

Enzymatic production of 2-monoacylglycerol from olive pomace oil

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in
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**Supervisor
Prof. Dr. Sibel FADİLOĞLU**

**by
Hatice Neval MUCUK
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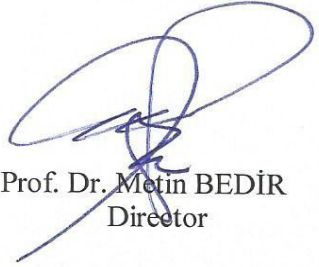
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Name of the student: Hatice Neval MUCUK

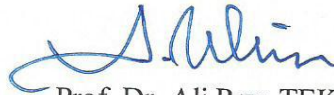
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Prof. Dr. Metin BEDİR
Director

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.



Prof. Dr. Ali Rıza TEKİN
Head of Department

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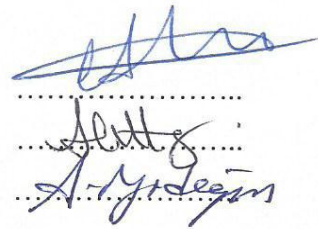
Prof. Dr. Sibel FADİLOĞLU
Supervisor

Examining Committee Members

Prof. Dr. Sibel FADİLOĞLU

Prof. Dr. Fahrettin GÖĞÜŞ

Assoc. Prof. Ayşegül İYİDOĞAN



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Hatice Neval Mucuk

ABSTRACT

ENZYMATIC PRODUCTION of 2-MONOACYLGLYCEROL from OLIVE POMACE OIL

MUCUK, Hatice Neval
M.Sc. in Food Engineering
Supervisor: Prof. Dr. Sibel FADILOĞLU
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Alcoholysis reaction of olive pomace oil (OPO) was performed by using an immobilized lipase from *Candida antarctica*, commercially called Novozyme 435 to produce 2-monoacylglycerol (2-MAG).

Response surface methodology was applied to evaluate the effect of several variables in the alcoholysis reaction and optimize the reaction conditions by using a four factor, five level central composite rotatable design. The four major factors were chosen as reaction temperature (35–60 °C), time (4–10 h), enzyme load (10–18 wt %) and substrate mole ratio (ethanol:oil, 40:1–90:1). Best fitting models were successfully established for the produced 2-Monoacylglycerols by multiple regressions with backward elimination. The optimum reaction conditions were found as reaction temperature 35 °C; time 6 h; substrate mole ratio 65:1; and enzyme load 15%. Under these optimum conditions, the 2-MAG yield was predicted as 87.8%. The value of the optimum reaction yield predicted by the model (87.8 %) was close to its actual value obtained through experimentation (83.5 %). Recovery and purification of 2-MAGs from alcoholysis reaction mixture were performed by using two methods: solvent extraction and low temperature crystallization. After solvent extraction, 2-MAG was obtained at 97% purity with 87.7% recovery yield while these values were 93.7% and 69.2%, respectively, after low temperature crystallization.

Key words: monoacylglycerol, alcoholysis, olive pomace oil, *Candida Antarctica*, response surface methodology

ÖZET

PİRİNA YAĞINDAN ENZİMATİK YOLLA 2-MONOASİLGİSEROL ÜRETİMİ

MUCUK, Hatice Neval

Yüksek Lisans Tezi, Gıda Mühendisliği Bölümü

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Bu çalışmada, 2-monoasilgliserol (2-MAG) üretmek için pirina yağının alkoliz reaksiyonu *Candida antarctica* kaynaklı ve ticari olarak Novozyme 435 olarak adlandırılan immobilize lipaz kullanılarak gerçekleştirildi.

Yanıt yüzey yöntemi, alkoliz reaksiyonunda çeşitli değişkenlerin etkisini değerlendirmek ve reaksiyon koşullarını optimize etmek için dört faktörlü beş seviyeli merkezi birleşik dairesel tasarım kullanılarak uygulanmıştır. Dört ana faktör olarak; reaksiyon sıcaklığı (35–60 °C), zaman (4–10 saat), enzim miktarı (kütlece %10–18) ve substrat mol oranı (etanol:yağ, 40:1–90:1) seçildi. En uygun modeller üretilen 2-monoasilgliseroller için geri elimineli çoklu regresyon ile başarıyla uygulandı. 2-monoasilgliserollerin üretimi için optimum reaksiyon koşulları; reaksiyon sıcaklığı 35 °C, reaksiyon zamanı 6 saat, substrat mol oranı 65:1 ve enzim miktarı %15 olarak bulundu. Bu optimum koşullar altında 2-MAG verimi % 87.8 olarak tahmin edildi. Optimum reaksiyonun model tarafından belirlenen verimi (% 87.8) deneyler sonucu elde edilen gerçek değerine yakın olarak bulundu (83.5 %). Alkoliz reaksiyon karışımından 2-monoasilgliserollerin geri kazanımı ve saflaştırılması iki metot kullanılarak yapıldı: çözücü ekstraksiyonu ve düşük sıcaklık kristalizasyonu. Çözücü ekstraksiyonu sonrası % 97 saflıkta ve % 87.7 geri kazanım verimiyle 2-MAG elde edildi. Düşük sıcaklık kristalizasyonu sonrası ise bu değerler sırasıyla % 93.7 ve % 69.2 olarak bulundu.

Anahtar kelimeler: monoasilgliserol, alkoliz, pirina yağı, *Candida Antarctica*, yanıt yüzey yöntemi

To my family

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LIST OF SYMBOLS/ABBREVIATIONS

MAG	Monoacylglycerol
DAG	Diacylglycerol
TAG	Triacylglycerol
FAEE	Fatty acid ethyl ester
OPO	Olive pomace oil
FA	Fatty acid
FFA	Free fatty acid
HPLC	High performance liquid chromatography
GC	Gas chromatography
CCRD	Central composite rotatable design
RSM	Response surface methodology
TLC	Thin layer chromatography
FID	Flame ionization dedector
MO	Monoolein
GRAS	Generally recognized as safe
PUFA	Poly unsaturated fatty acid
ANOVA	Analysis of variance

CHAPTER 1

INTRODUCTION

Surfactants are widely used and find wide range of applications due to their significant capability to affect the characteristics of surfaces and interfaces (Schramm, 2000). A surfactant contains both lipophilic and hydrophilic parts in its molecule so it is an amphiphilic substance. Surfactants find wide range of applications in agricultural, food, cosmetic and pharmaceutical industries (Fathabad, 2011). The food industry is one of the largest users for surfactants. They are widely used to perform several functions such as emulsification, defoamer, moisturizer and flavouring agent in the food industry.

One of the most common emulsifier is monoacylglycerol (MAG). Monoacylglycerols are nonionic surfactants and emulsifiers with hydrophilic and hydrophobic parts in molecules (Hasenhuettl and Hartel, 2008). Monoolein (MO), or glycerol monooleate, is commonly used as an emulsifying agent and as a food additive since the 1950s. It is a nontoxic, biodegradable, and biocompatible material classified as GRAS (generally recognized as safe) (Ganem-Quintanar et al., 2000).

Commercial MAGs are generally synthesized by the glycerolysis of fats and oils. Inorganic alkaline catalysts, like sodium, potassium or calcium hydroxide, are used to accelerate the glycerolysis reaction at high temperatures (220–250°C) (Pawongrat et al., 2007). This conventional method has some limitations. The burnt taste and undesirable dark color are obtained because of high temperature usage. Another disadvantages of the conventional method contain low MAG yield (maximum MAG yield of 40-50%), and application of expensive molecular distillation (Yang et al., 2005). Furthermore, undesirable acyl migrations can be accelerated by using this conventional method. In acyl migration fatty acids migrate from one position of the glycerol backbone to other position [generally from the *sn*-2 position to the *sn*-1(3) positions]. In addition to this, it is not feasible to heat-sensitive oils and fats considering the nutritional or biological properties of them (Damstrup et al., 2005).

Because of the disadvantages of the conventional process, lipases can be used as biocatalysts during the esterification reaction. Enzymatic synthesis of monoacylglycerols have many advantages when compared to the chemical method, for example, mild reaction conditions, high catalytic performance, stereo- and positional specificities and lower energy consumption (Lee et al., 2004).

Olive pomace oil is obtained by using solvent-extraction method from the olive pomace that is the solid by-product that forms after olive oil processing. It has a lower quality due to drying applications at high temperatures and long storage periods of pomace (Çiftçi, et al., 2009). Therefore it is used in soap making or other industrial applications. Also after refining process at suitable conditions and blending with a sufficient amount of virgin olive oil, it can be used for human consumption (Meziane, 2013). Its chemical composition is similar with the refined olive oil (Kiritsakis et al., 1998). For 2-monoolein production, olive pomace oil can be a suitable substrate due to its high oleic acid content at sn-2 position and low cost which provides economical surfactant production.

Response surface methodology (RSM) has been a popular and effective statistical method to evaluate and optimize of complicated processes (Bektaş et al., 2008). When RSM is compared to the other classic optimization methods, it has several advantages such as reducing the number of experimental trials, giving a lot of info from a small number of experiments (Cheong et al., 2007). Also, it is a faster and economical method for optimization of a process (Bezerra et al., 2008). It has been successfully used for optimization of enzymatic alcoholysis reactions (Montoya et al., 2011; Domingos et al., 2008). In this work, RSM was applied to determine the effect of several variables; temperature, time, substrate mole ratio, enzyme load etc. on the alcoholysis reaction and for optimization of reaction conditions.

The aim of this study is to produce 2-monoacylglycerol from olive pomace oil. For this purpose enzymatic alcoholysis reactions of olive pomace oil with ethanol were carried out. The reaction parameters such as substrate concentration, temperature, reaction time and enzyme were optimized for alcoholysis reaction. After alcoholysis reaction, solvent extraction and crystallization methods were used to purify 2-MAGs from the ethanolysis reaction mixture.

CHAPTER 2

LITERATURE REVIEW

2.1 Surfactants

Surface active agents or surfactants have a large application area in science and industry. For example in detergents, personal care products and cosmetic, food processing, paints, plastics, pharmaceuticals. The food industry is one of the largest users for surfactants. In the food industry, they are widely used to conduct different functions such as emulsification, defoamer, moisturizer and flavouring agent.

2.1.1 What are surfactants?

Surfactants are organic compounds which contain at least one lyophilic ('solvent-loving') part and one lyophobic ('solvent-fearing') part in the molecule. 'Hydrophilic' and 'hydrophobic' terms are used respectively, when the solvent is water or an aqueous solution in which the surfactant is to be used. Simply, a surfactant consists of at least one non-polar part as the tail and one polar (or ionic) head part (Karsa, 2006).

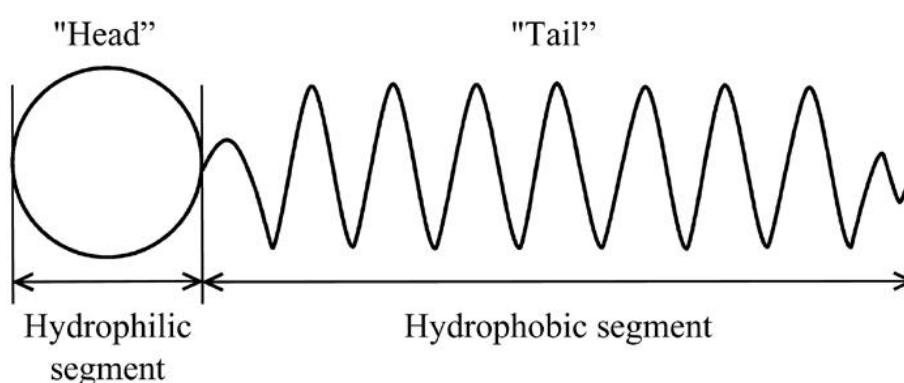


Figure 2.1 The basic molecular structure of a surfactant molecule (Szymański, 2008)

The relative sizes and shapes of the hydrophilic and hydrophobic parts of the surfactant molecule determine many of its properties (Gecol, 2006). Surfactants have the capability to adsorb onto the surfaces or interfaces of the system. They alter to a

marked degree the surface or interfacial free energies of those surfaces (or interfaces) when present in a system at low concentration (Rosen and Kunjappu, 2012).

2.1.2 Classification of surfactants

Surfactants can be categorized according to the character of their hydrophilic group. There are four basic classes of surfactants which are defined as follows:

2.1.2.1 Anionic

In this type of surfactants, the hydrophilic head of surfactant is negatively charged in solution. They are the most widely used type of surfactants in industrial applications. Because their production cost is relatively lower. Anionic surfactants constitute for about 50 % of the world production (Tadros, 2005).

2.1.2.2 Cationic

The head group of the cationic surfactant has positive charge in solution. Nitrogen compounds comprise the very large portion of cationic surfactants. Generally, the cationic surfactants are more expensive than anionic ones, because during the synthesis high pressure hydrogenation procedure is carried out. For this reason, they are only used for two purposes in which there is no cheaper substitute. They are used as bactericide or as positively charged substance which is able to adsorb on negatively charged substrates to provide antistatic and hydrophobant effect (Mishra et al., 2009).

2.1.2.3 Nonionic

Nonionic surfactants do not have any electrical charge. They are resistant to deactivation of water hardness. Because they have nondissociable hydrophilic group, such as alcohol, phenol, ether, ester, or amide. They are excellent grease/oil removers and emulsifiers (Mishra et al., 2009).

2.1.2.4 Amphoteric (or zwitterionic)

These surfactants contain both cationic and anionic parts. According to the acidity or pH of the solution, they can be charged negatively (anionic), positively (cationic) or they cannot electrically charged (nonionic) in solution. In acidic solutions, the molecule charges positively and behaves like a cationic surfactant on the other hand they become negatively charged in alkaline pH solutions and behave like an anionic

one. Amphoteric surfactants are used in shampoos and other cosmetic products due to their excellent dermatological properties (Mishra et al., 2009 and Tadros, 2005).

2.1.3 Application of surfactants

Surfactants are widely used for industrial, agricultural, food, cosmetic and pharmaceutical applications. Laundry detergents, household and personal-care products account for over half the use of surfactant (Yangxin et al., 2008). Their usage in soaps and detergents is the primary traditional application for surfactants (Myers, 2006).

Surfactants are possibly the most versatile components among all the ingredients of cosmetic products. They provide to make stable and homogeneous formulations in cosmetic products (Prieto-Blanco et al., 2007). Surfactants have different functions as emulsifier, foaming agent, solubilizer, wetting agent, antimicrobial agent, mediator of enzyme action in different cosmetic forms such as cream, lotion, gel, liquid, paste, powder, stick, film and spray. (Klekner and Kosaric, 1993).

Surfactants have an important role in agrochemical applications. Most agrochemicals are simply diluted into water at various ratios and applied as liquid sprays. Surfactants are added to those solutions for different reasons. The most obvious reason is to provide the spray solution to adhere to the target surface and spread over a large area (Tadros, 1995). Also, surfactants facilitate the transfer of the active compounds into the plant through pores and membrane walls which improves the efficiency (Myers, 2006).

In pharmaceutical industry, surfactants have a wide range of applications. They improve drug solubility and stability in a liquid preparation, stabilize and modify the texture of a semisolid preparation and change the flow properties of a granulate, which aid in the processing of the final tablet dosage form.

In textile and fiber industry, surfactants play an important role during the dyeing process. They provide uniform dyeing and better dyeing penetration. Also in textile finishes, they improve the fabric hand or feel when used as a fabric softener and during textile processes they control the foam formation (Myers, 2006).

