be 1000 ppm (Nakajima et al., 2008). Thus, addition of this modified lipid may increase the moisture content and water activity of the food products. There are other studies indicated similarly that the addition of fat replacers increased the moisture content and water activity of the reduced-fat products since fat mimetics generally absorbed more water (Akoh, 1998a; Pimdit et al., 2008). The ability of the products to solubilize hydrophilic substances is a positive property that specifically solubilizes functional substances in oil. However, its higher hydrolyzability is also a negative property. Since this product binds excessive water and may denature at high temperatures, it is not suitable for frying, however, are suitable for baking and retorting as fat mimetics (Akoh, 1998a).

4.6.7 Microstructure

The grey and normal scale micrographs, taken at the same magnification (20X), were given in Figure 4.29. The micrographs of the products and refined olive pomace oil

had some differences. While needle-like shape crystals were observed in refined olive pomace oil, large symmetrical spherical crystals were observed after incorporation of *N*-acetyl-L-leucine (or *N*-acetyl-L-valine) into the 2-monoacylglycerols. The crystal sizes of the products were 10 times higher than refined olive pomace oil. The average crystal size of refined olive pomace oil was 5 µm while crystal sizes of products were 50 µm. Moreover, it was observed that there were different sizes of crystals in ROPO. However, the sizes of the spherical crystals were homogenous. In literature, it was reported that increase in the difference in composition of oils results in small size crystals in the oil (Van vliet, 2013).