The food industry is one of the largest users for surfactants. Besides their use as a part of cleaning formulations in the food industry, surfactants are also used directly as emulsifiers and fat substitutes in food products. There are different kinds of surfactants in use and they have a large application area in the food industry to conduct different functions such as emulsification, defoamer, moisturizer and flavouring agent. The presence of surfactants can be critical to get the product with desired properties in the food preparations such as foam or sponge cake, bread, mayonnaise, ice cream, chocolate, margarine, beverages, soups and sauces, etc. (Myers, 2006).

2.2 Monoacylglycerols

Monoacylglycerols (MAGs), or popularly called monoglycerides, are a generic name of a group of esters. The term monoglycerides is used when only one hydroxyl group of the glycerol is esterified with fatty acid via an ester linkage. They are one of the most popular emulsifiers or surfactants widely used in food, cosmetics and pharmaceutical industries (Zhong et al., 2009).

2.2.1 Structure and Nomenclature

Glycerol is a small chain alcohol containing three carbon atoms, each of which has a hydroxyl (-OH) group bound to it (Pagliaro and Rossi, 2008). Since glycerol has three hydroxyl groups, three types of structurally different MAGs can exist, depending on which hydroxyl group is esterified: *sn*-1, the mid carbon position *sn*-2, or the last carbon position *sn*-3 (Nichols and Sanderson, 2003). Normally the 1- and 3- isomers are not different from each other. So they are called as ' α -monoacylglycerols', while the 2- isomers are β -monoacylglycerols.

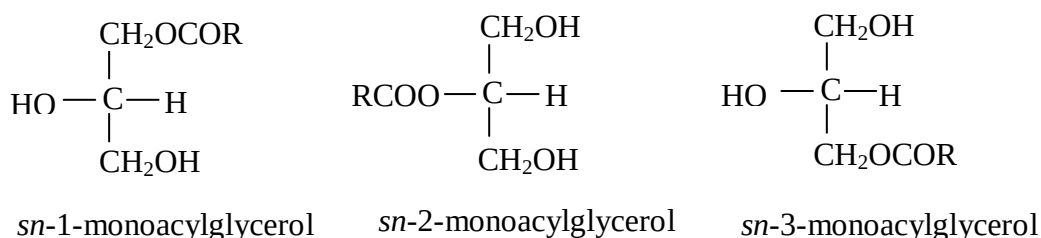


Figure 2.2 Three stereochemical forms of monoacylglycerols

2.2.2 Nutrition and Biological Effects of Monoacylglycerols

Monoacylglycerols are one of the first intermediate products which are released by lipolytic enzymes in the stomach and proximal small intestine during triacylglycerol digestion. Enzymes involved in the TAG lipolysis are gastric and pancreatic lipases and the lipolytic products are free fatty acids from sn-1,3 position and sn-2 MAGs. sn-2 MAGs are then absorbed through intestinal mucosa and hydrolyzed by monoacylglycerol lipase in blood to free fatty acids and glycerol (Iqbal and Hussain, 2009). On the other hand, the intestinal cells can directly take up 2-MAGs and convert them to the triacylglycerols via monoacylglycerol pathway. Then these TAGs are transported in lymph to the liver. Recently, it is demonstrated that monoacylglycerol lipase has a role in cancer progress. It has been shown that this enzyme controls the level of free fatty acids in cancer cells (Qin and Ruan, 2014).

Medium-chain MAGs are known for bactericidal or antimicrobial effects. For example, Petschow et al. (1996) reported that saturated MAGs which have carbon chain length between C-10:00 to C-14:00, at 1mM provided a 4-log-unit or greater reduction in the number of viable *Helicobacter pylori* after exposure for 1h. Isaacs et al., (1995) found that MAGs which have chain lengths between 8 to 12 carbons had more strongly antiviral and antibacterial effect than long chain MAGs when added to milk and infant formulas.

2.2.3 Production of Monoacylglycerols

MAGs can be produced by various methods. Today, industrially MAGs are synthesized by chemical glycerolysis of fats and oils in either batch or a continuous method (Zhong et al., 2014). Furthermore, they can be synthesized by enzyme catalyzed reactions and the interest in enzymatic production of MAGs is growing (Yang et al., 2005).

2.2.3.1 Chemical Synthesis

Chemical glycerolysis is a conventional and widely applied industrial method to produce MAGs (Kaewthong et. al., 2005). It is an interesterification process between glycerol and fats or oils (Figure 2.3). Inorganic alkaline catalysts, like sodium, potassium or calcium hydroxide, and high temperatures (220-250 °C) are used to accelerate the reaction under a nitrogen gas atmosphere (Adamczak, 2003). Glycerol

and fat immiscible to each other at ambient temperature becomes miscible at such high temperatures (Pagliaro and Rossi, 2008). The reaction is operated in either batch or continuous method. Better quality MAGs are obtained in continuous process when compared to the batch method with respect to lesser damage of fat with shorter heating and reaction time (Bornscheuer, 1995). During the glycerolysis reaction, the acyl residues or fatty acid chains separate from the triglyceride and randomly form mono-, di- and triglycerides. Furthermore, the product mixture often contains minor amounts of free glycerol and free fatty acid (Senanayake and Shahidi, 2005). After the reaction, the product usually contains 35–60% monoacylglycerols (Langone et al., 2002). MAG content can be increased by removal of excess glycerol, TAG, DAG, FFA and undesirable by-products (Zhong et al., 2014). For this purpose, after chemical glycerolysis the reaction mixture is subjected to molecular distillation. Molecular distillation (MD) is a separation method involving lowering the boiling point of compounds by working under vacuum conditions (Lin and Yoo, 2009). The separation mechanism of distillation is based on the boiling point differences of the substances. MAG can be separated in a short time from the higher boiling point DAG and TAG under low pressure conditions.

The acylglycerols are feed into an evaporator under vacuum and here the feed is directly spread into a very thin film. Then the feed is forced quickly down the evaporator surface. More volatile MAG components are driven to the closely positioned internal condenser where they are concentrated in a distillate by the heat and a high vacuum. DAG and TAG which are less volatile continue down the cylinder and are collected as the residue. After the molecular distillation, highly pure MAG is obtained with a very low content of DAG (less than 4% wt). Commercial MAGs produced by this method is called ‘distilled monoglycerides’. The isomeric composition of the MAG is 95–91% α -MAG and 5–9% β -MAG (Fregolente et al., 2006).

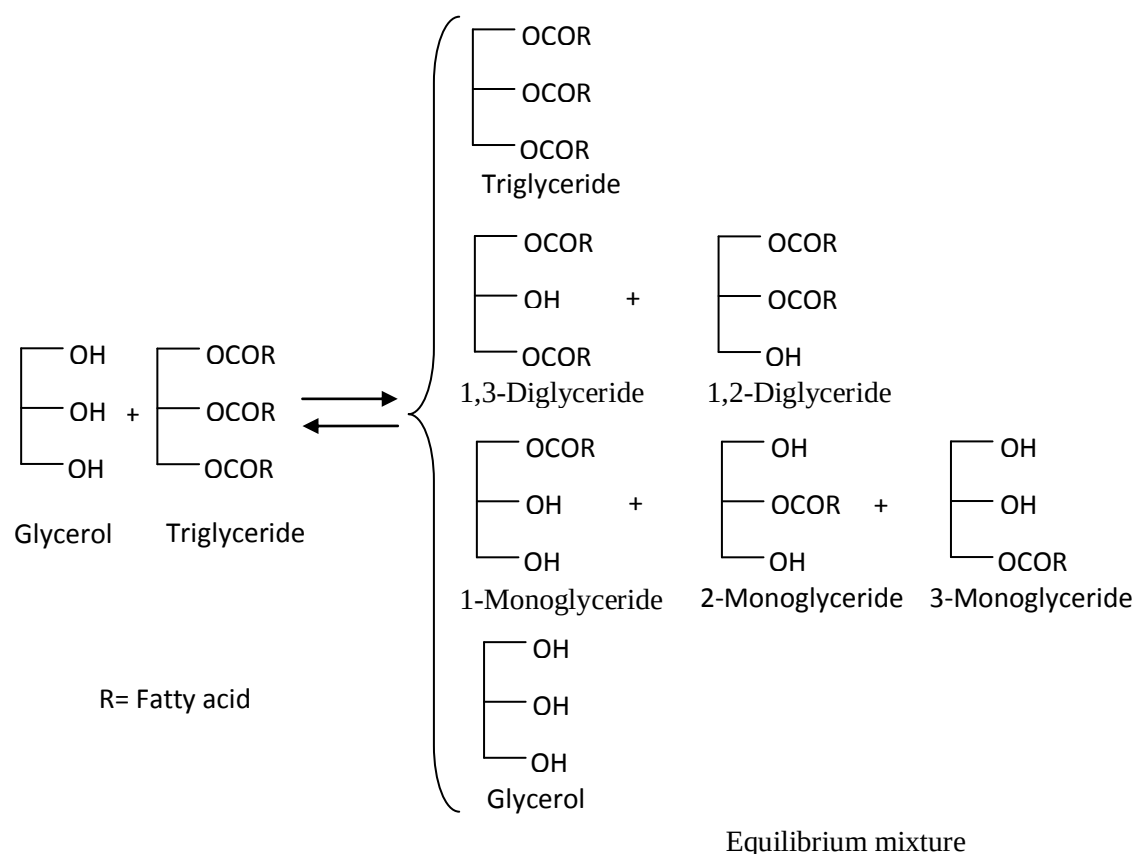


Figure 2.3 Starting components and end products produced through the glycerolysis reaction (Krog and Sparso, 2004)

2.2.3.2 Enzymatic Synthesis

Enzymatic synthesis of monoacylglycerols has attracted much attention for the last years. Conventional chemical process has several disadvantages when compared to enzyme-catalyzed production of MAGs (Valério et al., 2010). The high temperatures lead to formation of burnt taste and undesirable dark color. Low MAG yield and adoption of expensive molecular distillation are other limitations of chemical processes (Yang et al., 2005). Also conventional chemical process may accelerate undesired acyl migrations that cause migration of fatty acid residues from one position to another position of the glycerol backbone (Damstrup et al., 2005). In addition, the high-temperature chemical method is not applicable to heat-sensitive oils and fats (Pawongrat et al., 2007).

Through the disadvantages of the conventional chemical method, lipases can be used as biocatalysts. Lipases offer a variety of benefits to improve the process and product

quality (Langone et al., 2002). In the lipase-catalyzed production, milder reaction conditions are used and this improves the quality of product and makes production of heat-sensitive MAG feasible (Damstrup et al., 2005). The use of lipases for MAG production offers environmental advantages and a reduction in energy consumption by avoiding the extreme temperatures and alkaline catalysts. Moreover, various specificities (stereo-, positional-, and fatty acid-) of lipase provide to synthesize unique MAGs that cannot be prepared by the chemical methods (Lee et al., 2004).

There are different methods for enzymatic synthesis of MAGs. These are hydrolysis or alcoholysis of triglycerides, glycerolysis of triglycerides and esterification or transesterification of glycerol with fatty acids or esters (Ghattas et al., 2014).

2.2.3.2.1 Hydrolysis or alcoholysis of triglycerides

In the production of MAG by enzyme catalyzed hydrolysis, control of the reaction is important to avoid complete hydrolysis. The application of 1,3-specific lipases yielding 2-MAG is a easiest way. In this type reaction, the main problems are low overall MAG yield and acyl migration (Bornscheuer, 1995).

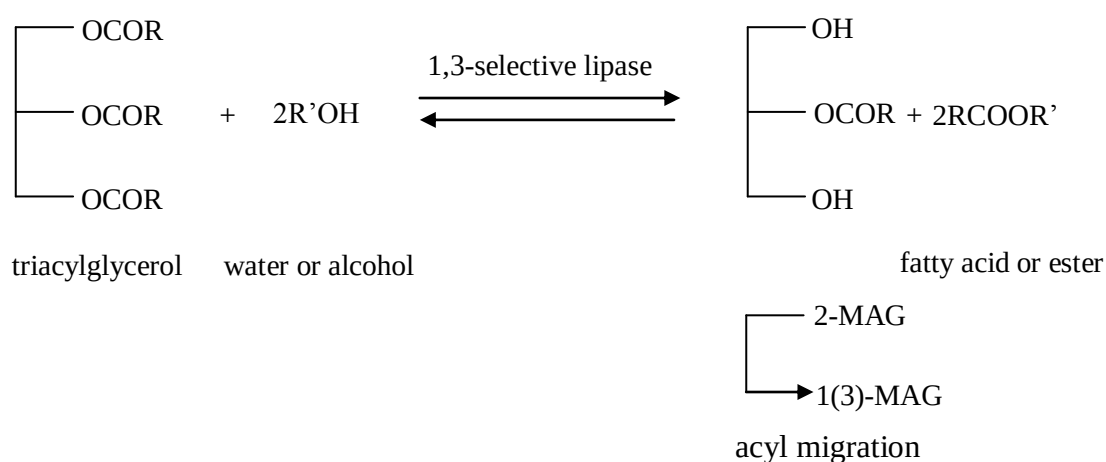


Figure 2.4 Hydrolysis and alcoholysis of a TAG to produce 2-MAG (acyl migration may lead to 1(3)-MAG) (Bornscheuer, 1995)

Alternatively, alcoholysis which is the reaction analogous to hydrolysis can be carried out to slow down the acyl migration (Adlercreutz, 1994). Also the removal of alcohol is easier than water after the reaction and alcoholysis reaction is often faster than hydrolysis. Because acidity of reaction medium has no change and there is less inhibition of lipase by free fatty acid during the alcoholysis reaction.

2.2.3.2.2 Glycerolysis of triglycerides

Glycerolysis is based on the transfer of acyl groups between glycerol and a TAG (Willis and Marangoni, 2002). In the glycerolysis reaction of TAG, all fatty acids bond to the TAG are converted to products. Thus, 3 mol monoacylglycerols could be formed from 1 mol TAG (Bornscheuer, 1995). Non-selective or 1,3-specific lipases can be used. A 1,3-specific lipase yield 1 mol 2-MAG and 2 mol 1(3)-MAG. But in practice, equilibrium mixture contains 9:1, 1(3)-MAG: 2-MAG because reaction conditions usually promote acyl migration. Using low temperatures in the lipase-catalyzed glycerolysis is the main advantage of this process when compared to the chemical glycerolysis (Pinyaphong et al., 2012).

Different reaction systems have been investigated in the presence or absence of solvents, with immobilized or nonimmobilized enzymes and in microemulsion or other systems for glycerolysis of TAGs (Yang et al., 2005). In general, glycerolysis reaction of triglycerides gives product with low MAG content (30-50%) due to an unfavored equilibrium. In order to break the reaction equilibrium to obtain a high yield of MAGs, glycerolysis can be performed in a solid phase system. In this method, formed MAGs are instantaneously crystallized by the reduction of reaction temperature to below the melting temperature of monoacylglycerols (McNeill et al. 1990; McNeill and Yamane, 1991). However, it is difficult to carry out the reaction in a continuous manner and the reaction is very long (up to one week). Also, the reuse of enzymes can be difficult in the enzyme catalyzed reactions (Bornscheuer, 1995).

2.2.3.2.3 Esterification or transesterification of glycerol with fatty acids or esters

Lipase-catalyzed esterification or transesterification of glycerol with fatty acids or esters is another way to produce monoacylglycerols (Langone et al., 2002). During the esterification reactions, formation of water and alcohol can damage the reaction equilibria and promote acyl migration. So, reaction efficiency is adversely affected. To avoid these problems and maximize the formation of monoacylglycerols, water or alcohol must be removed by addition of molecular sieves or using vacuum during the reaction (Feltes et al., 2013 and Bornscheuer, 1995).

2.2.4 Applications of Monoacylglycerols

Mono- and diacylglycerols are the oldest and most commonly used food emulsifiers. Mono- and diacylglycerols and their derivatives are considered as the most important emulsifier group. Because they are responsible approximately 75% of the world production of emulsifiers for food purposes (Akoh et al., 2002). They find a variety of applications in different industries. Both MAG and DAG can be used as additives in the food industry for human consumption and they are recognized as GRAS (Feltès et al., 2013).

Monoacylglycerols show great emulsifying properties due to the MAG molecular structure (Xu et al., 2006). They are non-ionic molecules consisted of both hydrophobic and hydrophilic parts (Zhong et al., 2014). Due to this combination monoacylglycerols are very important additives to provide food products at uniform quality among other applications. The main application area of monoacylglycerols in food industry is in margarines, spreads, bakery products, cake mixtures and confectionary due to their stabilizing, emulsifying and conditioning properties (Xu et al., 2006).

Also MAGs are used to modification of starch or protein-containing products via complex formation with amylose or proteins, or to modify physical characteristics of fats by controlling fat crystal polymorphism (Akoh et al., 2002). Thus, their functionality is dependent not only on their surfactant properties but also on physical characteristics such as crystalline behaviour, solubility in oils and fats, and interactions with water (Boyle, 1997).

MAGs of stearic, palmitic, oleic and linoleic acids are incorporated into margarine as emulsifying agents in proportions of 0.2 to 0.3% by weight (Mouloungui et al., 1998). Typical table margarine is water in oil (w/o) emulsion. Addition of MAGs provides good distribution of water droplets in the fat phase, smooth spreading consistency, stable crystall structure and pleasant melt-in-the-mouth sensation. The benefits of MAG addition to low fat spreads with a fat content 35-45% are the same as in table margarines, but unsaturated MAGs are often used. Less number of molecules are needed to stabilize fine water droplets in the oil phase due to unsaturated MAGs have a higher activity to reduce surface tension than saturated MAGs (Moonen and Bas, 2004).

MAGs are also widely used in bakery applications, frozen desserts, chewing gum and chews. In bakery applications MAGs have useful influence on rheological properties of the bread dough, increase the durability of bread and affect a solubility of the protein fraction (Ravi et al., 2000). Breads containing monoacylglycerols have an improved shelf life. Because adding monoacylglycerols retards the stalling rate of breads. Add to their antistalling property, MAGs in bakery products result in reduction of interfacial tension, improved dispersion of ingredients and increased aeration (Orthoefer, 2008). Monoacylglycerols increase the fermentation stability of the dough. For example, fully fermented dough is resistant to collapse by mechanical shock during transport in bakeries via trays or belts from the proofing cabinet to the oven (Moonen and Bas, 2004). In addition to fermentation stability, complexation of monoacylglycerol with starch or, more specifically, amylose, is the most important factor for enhancing the shelf-life (crumb softness) of bread and cakes. Interactions of monoacylglycerols with starch are also critical in the production of dry pasta. Addition of hydrated monoacylglycerols to the pasta dough provides less cooking loss and decreases stickiness in the finally cooked pasta (Moonen and Bas, 2004; Mouloungui et al., 1998).

Monoacylglycerols are also used for their lubricating and texturing properties in the production of potato flakes as well as in pre-cooked rice (Mouloungui et al., 1998). Due to their crystal polymorphism and similarity to triacylglycerols, MAGs can be used as fat extenders for low-fat dairy applications, providing better melting characteristics than non-lipid fat replacers (Giese, 1996).

In cosmetic applications, MAGs of saturated fatty acids are commonly used. The features of interest of these MAGs include the stability to oxidation, chemical inertness in the presence of active substances, emulsifying and emollient action, water dispersing activity and penetrating action (Mouloungui et al., 1998). Monoacylglycerols are part of the composition of natural lipids of the skin, thus possess high compatibility with skin and mucosa. Incorporation of MAGs in cosmetics and body care products does not appear to have any contra-indication or irritating action (Mouloungui et al., 1998; Bergsson et al., 2002). Furthermore, their compatibility with skin helps improve the feel of various skin care products. In addition this, they reduce defatting of the skin which caused by surfactant based

cleansers (Zocchi, 2009). MAGs find the widest use in the formulation of ointments and lotions for their ability to stabilize emulsions. Other products, where MAGs are applied, are hair-colouring and hair-setting products (Mouloungui et al., 1998).

MAGs also find application in pharmaceutical industry. When present in an aqueous medium, monoacylglycerol molecules spontaneously self-assemble into various liquid crystalline structures. The various phases can be used to obtain different functionalities, for example protecting molecules from chemical degradation, solubilising drugs and nutrients or controlling the release of drugs (Sagalowicz et al., 2006). Due to these properties, MAGs can be employed as vectors for active substances. Moreover they are able to modify the permeability and composition of cell membranes and even if their target is intracellular, the interaction with this barrier plays a fundamental role (Chang and Bodmeier, 1997; Schreier et al., 2000).

A number of medium-chain saturated fatty acids and their monoacylglycerols have been suggested as components of antimicrobial hydrogel formulations to prevent transmission of pathogens to mucosal membranes, particularly sexually transmitted viruses, such as herpes simplex virus and human immunodeficiency virus, and bacteria, such as *Chlamydia trachomatis* and *Neisseria gonorrhoeae* (Kristmundsdóttir et al., 1999; Thorgerisdóttir et al., 2005; Bergsson et al., 1998; Bergsson et al., 1999). Another interesting possibility is the use of monoacylglycerol/fatty acid suspensions as immunological adjuvants that can enhance the immunogenicity of antigen formulations (Schröder and Svenson, 1999).

Due to plastifying, lubricant and antistatic properties, MAGs are employed in plastics industry, for example in the manufacture of methacrylic resins. In the fibre and textile industry MAGs are utilized as aqueous emulsions with antistatic properties, especially in the manufacture of polyolefin fibres. They improve water- and oil-repellent behaviour and enhance colour-fastness and contrast in fabrics (Zhong et al., 2014).

2.3 Lipases

Lipases are the enzymes which are the part of the family of hydrolases. These enzymes are extensively used to catalyze the total or partial hydrolysis, alcoholysis, esterification and transesterification of carboxylic esters to produce diacylglycerols,

monoacylglycerols, fatty acids and glycerol (Houde et al., 2004). Lipases are very useful biocatalyst for modification of fats and oils through their capability of catalyzing various reactions. Also, lipase catalyzed reactions generally proceed with high regio- and stereo- selectivities. This makes the lipases an important group of biocatalyst in organic chemistry (Jaeger et al., 1999; Aravindan et al., 2007).

In addition, lipases perform an important role in the digestion of lipids. They serve as digestive enzymes to provide both the lipids transmit from one organism to another and their deposition and the mobilization which are used as an energy source in the organism. Additionally, they are participated in the metabolism of intracellular lipids and in the functioning of biological membranes (Villeneuve et al., 2000).

2.3.1 Sources of Lipases

Lipases are found widely in nature and can be found in different animals, plants and microorganisms with variations in their catalytic activity (Barros et al., 2010). It is known that various mammalian tissues, organs and fluids, such as heart, pancreas, muscle and serum, contain lipases. Pancreatic lipase has been used in many studies within the other animal lipases (Hübscher, 1970). Also it is known that various fruits, vegetables, plant tissues and seeds, like cotton, wheat, soy bean and castor bean, show lipase activity (Shahani, 1975).

In recent years, lipases produced by microorganisms have attracted attention in biotechnological applications and organic chemistry. Microbial lipases, mainly bacterial and fungal, are commercially important. Because their production cost is low and they show great stability and wider availability than plant and animal lipases (Gupta et al., 2004). Intracellular and extracellular lipases are produced by different microorganisms. In the applications, extracellular microbial lipases are used more commonly. Because their bulk production is much easier (Aravindan et al., 2007). Different microorganisms produce lipases such as *Candida*, *Rhizopus*, *Penicillium*, *Aspergillus*, *Geotrichium*, *Mucor*, *Pseudomonas*, *Achromobacter*, and *Staphylococcus* (Ghosh et al., 1996).

2.3.2 Reactions of Lipases

Lipases are capable to catalyze different hydrolytic and synthetic reactions by using different natural and synthetic substrates in aqueous and non-aqueous mediums

(Song et al., 2008) (Figure 2.5). Lipase catalyzed reactions may be classified in two main categories: hydrolysis and synthesis.

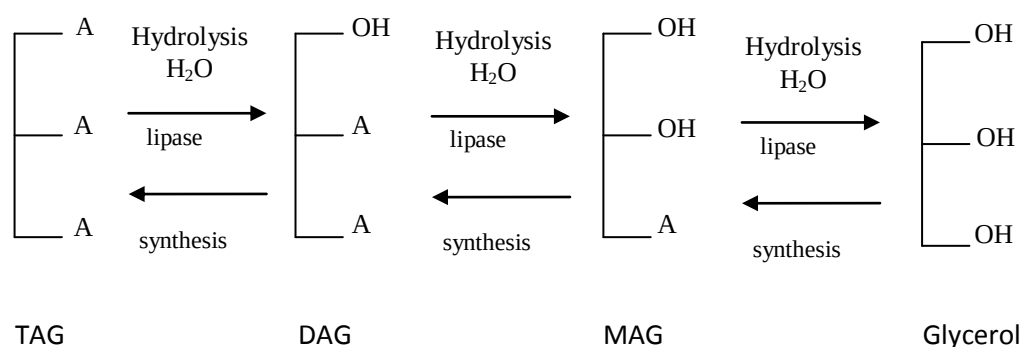


Figure 2.5 Enzymatic reaction of a nonspecific lipase catalyzing hydrolysis or synthesis of a TAG substrate. A: Fatty acid (Çiftçi and Fadiloğlu, 2010)

2.3.2.1 Hydrolysis

In the hydrolysis reaction, partial acylglycerols, fatty acids and glycerol form from the reaction of fats and oils in presence of water (Figure 2.6). Fats and oils can be hydrolyzed by using three different methods: fat splitting, alkaline hydrolysis and enzymatic hydrolysis (Gandhi, 1997).

In the fat splitting method, high temperature (240-260 °C) and pressure (60 bar) are used (Çiftçi and Fadiloğlu, 2010). Under these high temperature conditions the products become discoloured and these conditions cause degradation of some fatty acids. There are also limitations of alkaline hydrolysis method such as, high energy costs and requirement to acidify formed soaps (Gunstone, 1999).

Enzymatic hydrolysis is more appealing when compares to the other methods. Because enzyme catalyzed hydrolysis reaction proceeds at ambient or moderate temperatures and pressures. It also provides production of fatty acids from unstable oils which include highly unsaturated and conjugated fatty acids. In addition, this method reduces the by-product formation, due to enzyme specificity (Gandhi, 1997).

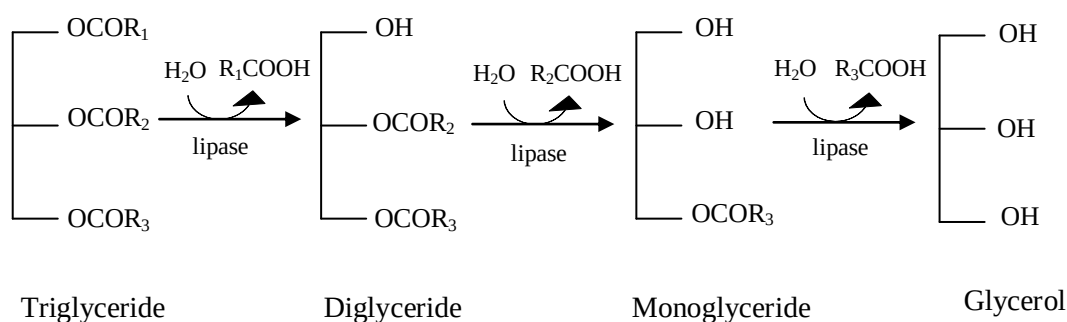


Figure 2.6 Lipase-catalyzed hydrolysis of a triglyceride (Adlercreutz, 1994)

2.3.2.2 Synthesis

2.3.2.2.1 Esterification

Esterification is the reverse reaction of hydrolysis and catalyzed by lipases under conditions of low water activity such as water-poor organic solvents or even solvent free systems (Villeneuve et al., 2000). Water is the by-product of reaction and excess of water shift the reaction to hydrolysis instead of synthesis. On the other hand, lipases need a certain amount of water for activation. Therefore controlling the water content of reaction medium is very critical. Optimum water content depends on the reaction medium such as substrate, solvent and lipase type (Çiftçi and Fadiloğlu, 2010).

2.3.2.2.2 Interesterification

Interesterification is the common term for defining the important class of organic reactions. Lipase-catalyzed interesterification is one of the major reactions used in industrial applications for modification of nutritional and functional properties of oils and fats (Amir et al., 2012). Application of lipases in the interesterification reaction of fats and oils offers some advantages like mild conditions and reduced side reactions (Villeneuve et al., 2000). In addition, depending on specificity of lipases, specific products with desired properties can be produced (Çiftçi and Fadiloğlu, 2010).

Reactions under this category can be classified into three groups: alcoholysis, acidolysis and transesterification.

i) **Transesterification:** Transesterification is a process based on the transfer of acyl residues between two TAGs, resulting in the formation of new triacylglycerols. Generally, it is used to alter the physical properties of fats and oils by changing the fatty acids' positional distribution in the triacylglycerols (Willis and Marangoni, 2002).

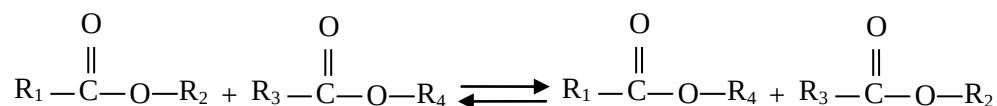


Figure 2.7 Lipase-catalyzed transesterification reaction between two different triacylglycerols (Willis and Marangoni, 2002)

ii) **Acidolysis:** Acidolysis reaction is based on the acyl exchange between TAGs and FAs. It is an effective way to incorporate a specific or a range of fatty acids into triacylglycerols (Xu et al., 2006).

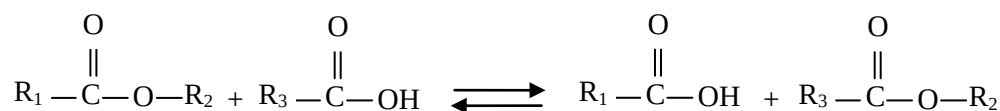


Figure 2.8 Lipase-catalyzed acidolysis reaction between an acylglycerol and an acid (Willis and Marangoni, 2002)

Acidolysis reaction is based on incorporation of fatty acids specially at *sn*-1 and *sn*-3 positions of TAG by using *sn*-1,3 specific lipases (Fomuso and Akoh, 1997). Acidolysis commonly proceeds in two steps. In first step, TAGs are hydrolyzed into diacylglycerols or even to monoacylglycerols. Then, new fatty acids are combined into TAGs. Acyl migration may occur because of the thermodynamically unstable nature of DAGs during the acidolysis reaction. This results in the formation of by-products and affects the product quality (Koh et al., 2010).