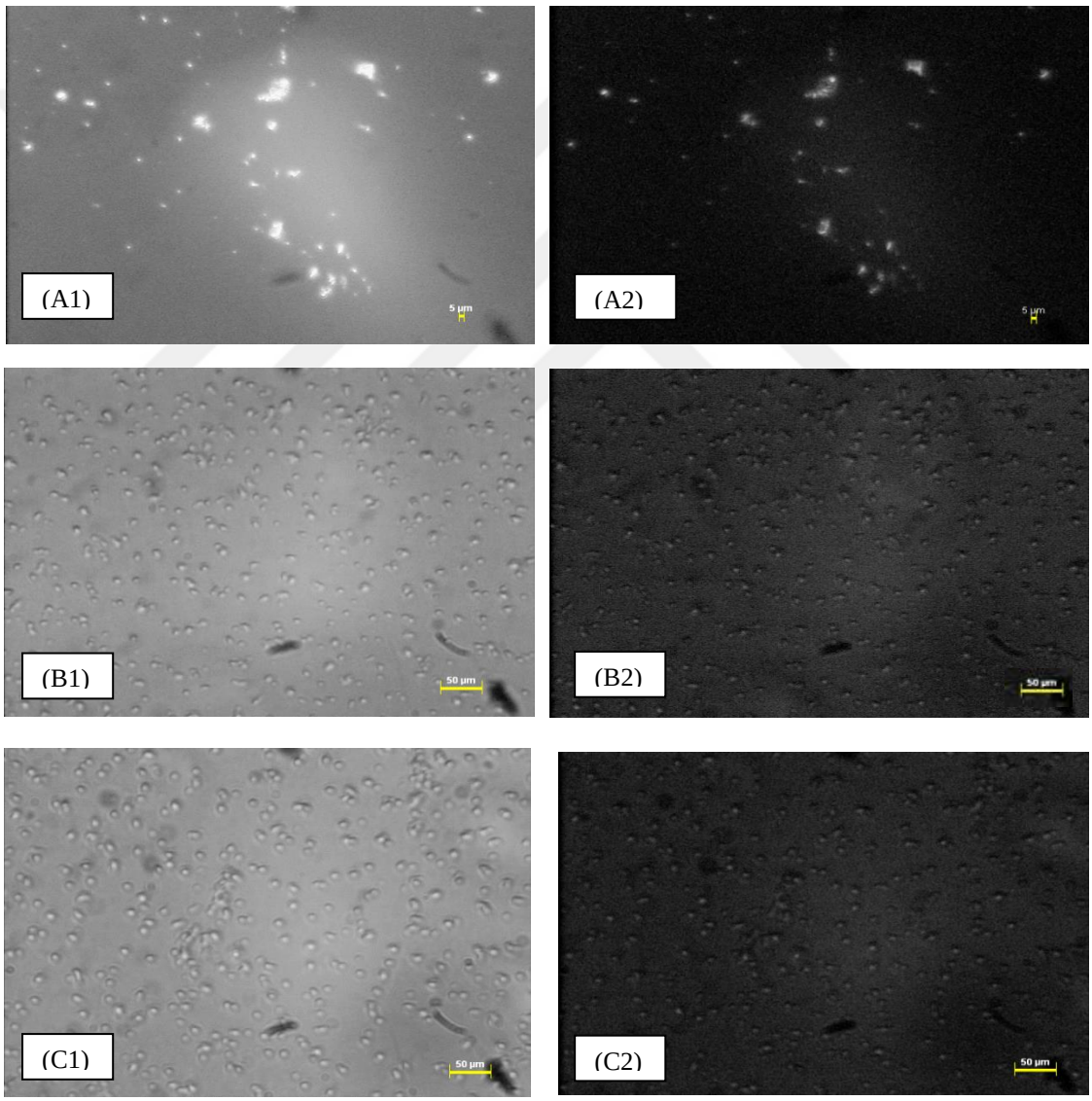


Figure 4.29 Polarized light micrographs. A, Refined olive pomace oil; B, Purified products obtained from the reaction of 2-MAG and *N*-acetyl-L-leucine; C, Purified product obtained from the reaction of 2-MAG and *N*-acetyl-L-valine. 1, normal scale micrographs; 2, polarized gray scale micrographs.

4.6.8 Solubility Properties

4.6.8.1 Solubility in Water (at 25ºC)

The purified reaction products and ROPO (2 mL) was disscolved in pure alcohol (4 mL). After that, the solution was titrated with distilled water until the turbidity was observed. The volume of water which was required to be added to the mixture was multiplied with 100. The result gave the 'solubility value in water'. The results were reported at Table 4.14. The solubility of ROPO and the two different products was statistically different from each other. Refined olive pomace oil had the statistically lowest solubility in water. The results were in good agreement with the results of vapour absorption capacity study which was mentioned at Section 4.6.6.

4.6.8.2 Crismer Value

Crismer value is one of the specification criteria used for international trade, mostly in Europe. Characteristic values are usually within a narrow limit. The miscibility of an oil is related to the solubility of glycerides, and is affected mainly by the unsaturation and chain length of the constituent fatty acids. The crismer values of refined olive pomace oil and *N*-acetyl-L-leucine (or *N*-acetyl-L-valine) incorporated products were given in Table 4.14. The crismer values of *N*-acetyl-L-leucine incorporated product (78.30) and *N*-acetyl-L-valine incorporated product (77.15) were statistically lower than the crismer value of refined olive pomace oil (80.12).

4.6.9 Caloric Value

One of the main objective of this thesis was to synthesize a novel product whose calorie was reduced comparable to refined olive pomace oil by addition of amino acids. In this respect, the caloric values of the products and refined olive pomace oil was determined using bomb calorimeter. The caloric value of refined olive pomace oil was determined as 9.49 kcal/g while caloric values of the *N*-acetyl-L-leucine and *N*-acetyl-L-valine incorporated products were determined as 7.72 and 6.20 kcal/g. The caloric values of the products were reduced 18% compared to refined olive pomace oil. The decrease was statistically significant due to the ANOVA results. It is a result of the fact that the caloric values of *N*-acetyl-L-leucine and *N*-acetyl-L-valine are 6.06 kcal/g and 3.63 kcal/g, respectively while caloric values of the most

abundant fatty acids of olive pomace oil, oleic, linoleic and palmitic acids are 9.43, 9.36 and 9.34 kcal/g, respectively. Moreover, since amino acids can not be metobolized completely, this newly synthesized lipid may have a usable energy value less than 7.7 kcal/g. Additionaly, Ingle et al. (1999) reported one key source of information showing that a reduced calorie medium-chain triglycerides has lower gross energy value that of determined by heat of combustion measured with bomb calorimetric methods. This modified lipid may be a potential product for low calorie fat replacers. The caloric value of the modified lipid seems to be comparable to some of the commercial low calorie fats whose calorie is between 5-8.3 kcal/g (Jones, 1996).