By using lipase-catalyzed acidolysis, cocoa butter equivalents (CBEs), human milk fat substitutes (HMFs) and structured lipids containing medium- or short-chain fatty acids have been produced (Çiftçi and Fadiloğlu, 2010).

iii) **Alcoholysis:** When an ester is reacted with an alcohol, this reaction is called alcoholysis. Alcoholysis has different commercial applications used for production

of monoglycerides, diglycerides and esters of fatty acids from triglycerides (Adlercreutz, 1994).

The two main applications of alcoholysis are glycerolysis and methanolysis. Glycerolysis is an alcoholysis reaction of triglycerides with glycerol to produce mono- and diglycerides. Alcoholysis of triglycerides with methanol (methanolysis) is a method to convert glycerol esters to methyl esters used as biodiesel (Willis and Marangoni, 2002).

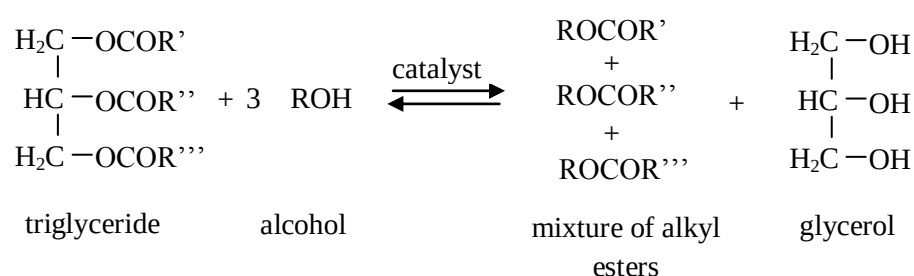


Figure 2.9 Alcoholysis reaction of TAGs (Schuchardt et al., 1998)

2.3.3 Specificity of Lipases

The main advantage of lipases is their specificity. This is often a main reason why an enzymatic reaction is chosen instead of a conventional chemical reaction (Adlercreutz, 1994).

Lipases show different specificities. These can be divided into five different groups:

2.3.3.1 Nonspecific Lipases

Some lipases do not have any positional or fatty acid specificity during the reactions. They catalyzed the cleavage of acylglycerol molecules randomly to generate free fatty acids and glycerol as well as mono- and diacylglycerols being intermediate products (Barros et al., 2010). This gives the same products which are similar with the produced by chemical catalysis. But in this method, there is less product degradation due to the lower reaction temperature when compared to the chemical methods. Examples of nonspecific lipase sources are *Candida rugosa*, *Staphylococcus aureus*, *Chromobacterium viscosum*, *Pseudomonas sp.* and *Thermomyces lanuginosus* (Ribeiro et al., 2011).

2.3.3.2 Positional Specificity

Regiospecificity is the capability of lipases to distinguish between two outer positions (sn-1 or sn-3 position and the inner position (sn-2 position) of glycerides. Lipases can be divided into two subgroups by considering their regiospecificity. The first group of lipases do not show any regiospecificity. They release fatty acids from all three positions of glycerol and catalyze complete breakdown of triacylglycerols. The second group of lipases called as sn-1,3 regiospecific only hydrolyze the sn-1 and sn-3 position of glycerides for releasing free fatty acids, 1,2(2,3)-diacylglycerols and 2-monoacylglycerols (Macrae and Hammond, 1985). By now no lipases are known showing a clear *sn*-2 specificity because of steric hindrance that prevents the fatty acid in position sn-2 from entering the active site. Just very few lipases show a preference for this position such as lipase from *Candida parapsilosis*, lipase A from *Candida antarctica* or a lipase from the bovine rumen metagenome (Willis and Marangoni, 2002).

1,3-specific lipases include lipases from *Aspergillus niger*, *Mucor javanicus*, *Rhizopus delemar*, *Rhizopus oryzae*, *Yarrowia lipolytica*, *Rhizopus niveus*, and *Penicillium roquefortii* (Ribeiro et al., 2011). The lipase specificity can be affected by microenvironmental conditions on the reactivity of functional groups or substrate molecules. For instance, immobilized *C. antarctica* lipase (Novozym 435) exceptionally alters the positional specificity. If there is a small amount (<1/3 molar amounts for TAG) of ethanol or methanol in the alcoholysis reaction medium, this lipase acts on 1,3- and 2-positions of TAG at the same velocity. On the other hand, if there are excess amounts (>20 molar amounts for TAG) of ethanol or methanol, this lipase shows specificity for 1,3-positions. Additionally, the lipase shows specificity for the 1,3-positions in esterification reaction of FFAs with glycerol (Hou, and Shimada, 2009).

2.3.3.3 Fatty Acid Specificity

Some lipases show preference for special fatty acids. The chain length and the number and position of double bonds of fatty acid affect the fatty acid specificity of lipases (Hou, and Shimada, 2009). For example, lipase obtained from *Geotrichum candidum* is highly specific towards fatty acids containing cis-9 double bonds, lipase from *Penicillium roquefortii* shows specificity to shorter chain fatty acids, while

lipase from *Penicillium cyclopium* is specific towards long chain fatty acids (Ribeiro et al., 2011).

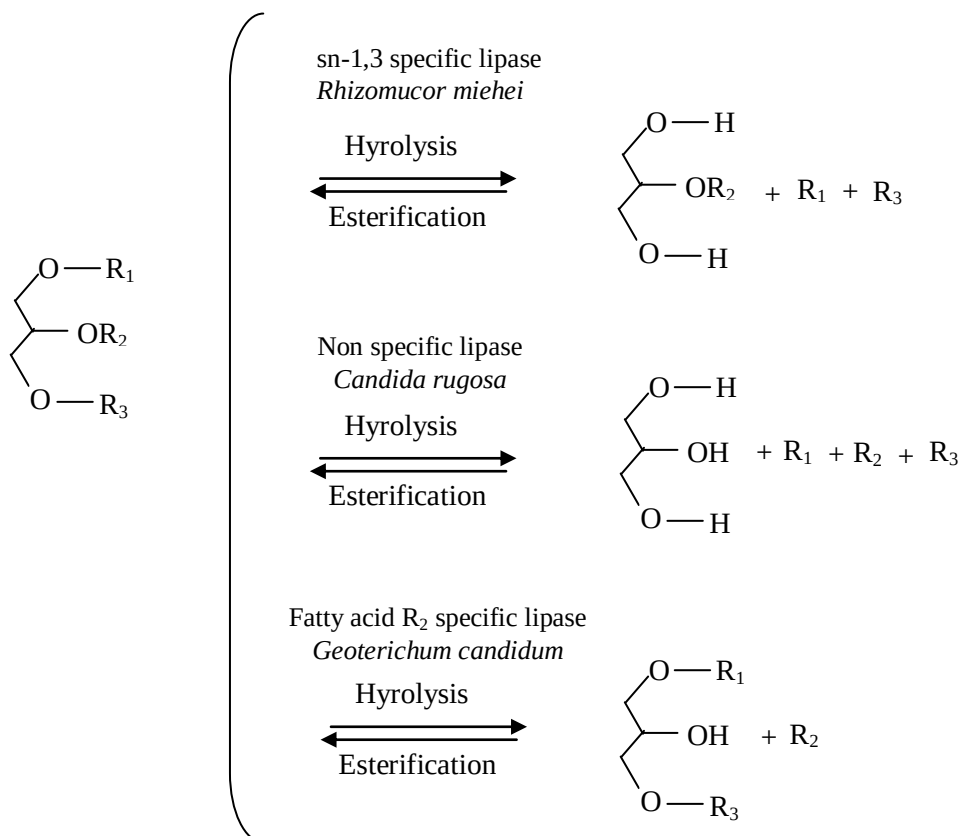


Figure 2.10 Lipase specificity in hydrolysis and esterification; R_1 , R_2 , R_3 fatty acids/ acyl moieties (Akoh and Min, 2002)

2.3.3.4 Stereospecificity

The capability of lipases to distinguish between the *sn*-1 and *sn*-3 positions of TAG is defined as stereospecificity. Positions 1 and 3 are hydrolyzed at different rates in reactions where the lipase is stereospecific. For example, *C. antarctica* lipase B, human lingual and dog gastric lipases are specific for *sn*-3 while lipases from *Pseudomonas fluorescens* and *T. Lanuginose* are specific for *sn*-1 position (Hou, and Shimada, 2009). Stereospecificity depends on the lipase source, acyl groups and lipid density at interface (Willis and Marangoni, 2002).

Table 2.1 The selectivity of lipases used for synthesis of TAG (Xu, 2000; Akoh and Min, 2002)

Lipase Source	Fatty acid specificity	Regiospecificity
Rhizomucor miehei	S>M, L	1>>3, 2
Candida antarctica	M, L>S	2
Rhizopus oryzae	M, L>S	1,3>>>2
Pseudomonas fluorescens	M, L>S	1>>3, 2
Rhizopus arrhizus	S, M>L	1,3
Porcine pancreatic	S>M, L	1,3
Aspergillus niger	S, M, L	1,3>>2
Rhizopus delemar	S, M, L	1, 2, 3
Candida rugosa	S, L>M	1, 2, 3

S: short chain fatty acid, M: medium chain fatty acid, L: long chain fatty acid

2.3.3.5 Substrate Specificity

In addition to TAGs which are the main substrate of lipases, lipases can cleave diacylglycerides, monoacylglycerides and phospholipids. Some lipases have specificity for special acylglycerols. For example lipase from *Penicillium camembertii* is specific towards mono- and diacylglyceride and acts only on mono- and diacylglycerides (Hou, and Shimada, 2009).

2.3.4 Factors Effecting Lipase Activity in Reaction Systems

Enzyme activity is defined as a measure of the rate at which an enzyme converts substrate to products in a biochemical reaction. Enzyme catalyzed reactions are affected by several factors. The main factors are temperature, pH, substrate concentration and composition, water content and water activity, product accumulation and solvent type.

2.3.4.1 Temperature

Generally, raising the temperature increases enzyme catalyzed reaction rates. Nevertheless, very high temperatures can decrease the reaction rates because of irreversible denaturation of the enzyme (Willis and Marangoni, 2002). Thermostability of lipases changes considerable depending on the enzyme source. Generally lipases from animal and plant have less thermostability than microbial extracellular lipases (Akoh and Min, 2002). In a solvent-free reaction system,

substrate should be kept in the liquid state. For this reason, temperature must be high enough. On the other hand, it is not necessary to study at high temperatures in the medium containing organic solvents. Because organic solvents easily solubilize hydrophobic substrates (Willis and Marangoni, 2002). Also, immobilization provides more temperature resistance when compared to free enzymes. Most of the immobilized lipases have the optimum temperature between 30 °C and 62 °C, while free lipases require slightly lower temperatures (30 °C – 40 °C). Immobilization limits motion and can decrease the unfolding and denaturation degree. So, immobilized lipases are more resistant to high temperatures (Akoh and Min, 2002).

2.3.4.2 pH

Lipases are affected by changes in pH of the reaction medium. All enzymes have an optimum pH where the enzyme is most active. The activity of enzyme decreases at higher or lower pH than the optimum pH (Çiftçi and Fadiloğlu, 2010). The optimum pH for most lipases is in the range of 7-9 while they can show activity over a wide range of acidic and basic pHs, between pH values 4 and 10. For instance, extracellular lipases from *Aspergillus niger* and *Chromobacterium viscosum* are active at acidic pH whereas lipase from *Pseudomonas* species have optimum pH around 8.5 (Willis and Marangoni, 2002).

During a lipase catalyzed interesterification reaction, enzyme can be inactivated rapidly if the pH is well removed from its optimum value (Willis and Marangoni, 2002). pH can affect binding of substrate to enzyme and the ionization of catalytic groups such as carboxyl or amino groups which are part of the enzyme's active site.

Also pH affects the stability of the tertiary or quaternary structure of enzymes and the velocity of the enzyme-catalyzed reactions, especially at extreme acidic or alkaline pHs (Powers, 2003).

2.3.4.3 Substrate concentration and composition

The reaction velocity increases until it reaches a maximum when the amount of enzyme is kept constant and the substrate concentration is gradually increased. After the maximum point the velocity remains constant.

Lipase catalyzed reactions are also affected by the composition of the substrate. For example, the existence of a hydroxyl group at the sn-2 position has a negative inductive effect (Willis and Marangoni, 2002). Presence of five and six double bonds in EPA and DHA, respectively, enhances steric hindrance effect, increasing rigidification and making the substrates less accessible to the enzyme. In reactions which containing organic solvents, oxidation of substrates, especially PUFAs, can decrease the lipase activity (Akoh et al., 2002).

2.3.4.4 Water content and water activity

Water content or water activity is an important factor for enzymatic interesterification reactions. Lipases need a small amount of water for their activities. They have an important property on the water- oil interface. The activity of lipases depends on this interface that provides a suitable environment for activation of enzyme. So, in water free medium activity of enzyme cannot be possible. The optimum water content for activity of lipase depends on the type of enzyme, substrate, organic solvent and immobilization support material. Lipases from molds show more tolerance than bacterial lipases to low water content. The optimum water content ranges from 0.004% to 11% (w/v) for lipase catalyzed interesterification reactions, whether most reactions require less than 1% water content for successful interesterification. In a reaction medium water content is the determining factor as to if the reaction equilibria will be toward hydrolysis or ester synthesis (Marangoni, 2002).

2.3.4.5 Product accumulation

The production of mono- and diglycerides may cause an increase in the rate of the reaction while presence of high levels of free fatty acids can inhibit the initial hydrolysis of triacylglycerols during interesterification of two triacylglycerols or during hydrolysis. Lipase loses its activity at high free fatty acid concentrations which can be caused by many reasons. High levels of free fatty acids would produce high levels of free or ionized carboxylic acid groups. These groups would increase the acidity of the microaqueous medium surrounding the lipase or cause desorption of water from the interface. Add to this, with short- and medium-chain fatty acids, there could be partitioning of fatty acids away from the interface into the surrounding

water shell due to their increased solubility in water. This would limit access by the substrate to the interface (Willis and Marangoni, 2002).

2.3.4.6 Solvent type

Employing enzymes in organic solvents has many potential advantages rather than in aqueous medium to catalyze organic reactions. For example, solvents increase solubility of non-polar substrates, shift thermodynamic equilibrium to favour synthesis and provide easy product recovery. Enzyme recovery can be easily performed by filtration because enzymes are insoluble in common organic solvents (Kanerva et al., 1990).

The type of solvent influences the catalytic efficiency of enzyme in lipase-catalyzed reactions. The substrate solvation can have an important influence on the reaction rate. Good substrate solvation in the bulk solvent decreases free energy of substrate and, so, slows down its conversion (Adlercreutz, 2013). Also, the solvent can directly interact with the enzyme which causes enzyme inhibition or inactivation. While choosing a solvent for a specific reaction, many factors should be considered, such as reactant solubility, boiling point, density, viscosity, surface tension, toxicity, flammability, waste disposal and cost (Akoh and Min, 2002).

2.3.5 Application of Lipases in Food Industry

Lipases are capable of catalyzing different types of reactions like hydrolysis, esterification, interesterification, acidolysis, alcoholysis, and aminolysis both in aqueous and organic mediums. So, lipases have a large application area in the pharmaceutical, leather, agrochemical, detergent, cosmetic, textile, and food industries (Ray, 2012). Food industry has an enormous potential for lipase application. Table 2.2 shows the lipase applications in the food industry.

Lipases are currently used to hydrolyze milk fat, enhance cheese flavour, accelerate cheese ripening, and for the lipolysis of butter fat and cream. Also lipases have an important role in the production of cheese-like products such as enzyme modified cheese (EMC), cheese flavours and cheese powders. They are used as an ingredient to give cheese flavour to frozen foods, soups, sauces, snacks and salad dressings (Aravindan et al., 2007; Ghosh et al., 1996; Houde et al., 2004).