CONCLUSIONS

In this thesis, an enzymatic production of a modified lipid which has low calorie value and high nutritional value and shows oil properties was developed by the incorporation of *N*-acetyl-L-leucine and *N*-acetyl-L-valine into 2-MAG. This reaction was limited by the fact that lipases are inactivated by polar solvents in which amino acids are soluble. A new developed method was presented for the esterification of 2-monoacylglycerol with *N*-acetyl-L-leucine and *N*-acetyl-L-valine overcoming that problem.

First step was the enzymatic synthesis of 2-MAG from refined olive pomace oil. The immobilized lipase from *C. antarctica* was found to be the most effective catalyst in the synthesis 2-MAG from ROPO using enzymatic ethanolysis reaction. Optimization of the ethanolysis reaction was performed by using response surface methodology. The main effects of 4 reaction parameters (enzyme load, temperature, time, and ethanol:oil molar ratio) and interactions between them were successfully elucidated using response surface methodology. Of the variables investigated, ethanol:oil molar ratio exhibited the greatest effect on synthesis of 2-MAG. Response surfaces presented that there was a complex interaction between the studied variables. Optimum reaction conditions were temperature 45 °C, ethanol:oil molar ratio 50:1, enzyme load 10 wt%, and time 5 h. Purification of 2-MAG from the ethanolysis mixture was performed by low-toxicity solvents with a recovery yield and purity of 80.10% and 96.00%, respectively.

Second step was the enzymatic esterification of 2-MAG with *N*-acetyl-L-leucine and *N-acetyl*-L-valine. The most effective esterification was carried out using lipase from *T. lanuginosus* at ethanol:hexane (80:20, v:v) reaction medium. Reaction conditions at which maximum incorporation occurs were optimized by studying the effects of the independent parameters (temperature, enzyme load, time) using three factors five levels face centered central composite design response surface methodology. The optimum reaction conditions were determined as temperature 54ºC, time 8 h, enzyme

load 15 (wt%) for the predicted maximum total reaction product of *N*-acetyl-L-leucine (29.3%). For the predicted maximum total reaction product of *N*-acetyl-L-valine (16.3%), the optimum reaction conditions were determined as temperature 55ºC, time 24 h, enzyme load 30.00 (wt%). In the experiments performed at optimum conditions, the incorporation yields of *N*-acetyl-L-leucine and *N*-acetyl-L-valine were 28.51 and 18.22%, respectively being closely correlated to the predicted results.

The products were purified and analyzed by LC-MS-MS. The results showed that novel modified lipid which have low caloric value and essential amino acid derivatives in their structure were synthesized successfully. The caloric value of the new product was reduced by 18% and this product behaved as oil above 10°C according to SFC results. These results indicated that a novel modified lipid was produced from 2-monoacylglycerol successfully. This modified lipid behaved 'as an oil' and may have beneficial effect on human health as a result of its high oleic and essential amino acid derivative content. This novel modified lipid may be utilized as a low calorie fat replacer in healthcare, pharmaceutical and healthy food industries. However, further research is required to increase the yield of this valuable product and also determine the digestibility and absorptivity of this modified lipid.

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APPENDIX

A. Statistical analysis of the physical, chemical and physicochemical properties of the ROPO and purified products

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Density	,000	2	6	1,000
Viscosity	,000	2	6	1,000
Specific heat capacity	,000	2	6	1,000
Thermal expansion	,000	2	6	1,000
Thermal conductivity	,000	2	6	1,000
Solubility in water	,000	2	6	1,000
Crismer Value	1,141	2	6	,380
Calorie	1,493	2	6	,298

ANOVA

		Sum of Squares	df	Mean Square	F
Density	Between Groups	,002	2	,001	108453,000
	Within Groups	,000	6	,000	
	Total	,002	8		
Viscosity	Between Groups	171,006	2	85,503	855031,000
	Within Groups	,001	6	,000	
	Total	171,007	8		
Specific heat capacity	Between Groups	,015	2	,007	73,000
	Within Groups	,001	6	,000	
	Total	,015	8		
Thermal expansion	Between Groups	,025	2	,013	127,000
	Within Groups	,001	6	,000	
	Total	,026	8		
Thermal conductivity	Between Groups	,000	2	,000	1,000
	Within Groups	,001	6	,000	
	Total	,001	8		
Solubility in water	Between Groups	2,480	2	1,240	12400,000
	Within Groups	,001	6	,000	
	Total	2,481	8		
Crismer Value	Between Groups	10,931	2	5,466	70,093
	Within Groups	,468	6	,078	
	Total	11,399	8		
Calorie	Between Groups	8,566	2	4,283	1713,280
	Within Groups	,015	6	,002	
	Total	8,581	8		

ANOVA

		Sig.
Density	Between Groups	,000
	Within Groups	
	Total	
Viscosity	Between Groups	,000
	Within Groups	
	Total	
Specificheatcapacity	Between Groups	,000
	Within Groups	
	Total	
Thermal expansion	Between Groups	,000
	Within Groups	
	Total	
Thermal conductivity	Between Groups	,422
	Within Groups	
	Total	
Solubility in water	Between Groups	,000
	Within Groups	
	Total	
Crismer Value	Between Groups	,000
	Within Groups	
	Total	
Calorie	Between Groups	,000
	Within Groups	
	Total	

Post Hoc Tests

Homogeneous Subsets

Density

Duncan[a]

		Subset for alpha = 0.05		
Samples	N	1	2	3
1,0000	3	,916200		
3,0000	3		,944600	
2,0000	3			,952300
Sig.		1,000	1,000	1,000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3,000.

Viscosity

Duncan[a]

Samples	N	Subset for alpha = 0.05		
		1	2	3
1,0000	3	78,4000		
3,0000	3		87,5400	
2,0000	3			87,7500
Sig.		1,000	1,000	1,000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3,000.

Specific heat capacity

Duncan[a]

Samples	N	Subset for alpha = 0.05	
		1	2
3,0000	3	,3700	
2,0000	3	,3800	
1,0000	3		,4600
Sig.		,267	1,000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3,000.

Thermal expansion

Duncan[a]

Samples	N	Subset for alpha = 0.05		
		1	2	3
1,0000	3	3,5200		
3,0000	3		3,5800	
2,0000	3			3,6500
Sig.		1,000	1,000	1,000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3,000.

Thermal conductivity

Duncan[a]

Samples	N	Subset for alpha = 0.05
		1
2,0000	3	,1500
3,0000	3	,1500
1,0000	3	,1600
Sig.		,281

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3,000.

Solubility in water

Duncan[a]

Samples	N	Subset for alpha = 0.05		
		1	2	3
1,0000	3	1,5000		
3,0000	3		2,5000	
2,0000	3			2,7000
Sig.		1,000	1,000	1,000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3,000.

Crismer Value

Duncan[a]

Samples	N	Subset for alpha = 0.05		
		1	2	3
3,0000	3	77,4833		
2,0000	3		78,3000	
1,0000	3			80,1200
Sig.		1,000	1,000	1,000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3,000.

Calorie Value

Duncan[a]

Samples	N	Subset for alpha = 0.05		
		1	2	3
3,0000	3	7,2000		
2,0000	3		7,7200	
1,0000	3			9,4800
Sig.		1,000	1,000	1,000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3,000.