Lipases are used in the bakery products for several purposes. Primarily they were used to improve the flavour content of bakery products by releasing short-chain fatty acids during esterification. Near the flavour enhancement, lipases provide prolongation in shelf life of the most bakery products. Also texture and softness of bakery products can be improved by using lipases. Because the use of lipases enables bakeries to control and enhance non-enzymatic browning, increase loaf volume and improve crumb structure (Aravindan et al., 2007; Hasan et al., 2006).

Lipases are used to enhance flavour to provide a creamy flavour in coffee whiteners (Ferreira-Dias et al., 2013). They are also used to produce buttery texture of toffees and caramels and reduce excessive sweetness (Gandhi, 1997). During black tea production, membrane lipids are breakdown by lipases to start the formation of volatile products. These volatile products give the characteristic flavour to the tea (Hasan et al., 2006). Lipases are used during the fish meat processing for the fat removal that is called biolipolysis procedure. After this procedure, it is possible to produce fat-free meats. Also lipases have been used to improve the aroma and accelerate the fermentation of alcoholic beverages such as apple wine (Gandhi, 1997; Hasan et al., 2006).

Table 2.2 Lipase Applications in the Food Industry (Aravindan et al., 2007)

Food Industry	Action	Product of Application
Dairy foods	Hydrolysis of milk fat, cheese ripening, modification of butter fat	Development of flavouring agents in milk, cheese and butter
Bakery foods	Flavour improvement	Shelf-life propagation
Beverages	Improved aroma	Alcoholic beverages, e.g. sake, wine
Food dressings	Quality improvement	Mayonnaise, dressings and whippings
Health foods	Transesterification	Health foods
Meat and fish	Flavour development	Meat and fish product, fat removal
Fats and oils	Transesterification, hydrolysis	Cocoa butter, margarine, fatty acids, glycerol, mono and diglycerides

In oleochemical industry, application of lipases decreases energy consumption and minimizes thermal degradation during the reactions of glycerolysis, hydrolysis, acidolysis and alcoholysis (Ghosh et al., 1996; Sharma et al., 2001). They can be used in synthesis of monoacylglycerols, fatty acyl amino esters and sugar esters which are used as emulsifiers in food formulations such as ice-creams, mayonnaise, salad dressings (Gandhi, 1997).

In fats and oil industry, lipases allow us to convert the less valuable fats and oils into more useful ones by changing the distribution of fatty acid chains on the glycerol backbone, replacing of fatty acids with new ones and changing the length of the fatty acids or saturation degree (Houde et al., 2004; Sharma et al., 2001). Lipase catalyzed reactions such as hydrolysis, acidolysis, alcoholysis and transesterification are used to production of value added products which are called as structured lipids. Human milk fat substitutes and low calorie lipids can be produced by lipase catalyzed modification of triglycerides (Houde et al., 2004; Ghosh et al., 1996).

In confectionery industry, cocoa butter is an important fat with its high commercial value. It is high priced and so its availability is low. Lipase catalyzed reactions of less expensive fats provides a good alternative to produce cheaper cocoa butter substitutes with similar properties of cocoa butter (Houde et al., 2004). Lipase catalyzed interesterification can be used to produce polyunsaturated fatty acids (PUFAs) and fats and oils which contain nutritionally important PUFAs like eicosapentaenoic and docosahexaenoic acids. Polyunsaturated fatty acids and their mono- and diglycerides are used in pharmaceuticals and food additives (Aravindan et al., 2007; Sharma et al., 2001).

2.3.6 Immobilization of Lipases

The reaction systems of lipases are different from other hydrolases that work in aqueous phase. Lipases are soluble in aqueous solutions (as a result of their globular protein nature) while their natural substrates are soluble in an organic phase. In other words, lipases are activated only when adsorbed to an oil/water interface. Although use of a proper organic solvent or an emulsifier helps overcoming the problem of intimate contact between substrate and enzyme, the practical use of lipases in such pseudohomogeneous reaction systems causes some technological and economical problems such as high cost of enzymes, contamination of products with residual

enzyme activity, slow reaction rate and using the enzyme for a single reactor pass (Shamel et al., 2007; Knezevic et al., 2006).

Most of the above problems could be overcome using immobilized lipases. Immobilization of lipases can offer several advantages including repeated usage, improvement of enzyme stability against pH and heat, ease of product separation, high purity and product yields, and continuous operation. It also ensures more flexibility with enzyme/substrate contact by using different reactor configurations. For industrial applications enzyme reusability is an important factor for the process economy. So, lipase immobilization is considered to be a possible way to facilitate enzyme recovery for reuse and to setup a continuous process (Cho and Rhee, 1993; Mendes et al., 2011).

An immobilized enzyme preparation is affected by many factors. The selections of carrier and immobilization method are the most important factors. The design of an efficient system for immobilization of lipase is a rather difficult task. Because the selection of an immobilization method and a support material is dependent on several factors such as lipase type, the type of reaction system, the process conditions, cost of immobilization method and support material, loading capacity of carrier, toxicity of immobilization reagents and the aim of immobilization. However, no single support material or immobilization method meets all these requirements in an optimal condition (Knežević et al., 2004). Lipase can be immobilized by different methods such as adsorption, covalent attachment, cross-linking, adsorption followed by cross-linking, and physical entrapment (Silva et al., 2012).

2.4 Olive Pomace Oil: A Potential Source for Lipase-Catalyzed Production of 2-Monoacylglycerols

2.4.1 Olive pomace and composition

Olive pomace is the solid by-product of the olive fruit processing. Its production is estimated to be 80% of the total olives processed for olive oil extraction (Meziane, 2013). Olive pomace is the pulpy material remaining after removing most of the oil from the olive paste (Kiritsakis et al., 2010). Olive pomace is important, mainly for the countries of the Mediterranean region where olive trees are grown (Gomes and Caponio, 2000). The commercial value of the olive pomace depends mainly on its oil and water content, that in turn depends on the processing method (pressure,

centrifugation, selective filtration) and other operating conditions (Kiritsakis et al., 2010). Olive pomace holds a certain amount of olive oil (4-8%) and contains fragments of skin, pulp, pieces of kernels and vegetable water (25-55%) (Niaounakis and Halvadakis, 2006). The main constituents are cellulose, proteins and water. Olive pomace also contains polyphenols and other constituents (Kiritsakis et al., 1998). The composition of olive pomace obtained by pressure process is shown in Table 2.3.

Table 2.3 Composition of olive pomace obtained by a pressure-type olive oil mill (Kiritsakis et al., 1998)

Constituents	Content (%)
Water	25.0
Nitrogen compounds	4.4
Non-nitrogen extractable compounds	20.0
Cellulose	40.0
Ash	6.6
Oil	4.0

2.4.2 Olive pomace oil

Olive pomace oil is oil that obtained from olive pomace by solvent extraction. Olive pomace oil does not include any oil obtained by a reesterification procedure or any mixture with other kinds of oils (Firestone, 2005). It is marked as follows.

Crude olive pomace oil: Olive pomace oil intended for refining in order to be used for human consumption or used in technical applications as it is.

Refined olive pomace oil: Oil obtained from crude olive pomace oil after refining process, which does not lead to alterations in the initial glyceride structure.

Olive pomace oil: Oil that is a mixture of refined olive pomace oil and virgin olive oil.

2.4.3 Extraction of olive pomace oil

Olive pomace is further processed to extract olive pomace oil in many Mediterranean countries. Also it is used for animal feed supplement and energy production or

disposed on field. Whereas, its economic importance is primarily owing to residual oil it contains (Meziane, 2013).

Solvent extraction is used for the recovery of residual oil from the olive pomace. Before extraction with solvents, the olive pomace must be prepared to obtain more oil. The preparation consists of a coarse crushing that aims reducing the pomace to such a dimension that the contact with the solvent can be more complete. Then the ground pomace is dried (Boskou, 1996). The extraction of oil with a solvent has a higher yield when the moisture of the pomace is between 5 and 10%. In addition to this, if pomace is dried rapidly after the olive oil extraction process, a greater yield and a higher quality of olive pomace oil can be obtained. For this reason, it is important to dry olive pomace quickly as possible before spoilage begins to occur (Meziane, 2011).

The driers contain slowly rotating, large cylinders. They are equipped with an internal set of propellers or blades used to push the pomace to the opposite side in a current of hot air. A large mass of hot air blows in through a furnace. Olive pomace from which the oil has been extracted (exhausted olive pomace) is used for heating the air (Kiritsakis et al., 1998).

The extraction of oil is carried out in special tanks filled with hot and dry pomace where the pomace is mixed with solvent. The solvent that is used for extraction is commonly hexane. Generally the equipments used for the extraction of pomace oil are discontinuous and the solvent passes from one extractor to the next against the current in comparison with the current with which the tanks are filled with pomace. The oil from pomace can be extracted by using continuous system. In this case, olive pomace and solvent move against the current so that the pure solvent comes into contact with the already partially exhausted pomace while the fresh pomace, which is richer in oil, comes into contact with the more concentrated oil/solvent mixture (Boskou, 1996).

After extraction, the oil/solvent mixture which is called as micelle must be filtered if it is necessary to separate the solid parts that it carries (Boskou, 1996). Then, this product passes through the distillation unit, where it is indirectly heated by steam. The solvent vaporizes and is carried out to the condensers where it is recovered after

eliminating the water by decantation (Kiritsakis et al., 1998). The extracted oil is crude olive pomace oil. It is considered an inferior grade due to long storage periods of pomace and high temperature drying applications. It is used for soap making or industrial purpose because of the high content of unsaponifiable matters and free fatty acids that it contains. Also it is refined and blended with a certain amount of virgin olive oil. After that it can be commercialized for human consumption (Meziane, 2013 and Fadiloğlu, et al., 2003).

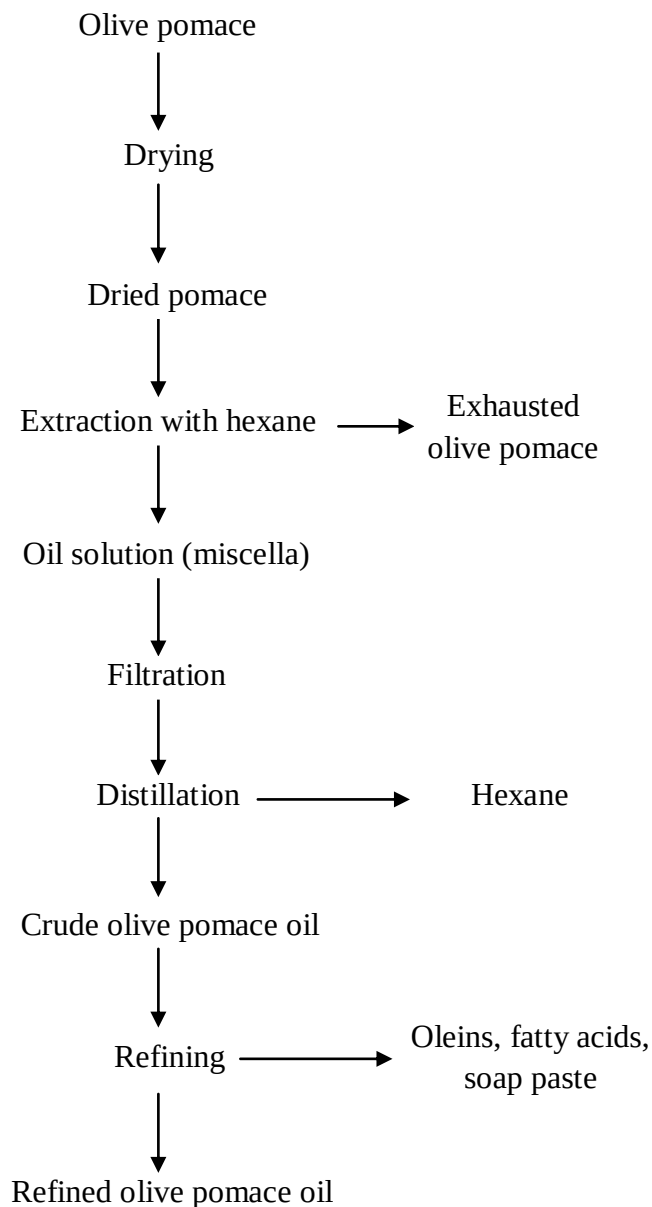


Figure 2.11 Treatments and by-products of olive pomace processing (Kiritsakis et al., 1998; Göğüş et al., 2009)

2.4.4 Deterioration of olive pomace oil

The crude, unrefined oil extracted from the pomace is dark green and has a medium to high free acidity. The moisture content of pomace favors triacylglycerol hydrolysis. So the oil in olive pomace undergoes rapid deterioration (Göğüş et al., 2004). Carola (1975) showed that the acidity of olive pomace oil may increase from 5 to 60% in a short time. This is due to the presence of the enzyme lipase originating either from the olive fruit or from microorganisms growing on the olive pomace. High moisture and temperature favour growing of these microorganisms during the storage. In addition, during the storage of the olive, oxidation products (aldehydes and ketones) are formed (Kiritsakis et al., 1998). So it is necessary to process olive pomace quickly before spoilage begins to occur (Meziane, 2011).

2.5 Experimental Design and Response Surface Methodology

Response surface methodology (RSM) is an effective and popular statistical method for developing and optimization of complex processes (Bektaş et al., 2008). When RSM is compared to the classic optimization methods, it has several advantages such as reducing the number of experimental trials, giving a large amount of information from a small number of experiments (Myers and Montgomery, 2002). Also it is a faster and economical method for optimization of a process (Bezerra et al., 2008). RSM has been successfully applied to optimize enzymatic alcoholysis reactions (Montoya et al., 2011; Domingos et al., 2008).

In RSM, both mathematical and statistical techniques are used together in the modeling and analysis of situations in which a response is affected by several variables, alone or in combination (Can and Özçelik, 2005). The central composite rotatable design (CCRD) is the preferred experimental design for fitting polynomial models to analyze response surfaces of multi-factor combinations. Also rotatable design has the property that the variance of predicted response does not change when the design is rotated around the centre point. Thus, CCRD with RSM is a very effective method for reducing the number of combinations required without compromising the validity of the results in studies where a large number of independent variables are included. If the proposed model is adequate, as revealed by the diagnostic checking provided by analysis of variance (ANOVA) and residual

plots, contour plots can be usefully employed to study response surface and locate the optimum (Wanasundara and Shahidi, 1998).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Crude olive pomace oil (OPO) was kindly obtained from Güvenal San ve Tic. AŞ., Gaziantep, Turkey. Before the reactions and analysis, OPO was centrifuged for 20 min. at 6000 rpm and precipitates were separated. Some properties of crude olive pomace oil are given in the Table 3.1.

Table 3.1 Some properties of crude olive pomace oil

Property	Value
FFA (%)	10.4
Peroxide value (meq O ₂ /kg)	15.08
Color	L*: 43.33, a*: -0.8, b*: 58.78
TAG (%)	79.31
MAG+DAG (%)	10.48
Fatty acid composition (%)	Palmitic acid, C _{16:0} : 13.27 Palmitoleic acid, C _{16:1} : 0.74 Margaric acid, C _{17:0} : 0.14 Heptadecenoic acid, C _{17:1} : 0.16 Stearic acid, C _{18:0} : 4.27 Oleic acid, C _{18:1} : 67.73 Linoleic acid, C _{18:2} : 10.82 Linolenic acid, C _{18:3} : 0.76 Arachidic acid, C _{20:0} : 0.64 Eicosenoic acid, C _{20:1} : 0.29 Eicosatrienoic acid, C _{20:3} : 0.25 Arachidonic acid, C _{20:4} : 0.80 Lignoceric acid, C _{24:0} : 0.13

Lipase from porcine pancreas (type II) was obtained from Sigma Aldrich. Immobilized lipase from *Candida antarctica* (Novozym 435, activity 7000 PLU/g) was kindly obtained from Novo Nordisk A/S (Bagsvaerd, Denmark). Thin layer chromatography (TLC) plates (Kieselgel G) were purchased from Merck (Darmstadt, Germany). Hexane, acetone, ethanol, chloroform, acetonitrile and other solvents were obtained from Sigma Aldrich. All solvents used were of HPLC grade and all other solvents and reagents were analytical or chromatographic grade. Standard 2-oleoylglycerol ($\geq 94\%$ purity) and oleic acid ($\geq 99\%$ purity) were obtained from Sigma Aldrich.

3.2 Production of 2-Monoacylglycerols

Alcoholysis of olive pomace oil was performed in 50 mL tightly closed-screw capped erlenmayer flasks. The specified amount of enzyme (wt%) and ethanol were added as given in Table 3.1. to the olive pomace oil (1 g). The values of time and temperature were set according to an experimental central composite design Table 3.2. The reactions were carried out in a rotary incubator (New Brunswick Scientific, Nova 40, USA) at 250 rpm. At the end of the reaction, lipase was removed from the reaction mixture by decantation. Ethanol was evaporated in a rotary evaporator (IKA RV 10 D S99, Germany) for a bath temperature of 40 °C and the mixtures stored at -20 °C before the analysis. The thin-layer chromatography-flame ionization detection (TLC-FID) Iatroscan system was used to analyze reaction products (TAG, DAG, MAG, FFA, FAEE and glycerol).

3.3 Methods of Analysis

3.3.1 Color

A HunterLab ColorFlex instrument (Hunter Associates Laboratory, Inc., Reston, VA, USA) was used for determination of the color values of OPO which are Hunter L^* , a^* , b^* values as measures of lightness, redness and yellowness, respectively. The measuring head was equipped with 51 mm diameter viewing port and used the system of diffuse illumination with 10° viewing geometry. The colorimeter was calibrated each time against a standard white and black tile.

3.3.2 Analysis of total, sn-2 and sn-1,3 fatty acid composition of OPO

The method which developed by Brockerhoff (1965) was used to detect the fatty acid composition at the sn-2 position of olive pomace oil. Olive pomace oil was hydrolysed with porcine pancreatic lipase that is a selective enzyme for sn-1,3 positions of TAGs. 0.4 g of OPO was weighed into a 100 mL erlenmayer flask, and 8 ml of Tris solution, 0.5 mL of CaCl₂, and 0.2 mL of Bile salt solutions were added. The mixture was incubated in a rotary incubator (New Brunswick Scientific, Nova 4, USA) at 40 °C and 150 rpm. 0.2 g of sn-1,3 specific porcine pancreatic lipase was added at the end of 10 min. After 20 min, reaction was ended by adding 15 mL ethanol and 5 mL 6N HCl. Then this mixture was taken into a separatory funnel and extracted by diethylether. The supernatant was taken and dried by a small amount of anhydrous magnesium sulphate addition.

The products of lipolysis reaction were separated by using thin layer chromatography method. TLC plates were developed with petroleum ether:diethyl ether: acetic acid (70:30:1, v/v/v). The sn-2 MAG band was scraped off and extracted with diethyl ether. 2-MAGs of OPO were converted to fatty acid methyl esters (FAME) prior to GC analysis. For methylation, 100 mg of the oil sample was mixed with 2 mL heptane. 0.2 mL 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 Gas Chromatography (GC) as described below. Also the fatty acid composition at sn-1,3 positions was 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 (Eqn. 4.1).

$$[\text{position 1 or 3}] = (3 \times [\text{FA\% in TAG}] - [\text{FA\% in position 2}])/2 \quad (\text{Eqn 4.1})$$

3.3.3 GC analysis

The fatty acid composition of olive pomace oil was analyzed after methylation. A 7890A gas chromatography equipment (Agilent Technologies, USA) with a flame ionization detector (FID), a split/splitless injector (operated with a split ratio of 1:50), and a capillary column HP-88 (88 % Cyanopropylaryl 100 m x 0.250 mm ID x 0.20 µm) was used. The carrier gas was helium. The injection temperature was 250 °C and

injection volume was 1 μ L. The oven temperature was set at initial 120 $^{\circ}$ C, held for 1 min, followed by increase of 10 $^{\circ}$ C/min up to 175 $^{\circ}$ C, held for 10 min, followed by increase of 5 $^{\circ}$ C/min up to 210 $^{\circ}$ C, held for 5 min followed by increase of 5 $^{\circ}$ C/min up to 230 $^{\circ}$ C and held for 5 min. The detector was programmed at 260 $^{\circ}$ C with 350 mL/min air flow, 35 mL/min hydrogen flow, and 15 mL/min helium makeup flow. The percentage of each fatty acids was determined from retention times based on comparison with fatty acid standards.

3.3.4 Analysis of TAG Content

The composition of olive pomace oil including triacylglycerol (TAG), diacylglycerol (DAG) and monoacylglycerol (MAG) was determined by SCL-10A HPLC system (Shimadzu, Japan) using the method proposed by the AOCS Official Method Ce 5b-89 (AOCS, 1993). The analyses were performed isocratically with a mobile phase consisting of 64:36 (v/v) acetone/acetonitrile. 100 μ L oil was diluted in 900 μ L acetone, filtered and then injected into the column (Sphereclone 5 μ ODS (2), 250x4.6 mm; Phenomenex, 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 set at 30 $^{\circ}$ C and elution was monitored with a refractive index detector (RID-10A, Shimadzu, Japan). The total analysis time was 45 min. All TAG content was given as weight percentage of the total weight of the sample.

3.3.5 Recovery and Purification of 2-MAGs

Recovery of the 2-MAG from the ethanolysis reaction mixture was made by using two different methods: solvent extraction and low temperature crystallization.

3.3.5.1 Solvent Extraction

Recovery of the 2-MAG from the ethanolysis reaction mixture by solvent extraction was performed using a modification of the method described by Rodriguez et al. (2012) and summarized in Fig. 3.1. Firstly, residual ethanol was evaporated from the reaction mixture in a rotary evaporator (IKA RV 10 D S99, Germany) at 40 $^{\circ}$ C. Then the mixture was dissolved in an ethanol/water mixture (90:10, v/v) in the proportion 1:9 (v/v). After that this mixture was extracted twice with a triplicate volume of hexane to extract FAEEs, DAGs and TAGs. The 2-MAGs were in the hydroethanolic phases. Thus, two hydroethanolic solutions (1 and 2) were obtained. Hydroethanolic

solution 3 is the mixture of these two solutions. In the last step, hydroethanolic solution 3 was extracted with a equal volume of hexane and solvents were evaporated from the final hydroethanolic solution. 2-MAGs were stored at -20°C before the analysis.

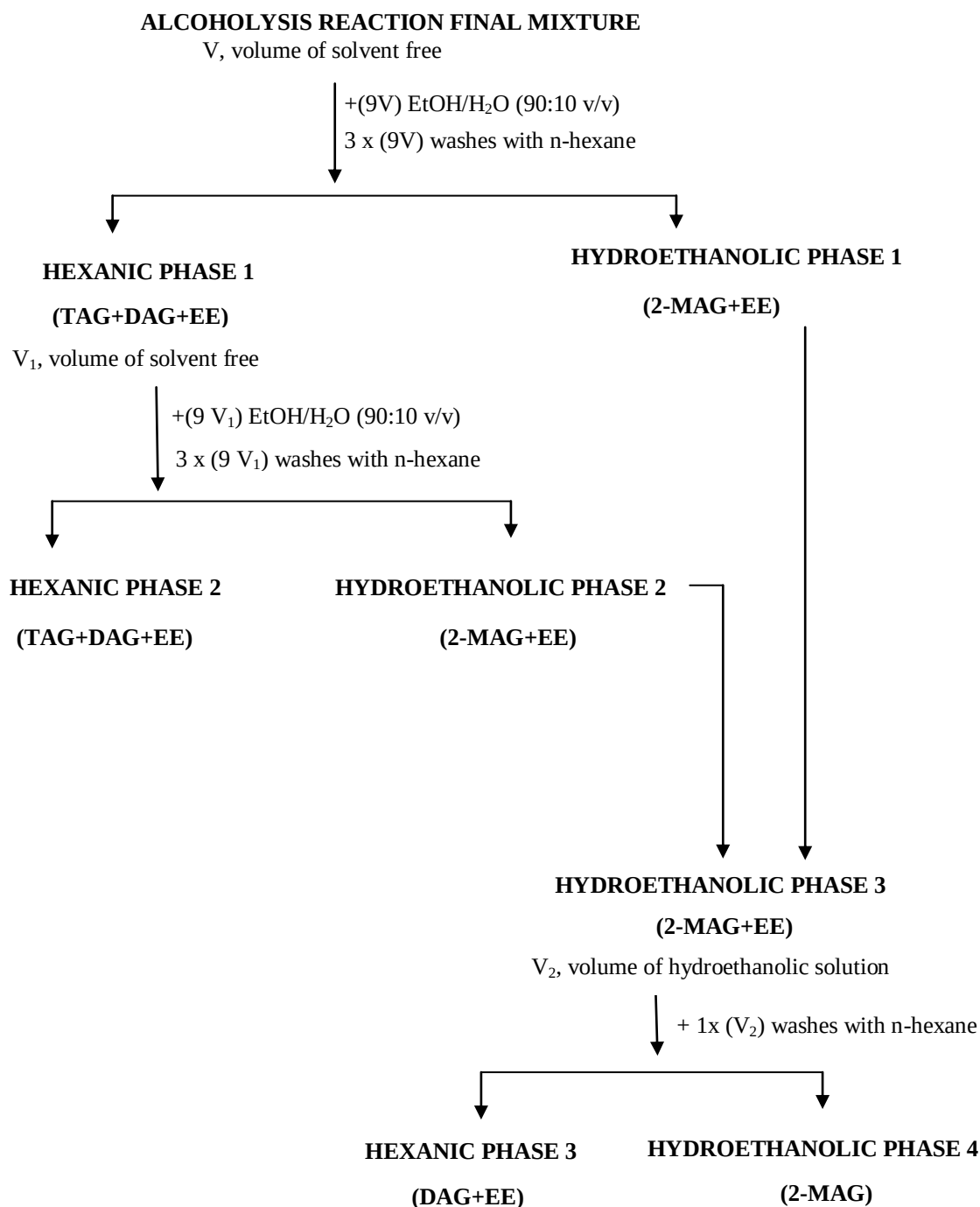


Figure 3.1 Experimental procedure for the recovery of 2-MAGs from the alcoholysis final mixture by solvent extraction

3.3.5.2 Low Temperature Crystallization

In crystallization, lipase and residual ethanol was removed from the reaction mixture. The resultant oily liquid was dissolved in tenfold hexane by weight at room temperature. The solution was cooled to -18 °C and left at this temperature for 1 hour. White crystals were formed at the end of the time. Then, the solution was centrifuged at -10 °C and 8000 rpm for 10 min and white crystals were separated. These white crystals include the monoacylglycerols.

3.3.6 Determination of the reaction products by TLC-FID

Reaction products were determined by using the thin-layer chromatography-flame ionization detection (TLC-FID) Iatroscan system. The procedure was applied 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 equipment (Iatroscan MK-5; Iatron Laboratories, Tokyo, Japan) to separate 2-MAG and 1(3)-MAG. The preparation of boric acid treated chromarods were performed by dipping the rods into 3% boric acid solution and then drying for 5 min at 120 °C. The samples were diluted in chloroform and rods loaded with the samples were eluted for 8 cm with 100% chloroform. The developed chromarods were dried at room temperature for 5 min. After that the chromarods were again developed with chloroform:(methanol: NH₄OH, 8: 2, v:v) (70:0.05, v:v) for 10 cm. Then the developed chromarods was dried at 120°C for 5 min in an oven. Scanning of chromarods was done under the conditions which were hydrogen gas flow rate 160 mL/min, air flow rate 2.0 L/min, scanning speed 30 s/scan. The 2-MAG yield was defined as the percentage of the theoric yield of 35.07% w:w. The theoretical yield was calculated due to the sn-2 fatty acid composition of olive pomace oil.

3.4 Experimental Design

Pre-studies for experimental design of ethanolysis reaction were done to decide the range of independent variables in the experimental design created by response surface methodology.

A five level, four factorial central composite rotatable design (CCRD) was applied to study the responses, percent of MAG yield, effect of reaction factors on the

production of MAG and optimize reaction conditions to obtain maximum MAG yield.

Table 3.2 A five-level, four factorial central composite rotatable design (CCRD) applied for alcoholysis of olive pomace oil

Run	Time (h)	Enzyme load (wt%)	Temperature (°C)	Substrate mol ratio (ethanol:oil)
1	7.00	14.00	47.50	65.00
2	7.00	14.00	22.50	65.00
3	4.00	10.00	35.00	40.00
4	7.00	14.00	47.50	65.00
5	4.00	10.00	60.00	40.00
6	4.00	10.00	35.00	90.00
7	7.00	14.00	47.50	65.00
8	13.00	14.00	47.50	65.00
9	10.00	10.00	60.00	90.00
10	10.00	10.00	60.00	40.00
11	4.00	18.00	60.00	40.00
12	1.00	14.00	47.50	65.00
13	4.00	18.00	35.00	90.00
14	10.00	18.00	35.00	40.00
15	10.00	18.00	35.00	90.00
16	10.00	18.00	60.00	40.00
17	7.00	14.00	47.50	65.00
18	7.00	14.00	47.50	65.00
19	7.00	14.00	47.50	15.00
20	4.00	10.00	60.00	90.00
21	10.00	10.00	35.00	90.00
22	7.00	14.00	72.50	65.00
23	10.00	10.00	35.00	40.00
24	4.00	18.00	60.00	90.00
25	10.00	18.00	60.00	90.00
26	7.00	14.00	47.50	65.00
27	7.00	6.00	47.50	65.00
28	7.00	14.00	47.50	115.00
29	4.00	18.00	35.00	40.00
30	7.00	22.00	47.50	65.00

The independent variables were selected as reaction temperature (Te), reaction time (Ti), enzyme concentration (En) and substrate mol ratio (Sb). Levels of the independent variables were defined as Te, 35-60 °C; Ti, 4-10h; En, 10-18 wt% and

Sb, 40:1-90:1 by considering the pre-studies. The employed CCRD consisted from 30 experiments which contain 6 centre point, 8 star point and 16 axial points (Table 3.2). The star points ensure estimation of curvature of models. Six replicate runs at the centre point of the design were applied to allow the estimation of pure error.

CCRD consists of three parts. These are factorial points (-1, 1), centre points (0, 0), and axial points (-2, 2). All variable that will be optimized was coded at these levels and the ranges are shown in Table 3.3.

Table 3.3 Independent variables and their levels for central composite design in optimization of lipase-catalyzed alcoholysis reaction

Independent variables	Symbol	Coded variable levels				
		-2	-1	0	+1	+2
Reaction time	Ti	1	4	7	10	13
Enzyme load	En	6	10	14	18	22
Temperature	Te	22.5	35	47.5	60	72.5
Substrate mol ratio	Sb	15	40	65	90	115

3.5 Data Analysis and Optimization by RSM

The data obtained from the experiments were analyzed by using 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 for optimization of reaction conditions. The significance level for all tests was set at 95% confidence level. The goodness of the models established were determined using coefficient of determination, R^2 , together with the absolute average deviation (AAD) values and ANOVA (Arifin et al., 2012).