CURRICULUM VITAE

PERSONAL INFORMATION

Surname, Name: Çavdar Keskin, Hasene
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EDUCATION

Degree	Instution	Graduation year	GPA
Ph.D.	University of Gaziantep		4.00/4.00
M.Sc.	University of Gaziantep	2011	3.88/4.00
B.Sc.	University of Gaziantep	2009	3.42/4.00

ACADEMIC/WORK EXPERIENCE

Year	Place	Enrollment
August 2013-September, 2013	Participation in the project COST Action: TD1203, 'Pectin extraction and characterization from grapefruit peel' Green Chemistry Centre, Department of Chemistry, York, England.	Researcher
June 2012-September 2012	Participation in the project 'Biosynthesis of broad spectrum of sterol derivatives and physical characterization of its nano micelling behaviors', Faculty of Engineering, Aarhus University, Aarhus, Denmark.	Visiting Scholar
November 2009-present	Department of Food Engineering, Gaziantep University, Gaziantep, Turkey	Research Assistant
June 2008-September 2008	Institute of Crop Science, Hohenheim University, Germany	Intern student

PROJECTS

2013	Effect of different enzymes on the synthesis of 2-MAG using enzymatic ethanolysis	BAP
2013-2015	Production of modified lipid with incorporation of hydrophobic and essential amino acids into refined olive pomace oil and its characterization	TUBITAK, 1001
2010-2012	Production of Low Caloric and Spreadable Lipid by Enzymatic Interesterification of Terebinth Fruits Oil	BAP

PUBLICATIONS IN INTERNATIONAL JOURNALS

1) **Keskin, H.**, Koçak Yanık, D., Gök, U., Göğüş, F. (2017). Optimization of microwave-assisted extraction of Pomegranate (Punica granatum L.) seed oil and evaluation of its physicochemical and bioactive properties. Food Technology and Biotechnology, 55(1), 86.
2) **Keskin, H.**, Koçak Yanık, D., Mucuk, H. N., Göğüş, F., Fadıloğlu, S. (2016). Valorization of Olive Pomace Oil with Enzymatic Synthesis of 2-Monoacylglycerol. Journal of Food Science, 81(4), 841-848.
3) Koçak Yanık, D., **Keskin, H**., Fadıloğlu, S., Göğüş, F. (2016). Acidolysis of terebinth fruit oil with palmitic and caprylic acids in a recirculating packed bed reactor: optimization using response surface methodology. Grasas y Aceites, 67(2), e131.
4) Panpipat, W., **Keskin, H**., Guo, Z. (2015). Mapping the structure-activity relationship of β-sitosteryl fatty acid esters in condensing phospholipid monolayers. Frontiers Chemical Science and Engineering, 9(1),105–113.

5) Gogus, F., Ozel, M. Z., **Keskin, H**, Yanık, K. D., Lewis A. C. (2015). Volatiles of fresh and commercial sweet red pepper pastes: processing methods and microwave assisted extraction. Journal of Food Properties, 18, 1625–1634.
6) Koçak, D., **Keskin H.**, Fadıloğlu, S., and Göğüs, F. (2013). Acidolysis Reaction of Terebinth Fruit Oil with Palmitic and Caprylic Acids to Produce Low Caloric Spreadable Structured Lipid. Journal of American Oil Chemist's Society, 90, 999-1009.
7) **Keskin, H.**, Koçak, D., Göğüs, F. and Fadıloğlu, S. (2012). Enzymatic Acidolysis Of Triolein with Palmitic and Caprylic Acids: Optimization of Reaction Parameters by Response Surface Methodology, Grasas y Aceites, 63, 274-283.
8) Koçak, D., **Keskin, H.**, Fadıloğlu, S., Kowalski, B. and Göğüs, F. (2011). Characterization of Terebinth Fruit Oil and Optimization of Acidolysis Reaction with Caprylic and Stearic Acids. Journal of American Oil Chemist's Society, 88,1531–1538.