The first- or second-order coefficients were generated by regression analysis with backward elimination. The quadratic response surface model was fitted to the equation (3.1)

$$Y_i = \beta_0 + \sum_{i=1}^4 \beta_i X_i + \sum_{i=1}^4 \beta_{ii} X_i^2 + \sum_{i=1}^3 \sum_{j=i+1}^4 \beta_{ij} X_i X_j + \varepsilon \quad (3.1)$$

where Y_i is the response for percent MAG yield, β_0 intercept, β_i first-order model coefficients, β_{ii} quadratic coefficients for the i th variable, β_{ij} interaction coefficients for the interaction of variable i and j , X_i are independent variables and ε is the random error.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Triacylglycerol and Fatty Acid Composition of Olive Pomace Oil

The TAG composition of olive pomace oil was determined using HPLC equipped with refractive index detector (Figure 4.1). The average molecular weight of olive pomace oil was determined using the triacylglycerol composition and found to be as 808.06 g/mol. Each individual TAG percentage in the oil is given in Table 4.1.

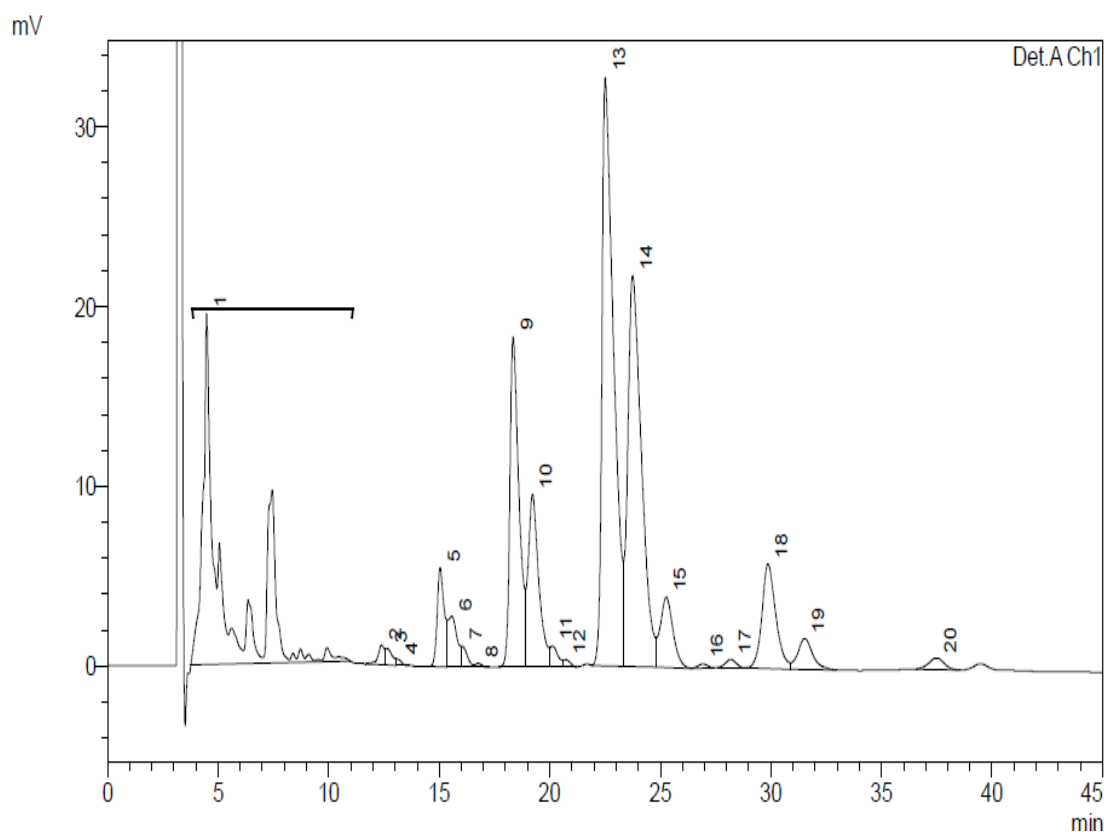


Figure 4.1 HPLC chromatogram of olive pomace oil. 1, FFA+MAG+DAG; 2, LLL; 3, OLLn+PoLL; 4, LnLP; 5, LLO; 6, LnOO+PoOL; 7, PLL+PoPoP; 8, POLn; 9, LOO; 10, LOP+SLL; 11, PLL; 12, PLP; 13, OOO; 14, POO; 15, POP; 16, PPP; 17, ALO; 18, SOO; 19, POS+SLS; 20, SPS. L, linoleic acid; O, oleic acid; Ln, linolenic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

Table 4.1 Triacylglycerol composition of olive pomace oil

TAG	Total%	TAG	Total%
LLL	0.58	PLP	0.27
OLLn+PoLL	0.48	OOO	31.28
PLLn	0.13	POO	24.53
LLO	3.2	POP	4.15
OOLn+PoOL	2.09	PPP	0.16
PLL+PoPoO	0.55	ALO	0.40
POLn	0.12	SOO	6.55
OOL+LnPP	13.49	SLS+POS	2.08
POL+SLL	8.33	SPS	0.82
PLL	0.79		
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 olive pomace oil

The fatty acid composition (%)			
Fatty Acid	Total FA in TAG	Total FA in <i>sn</i>-2	Total FA in <i>sn</i>-1,3
C16:0 (Palmitic acid)	13.27	13.90	12.95
C16:1 (Palmitoleic acid)	0.74	-	1.11
C17:0 (Margaric acid)	0.14	-	0.21
C17:1 (Heptadecanoic acid)	0.16	-	0.24
C18:0 (Stearic acid)	4.27	6.93	2.94
C18:1 (Oleic acid)	67.73	69.80	66.69
C18:2 (Linoleic acid)	10.82	9.37	11.55
C18:3 (α -Linolenic acid)	0.76	-	1.14
C20:0 (Arachidic acid)	0.64	-	0.96
C20:1 (Eicosenoic acid)	0.29	-	0.44
C20:3 (Eicosatrienoic acid)	0.25	-	0.37
C20:4 (Arachidonic acid)	0.80	-	1.20
C24:0 (Lignoceric acid)	0.13	-	0.20

The total fatty acid composition, fatty acid composition at *sn*-2 and *sn*-1,3 position of olive pomace oil was analyzed using GC. Oleic acid, palmitic acid and linoleic acid are the major fatty acids of OPO with the percentages 67.73, 13.27 and 10.82, respectively (Table 4.2). Olive pomace oil has high unsaturated fatty acid content (81.55%). The most abundant fatty acid at *sn*-2 position was oleic acid (69.80%). The major abundant fatty acids at *sn*-1,3 positions were oleic, palmitic and linoleic acids with the percentages of 66.69, 12.95 and 11.55. These results were similar to the studies of Zambiasi et al (2007).

4.2 Pre-studies for Experimental Design of Ethanolysis Reaction

Pre-studies for experimental design of ethanolysis reaction were done to decide the range of independent variables in the experimental design created by response surface methodology.

4.2.1 Time Studies

The influence of time on the ethanolysis reaction was studied between 2-24 h to decide the time range in the experimental design created by response surface methodology. The reaction parameters were selected as temperature 50 °C, ethanol:oil molar ratio 70:1 and enzyme load 10 (wt %) for time studies. 2-MAG % conversion with respect to time is shown in Figure 4.2. It is seen that the yield of 2-MAG increased with the increase in the time up to first six hours and became stable after the six hours. However, after twelve hours the 2-MAG yield decreased. The reaction reached equilibrium at 6 h.

4.2.2 Enzyme Load Studies

The effect of enzyme concentration on the ethanolysis reaction of olive pomace oil was studied between 5-25 (wt %). The reaction parameters were selected as temperature 50 °C, ethanol:oil molar ratio 70:1 and time 6 h for enzyme load studies. The effect of enzyme concentration on the 2-MAG yield is given in Figure 4.3. It is seen that increase in the enzyme concentration up to 15 (wt %) increased the 2-MAG yield. However, at the enzyme loads above 15 (wt %), a decrease is seen in 2-MAG yield.

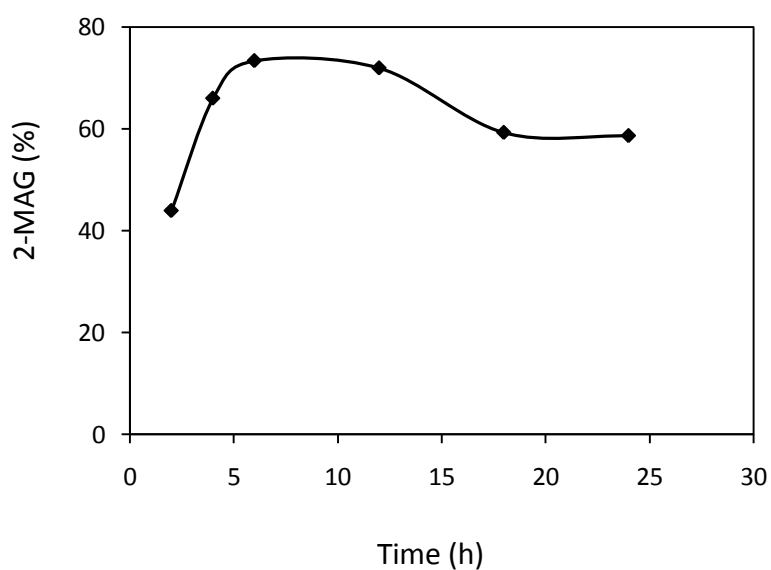


Figure 4.2 The effect of time on the ethanolysis reaction of olive pomace oil. Ethanolysis reactions were carried out using Novozyme 435 at temperature 50 °C, ethanol:oil molar ratio 70:1 and enzyme load 10 (wt %).

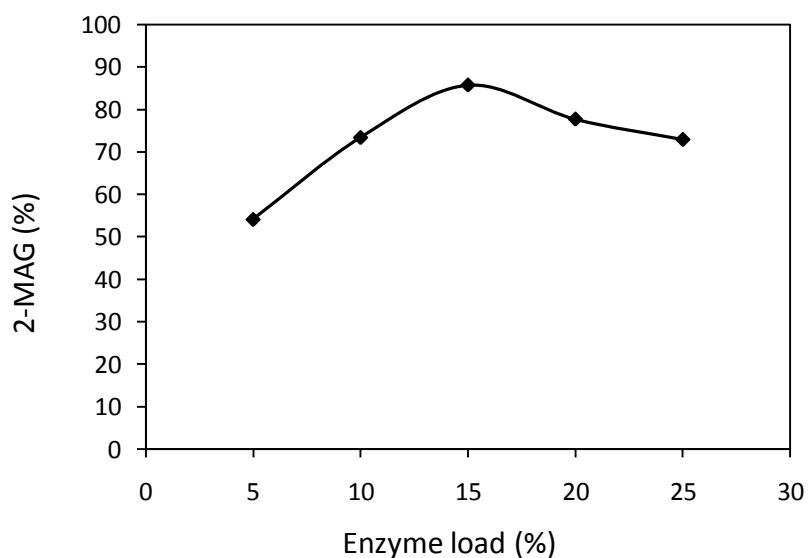


Figure 4.3 The effect of enzyme load on the ethanolysis reaction of olive pomace oil. Ethanolysis reactions were carried out using Novozyme 435 at temperature 50 °C, ethanol:oil molar ratio 70:1 and time 6 h.

4.2.3 Temperature Studies

The influence of the temperature on the ethanolysis reaction was studied between 30 and 70°C. The other reaction conditions were selected as enzyme load 15 (wt %), ethanol: oil molar ratio 70:1 and time 6 h. Figure 4.4 shows the change in 2-MAG yield with change in temperature. It is seen that at temperatures higher than 40 °C, the 2-MAG yield decreases. The highest 2-MAG yield was observed at 40 °C.

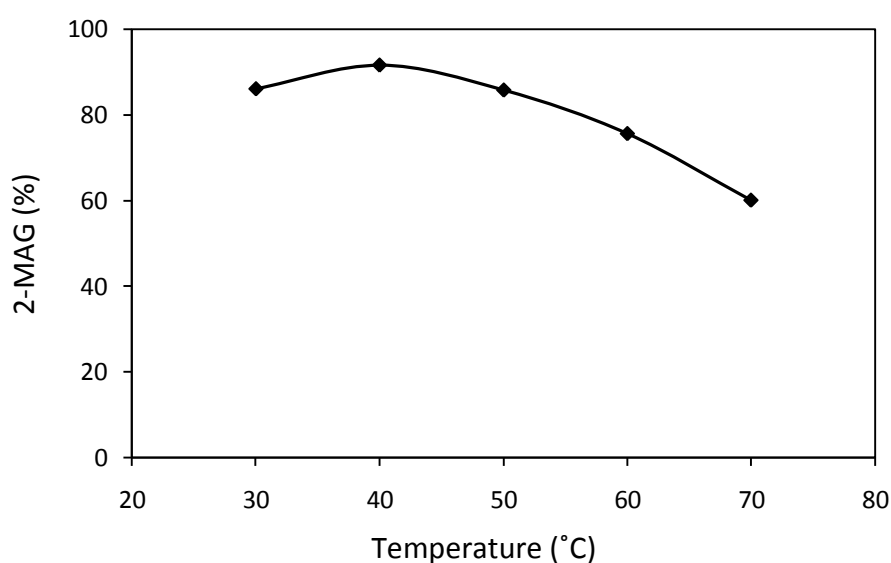


Figure 4.4 The effect of temperature on the ethanolysis reaction of olive pomace oil. Ethanolysis reactions were carried out using Novozyme 435 at ethanol:oil molar ratio 70:1, time 6 h and enzyme load 15 (wt %).

4.2.4 Ethanol:oil Ratio Studies

The effect of the ethanol:oil mol ratio on the ethanolysis reaction studied between 10:1 and 110:1. The other reaction parameters were fixed as time 6 h, enzyme load 15 (wt %) and temperature 40 °C. The change in the 2-MAG yield with respect to ethanol:oil mol ratio is shown in Figure 4.5. It is seen that the yield of 2-MAG increased with the increase in substrate mol ratio up to 90:1. However, after this point the yield decreased. The highest conversion rate was observed at 90:1.

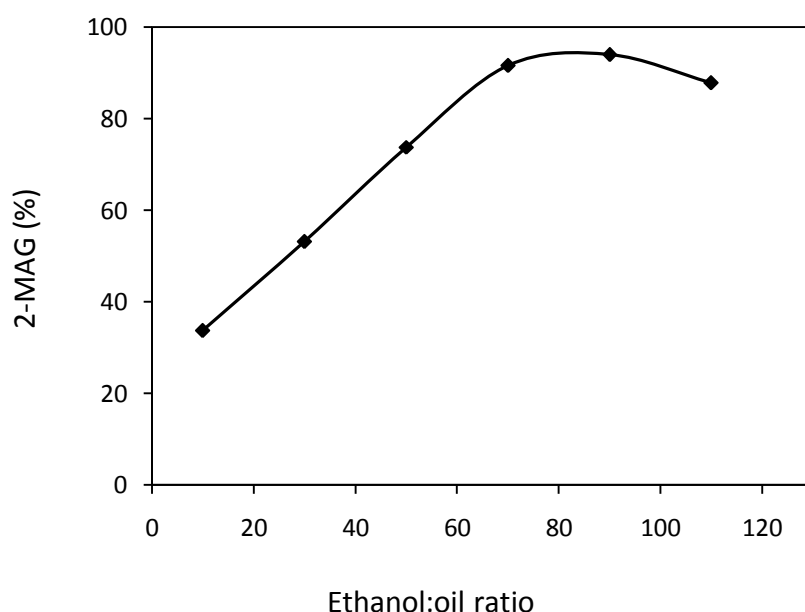


Figure 4.5 The effect of ethanol:oil mol ratio on the ethanolysis reaction of olive pomace oil. Ethanolysis reactions were carried out using Novozyme 435 at temperature 40 °C, time 6 h and enzyme load 15 (wt %).