PRESENTATIONS

1) **Keskin, Ç. H.,** Yanık, K. D., Göğüş F., Fadıloğlu, S. (2017). Characterization of a Novel Modified Lipid: Nα-acetyl-L-leucine Incorporated 2-monoacylglycerol, 15. Eurofedlipid Congress, Uppsala, Sweden.
2) **Keskin, Ç. H**., Yanık, K. D., Göğüş F., Fadıloğlu, S. (2017). A Literature Survey: Active Compounds and Health Effects of Plant Seed Oils, 15. Eurofedlipid Congress, Uppsala, Sweden.
3) **Keskin, H**., Yanık, K. D., Göğüş F., Fadıloğlu, S. (2016). Lipase Catalyzed Esterification of 2-Monoacylglycerol with Nα-Ac-Leu(Val)-OH, European Biotechnology Congress, Riga, Latvia.
4) **Keskin, H.**, Yanık, K. D., Göğüş F., Fadıloğlu, S. (2015) A Novel Approach: Lipase Catalyzed Esterification of 2-monoacylglycerol and Nα-acetyl-L-leucine, 13. Eurofedlipid Congress, Florence, Italy.
5) Gök, U., **Keskin, H.**, Yanık, K. D., Göğüş F. (2015) Microwave Assisted Extraction of Pomegranate (Punica granatum L.) Seed Oil using Response Surface Methodology, 13. Eurofedlipid Congress, Florence, Italy.

6) Koyuncu, Ç., **Keskin, H.**, Yanık, K. D., Göğüş F. (2015) Microwave Assisted Extraction of Pectin from Grapefruit Peel: Optimization of Extraction Conditions, 1st International Conference on Green Chemistry and Sustainable Technologies, İzmir, Turkey.
7) Mucuk, H. N., Yanık, K. D., Fadıloğlu, S. **Keskin, H.**, Göğüş, F. (2014) Chemical characterization of pistachio (Pistacia vera) hull and yield of hexane and ethanol extracts 1st International Conference on Green Chemistry and Sustainable Technologies, İzmir, Turkey.
8) Yanık, K. D., **Keskin, H.**, Göğüş F., Fadıloğlu, S. (2015) Yağ Yerine Kullanılan Maddeler: Yağ İkameleri ve Yağ Taklitleri, YABİTED, Turkish Lipid Group, II. Vegetable Oil Congress, Tekirdağ, Turkey.
9) **Keskin, H.**, Yanık, K. D., Göğüş F., Fadıloğlu, S. (2015) Utilization of Oliev Pomace Oil: A Review, 3. Intenational Symposium on Traditional Foods from Adriatic to Caucasus, Sarajevo, Bosnia and Herzegovina.
10) **Keskin, H.**, Yanık, K. D., Göğüş F., Fadıloğlu, S. (2015) 'Çevre Dostu' Amino Asit Bazlı Sürfaktantlar, 2. İç Anadolu Bölgesi Tarım ve Gıda Kongresi, Nevşehir, Turkey.
11) **Keskin, H.**, Mucuk, H. N., Yanık, K. D., Göğüş, F., Fadiloglu S. (2014) Utilization of Olive Pomace Oil with Enzymatic Production of 2-monoacylglycerol, ICBET 2014: XII International Conference on Bioscience Engineering and Technology, Istanbul, Turkey.
12) Guo, Z., Panpipat, W., Xia, D., Dong, M., **Keskin, H.**, Xu, X. (2014) Condensing Effects of β-sitosteryl Aliphatic Esters on Dipalmitoyl Phosphatidylcholine (DPPC) Monolayers: A Structure Activity Relationship Study, 11. Eurofedlipid Congress, Montpellier, France.
13) **Keskin, H.**, Mucuk, H. N., Yanık, K. D., Göğüş, F., Fadıloğlu, S. (2014) Synthesis of 2-Monoacylglycerol with Enzymatic Hydrolysis using Porcine Pancreatic Lipase, International Food Congress Novel Approaches in Food Industry, İzmir, Turkey.
14) Mucuk, H. N., **Keskin, H.**, Göğüş, F., Fadıloğlu, S. (2014) Enzymatic production of 2-monoacylglycerol from olive pomace oil, International Food Congress Novel Approaches in Food Industry, İzmir, Türkiye.

15) **Keskin, H.**, Mucuk, H. N., Yanık, K. D., Göğüş, F., Fadıloğlu, S. (2014) The effect of reaction variables on the synthesis of 2-Monoacylglcycerol from refined olive pomace oil, 2014, 3rd International ISEKI- Food Congress-Food Science and Technology Excellence for a Sustainable Bioeconomy, Athens, Greece.
16) Gögüş, F., Ozel, Z. M., **Keskin, H.**, Yanık, K. D., Lewis C. A. (2014) Effect of Production Methods on the Volatile Compounds of Sweet Red Pepper Paste, 3rd International ISEKI- Food Congress-Food Science and Technology Excellence for a Sustainable Bioeconomy, Athens, Greece.
17) Koçak, D., **Keskin, H.**, Göğüs, F. and Fadıloğlu, S. (2013). Acidolysis Reaction of Terebinth Fruit Oil with Palmitic and Caprylic Acids in a Recirculating Packed Bed Reactor: Optimization using Response Surface Methodology, 11. Eurofedlipid Congress, Antalya, Turkey.
18) **Keskin, H.**, Mucuk, H. N., Yanık, K. D., Göğüş F., Fadıloğlu, S. (2013) Traditional Bitter Orange (Citrus Aurantium L.) Molasses, 2. Intenational Symposium on Traditional Foods from Adriatic to Caucasus, Struga-Ohrid, Macedonia.
19) Mucuk, H. N., **Keskin, H.**, Yanık, K. D., Göğüş F., Fadıloğlu, S. (2013) A Traditional, Natural and Nutrious Food Product: Molasses, 2. Intenational Symposium on Traditional Foods from Adriatic to Caucasus, Struga-Ohrid, Macedonia.
20) **Keskin, H.**, Koçak, D., Göğüs, F. and Fadıloğlu, S. (2012). Trioleinin Enzimatik Asidoliz Metoduyla Modifikasyonu, Turkish Lipid Group, I. Vegetable Oil Congress, Adana, Turkey.
21) Koçak, D., **Keskin, H.**, Fadıloğlu, S., and Göğüs, F. (2012). Menengiç Meyve Yağının Palmitik ve Kaprilik Asit ile Enzimatik Asidolizi: Tepki Yüzey Metodu ile Reaksiyon Koşullarının Optimizasyonu, Turkish Lipid Group, I. Vegetable oil Congress, Adana, Turkey.
22) **Keskin, H**., Koçak, D., Göğüs, F. and Fadıloğlu, S. (2011). Interesterification of Triolein with Palmitic and Caprylic Acids: Optimization of Reaction Parameters by Response Surface Methodology, 9. Eurofedlipid Congress, Rotterdam, Holland.

23) **Keskin, H.**, Koçak, D., Göğüs, F. and Fadıloğlu, S. (2010). New Developments in the Synthesis of Reduced Calorie Fats, 1st International Congress on Food Technology, Antalya, Turkey.

OTHERS

1) **Keskin, H**., Bayram, M. (2015). Handling and Storage of Distillers Grains with Solubles (DDGS), Miller Magazine, 72, 100-108.

2) **Keskin, H**., Bayram, M. (2016). Silo Design Using Eurocode, Miller Magazine, 77, 110-128.

AWARDS

- 2012: Best Msc Thesis Award, Gaziantep University, Turkey
- 2012: Best Poster Award, I. Vegetable Oil Congress, Turkish Lipid Group, Turkey

Memberships

- International Food Technology
- Euro Fed Lipid
- Turkish Lipid Group
- International Association for the Exchange of Students for Technical Experience (IAESTE)
- Turkish Food Engineering Group