4.3 Diagnostic Checking of Fitted Models

RSM was implemented to model the response, 2-MAG yield. The data of response obtained from reactions under different conditions as defined in central composite rotatable design (CCRD) are shown in Table 4.3. The model predicted for 2-MAG% was significant at the 99% confidence level and the lack of fit was significant ($P < 0.05$) with coefficient of determination (R^2) 0.87. The high value of coefficient of determination, significance of model and non-significant lack of fit indicate that the model is a good fit. The best fitting quadratic model was determined by using regression and backward elimination by means of elimination of insignificant factors and interactions in the model.

4.4 Effects of Reaction Parameters: Statistical Evaluation of the Reaction Parameters and Response Surface Plotting

The effects of the reaction parameters on the enzymatic production of 2-monoacylglycerols were evaluated by statistical analysis and response surface plotting. By this way, it was possible to analyse the linear and quadratic effects of reaction parameters and interactions between them for response. Effects of the

parameters were interpreted relating the coefficients of the parameters and corresponding P values. Coefficients and P values were calculated by using Design Expert 7.0 software. The P value below 0.05 indicates a confidence interval of 95%. In this thesis, it was analyzed that effects had a 'P>F' value less than 0.05 are significant and effects had a 'P>F' value higher than 0.05 are nonsignificant. Coefficients and P values for response were presented in Table 4.4.

Response surface plots were obtained by using Design Expert 7.0 software. In Figure 4.6 response surface plots were represented for 2-MAG yield. The relationships between independent and dependent variables are shown in the three-dimensional representation as response surfaces. Response surface plots composed of three axis. The levels of parameters are shown on x and y axis and the 2-MAG yield is shown on z axis. Other two corresponding parameters which were not shown on the plots were kept constant at their center levels.

Table 4.3 2-MAG yield obtained by alcoholysis of OPO under the conditions generated by RSM

Run	2-MAG%	Run	2-MAG%
1	84.15	16	28.80
2	84.29	17	81.72
3	68.12	18	86.57
4	78.07	19	38.01
5	68.80	20	45.37
6	72.71	21	93.10
7	82.49	22	57.06
8	43.20	23	60.96
9	69.00	24	76.79
10	54.52	25	69.80
11	74.99	26	73.62
12	37.07	27	82.18
13	90.76	28	82.04
14	57.14	29	82.75
15	91.62	30	63.27

Table 4.4 Analysis of variance table and estimated coefficients for 2-MAG%

Source	Coefficients	Sum of squares	df	Mean square	F value	p-value Prob>F
Model		943.85	9	104.87	14.48	<0.0001 significant
Intercept	27.76					
Linear						
Ti	-0.64	9.74	1	9.74	1.35	0.2598 ²
En	1.15	31.49	1	31.49	4.35	0.0501 ²
Te	-2.69	173.61	1	173.61	23.97	<0.0001 ¹
Sb	2.93	206.21	1	206.21	28.48	<0.0001 ¹
Interaction						
Ti*En	-2.21	78.46	1	78.46	10.83	0.0036 ¹
Ti*Sb	2.89	133.23	1	133.23	18.40	0.0004 ¹
En*Sb	1.25	24.98	1	24.98	3.45	0.0781 ²
Quadratic						
Ti ²	-3.03	260.38	1	260.38	35.96	<0.0001 ¹
Sb ²	-1.28	46.73	1	46.73	6.45	0.0195 ¹
Residual		144.83	20	7.24		
Lack of fit		131.72	15	8.78	3.35	0.0939 not significant
Pure error		13.11	5	2.62		
Cor total		1088.67	29			

$R^2=0.87$, adj $R^2=0.81$, pred $R^2=0.63$

Ti: time, En: enzyme load, Te: temperature, Sb: substrate mol ratio

¹ Significant at 'P>F' less than 0.05, ² not significant at 'P>F' higher than 0.05

4.4.1 Effect of Substrate Mole Ratio on the Production of 2-MAG

Effect of substrate mol ratio had the highest coefficient for 2-MAG (Table 4.4). So, it can be said that substrate mol ratio was the most effective factor in the production of 2-MAG ($P<0.0001$). Positive sign of this coefficient shows that increasing the substrate mol ratio caused increase in 2-MAG production. This result was also supported by response surface plots. In Figure 4.6b, c, d, it is seen that increase in substrate mol ratio increases 2-MAG until a maximum point of 90. Irimescu et al. (2002) also found similar results in their study. Shimada et al. (2003) found the

substrate mol ratio was important factor affecting ethanolysis reaction and they reported that the increase in the amount of ethanol accelerated the velocity of ethanolysis reaction. Also our pre-studies for ethanolysis reaction showed that 2-MAG yield was low at low substrate mol ratios and glycerol was released under these conditions. According to this result it can be said that specificity of Novozyme 435 on sn-1,3 positions of triacylglycerols increases at higher ethanol:oil mol ratios. This result was similar with the results of Irimescu et al. (2002), Muñio et al. (2008) and Preffer et al. (2007). They explained a possible reason for this behaviour that ethanol may decrease flexibility of the tertiary structure of enzyme, hindering the substrate from accessing the catalytic binding pocket with the acyl group in the second position.

In addition to the main effect of substrate mol ratio, it had also an interaction effect with another factor, time. In Figure 4.6b, it is seen that while reaction reaches equilibrium in 6 hour at low substrate mol ratios, at higher substrate mol ratios reaction reaches equilibrium at the end of the 7 h. The term $Ti*Sb$ was statistically significant as it was seen in Table 4.4. Also, it can be said that from the Figure 4.6c 2-MAG yield increases with the increase in enzyme load at higher substrate mol ratio. But increase in the enzyme load does not affect the 2-MAG yield at low substrate mol ratios. This may be due to the saturation of enzyme with substrate at lower enzyme loads and so some of the substrates cannot find any active sites to which to bind. However, the term $En*Sb$ was not statistically significant as it was seen in Table 4.4.

4.4.2 Effect of Temperature on the Production of 2-MAG

When the effect of temperature coefficient was considered, temperature effect followed the substrate mol ratio in the decreasing effectiveness order for 2-MAG (Table 4.4). Temperature was more effective than enzyme load and time ($P < 0.0001$). It had a negative coefficient. Negative sign of this coefficient indicates that increasing the temperature cause decrease in 2-MAG production. This result was also supported by response surface plots. In Figure 4.6d, e, f, it is seen that increase in temperature decreases 2-MAG yield until a maximum point of 60 °C. The optimum temperature for ethanolysis reaction of olive pomace oil was found as 35 °C for maximum yield of 2-MAG. Irimescu et al. (2002) and Preffer et al. (2007) reported that the similar results for the ethanolysis reaction catalyzed by *Candida antarctica*

lipase. They reported that the reaction temperature is a very important factor. Because high temperatures increase the acyl migration rate and at temperatures greater than 40 °C the 2-MAG yield decreased due to the increasing acyl migration and partial deacylation to glycerol. In the plot of temperature versus enzyme load, it is clearly seen that when all other parameters kept constant, increase in temperature cause a linear decrease in the production of 2-MAG. This may be due to the unsuitability of higher temperatures for the ethanolysis of olive pomace oil.

4.4.3 Effect of Enzyme Load on the Production of 2-MAG

Result of ANOVA indicated that enzyme load was also effective on 2-MAG production. It was less effective than substrate mol ratio and temperature while more effective than time. However enzyme load was not statistically significant ($P>0.05$) (Table 4.4) and it had positive coefficient. The response surface plots demonstrated the statistical analysis results (Figure 4.6a, c, f). It can be said that from the Figure 4.6c if the substrate mol ratio was fixed at its lower level, increase in the enzyme load would not affect the 2-MAG yield. But, when the substrate molar ratio was fixed at the upper level, increasing the enzyme load leads to a higher 2-MAG yield. This occurs when there are no limiting factors such as presence of activators or inhibitors or mass transfer effect. Rodrigues et al. (2008) found that the similar results for the ethanolysis reaction.

In the ANOVA results, the interaction term $Ti*En$ was statistically significant while the interaction term $En*Sb$ was not statistically significant (Table 4.4). In Figure 4.6a, it is seen that increase in the enzyme load provides that reaction reaches to maximum in shorter time. Also it can be said that from the Figure 4.6f, increasing the enzyme load caused increase in the production of 2-MAG linearly when the other parameters were kept constant. 2-MAG reached its maximum point at enzyme load 18 wt%.

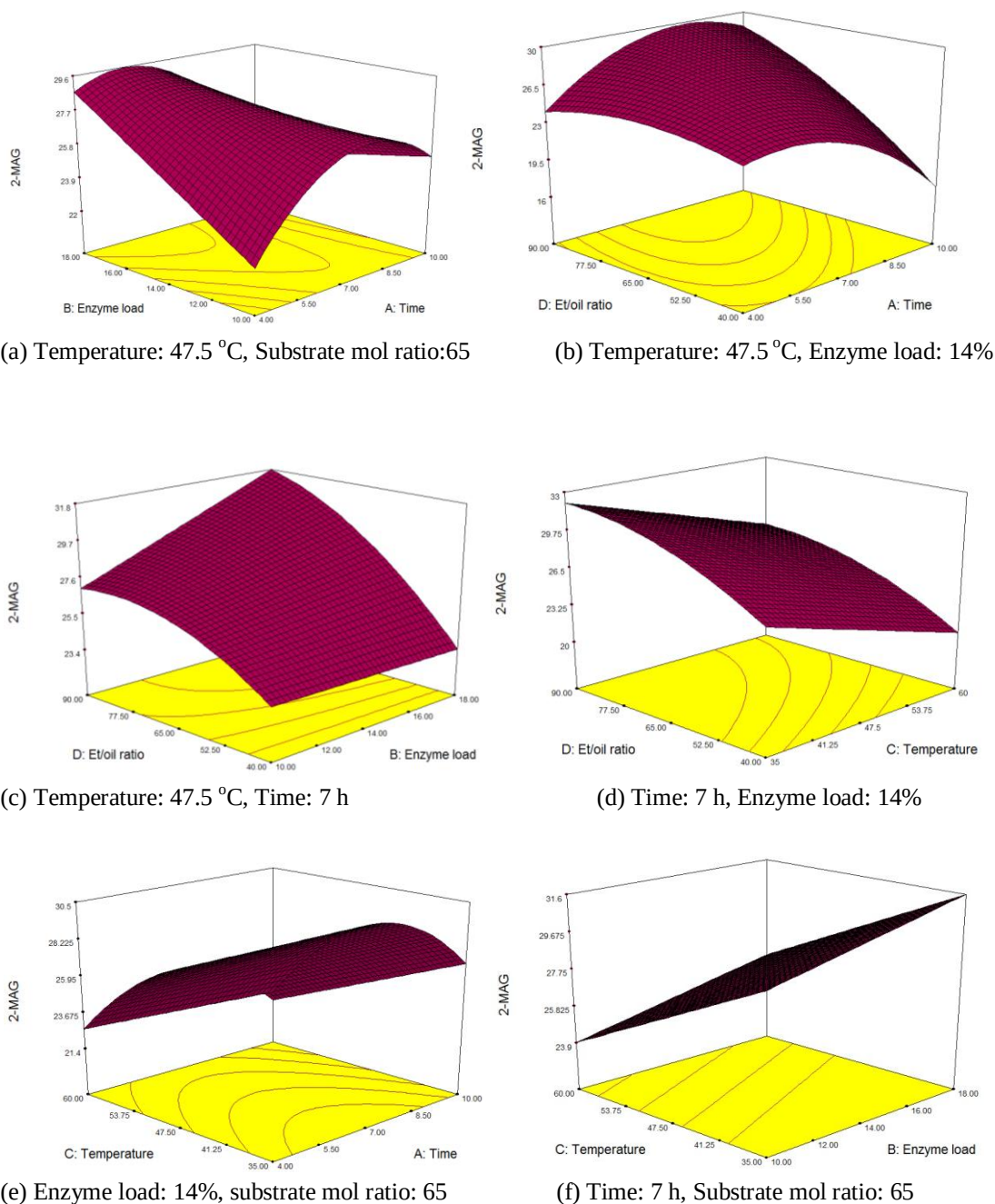


Figure 4.6 Response surface plots for 2-MAG%: (a) enzyme load versus time; (b) substrate mol ratio versus time; (c) substrate mol ratio versus enzyme load; (d) substrate mol ratio versus temperature; (e) temperature versus time; (f) temperature versus enzyme load

4.4.4 Effect of Time on the Production of 2-MAG

Result of ANOVA indicated that time was also effective on 2-MAG production. Increasing time caused increase in the yield of 2-MAG. However, this increase was not statistically significant ($P>0.05$) (Table 4.4). As seen in Figure 4.6e percent yield reached maximum at 7 h and thereafter there was a decrease in 2-MAG%. This trend

is similar to the studies of Munio et al. (2008), Irimescu et al. (2002) and Köse et al. (2002). Munio et al. (2008) reported that 2-MAG yield decreased at longer reaction times. This may be because: Novozyme 435 behaves as non specific and also acts on the 2-MAG, or acyl-migration occurs and 2-MAG are transformed to 1(3)-MAG and the lipase, since it is 1,3 specific lipase, acts on the latter to form glycerol. In addition to the main effect of time, it had also an interaction effect with another factors, enzyme load and substrate mol ratio. In Figure 4.6a,b, it is seen that while reaction reaches maximum in six hour at lower substrate mol ratios and enzyme loads, at higher substrate mol ratios and enzyme loads reaction reaches maximum at the end of the seventh hour. The terms $Ti*Sb$ and $Ti*En$ were statistically significant as it was seen in Table 4.4.

4.5 Optimization

In this study, it was aimed to produce 2-monoacylglycerols from olive pomace oil. For this purpose, the optimum conditions for the lipase-catalyzed alcoholysis reaction of olive pomace oil were predicted by using Design Expert Software 7.0. The reaction conditions were optimized for the maximal 2-MAG production. Enzyme load was kept at 15% due to the cost considerations in industrial point. The recommended optimal conditions were enzyme load 15%, time 6 h, temperature 35 °C and substrate mol ratio 65:1 with a desirability value of 0.98. Under the optimum conditions, the 2-MAG yield was 87.8. The value of the optimum reaction yield predicted by the model (87.8 %) was close to its actual value obtained through experimentation (83.5 %).

4.6 Recovery and Purification of 2-MAGs from ethanolysis reaction

At the end of alcoholysis reaction, reaction mixture includes 2-MAGs, ethyl esters (EEs), free fatty acids (FFAs), diacylglycerols (DAGs), non-reacting TAGs and ethanol. Reaction products were analyzed by the thin-layer chromatography-flame ionization detection system (Figure 4.7). The 2-MAGs must be purified from the reaction mixture. The recovery of 2-MAGs was performed by using two methods: solvent extraction and low temperature crystallization.

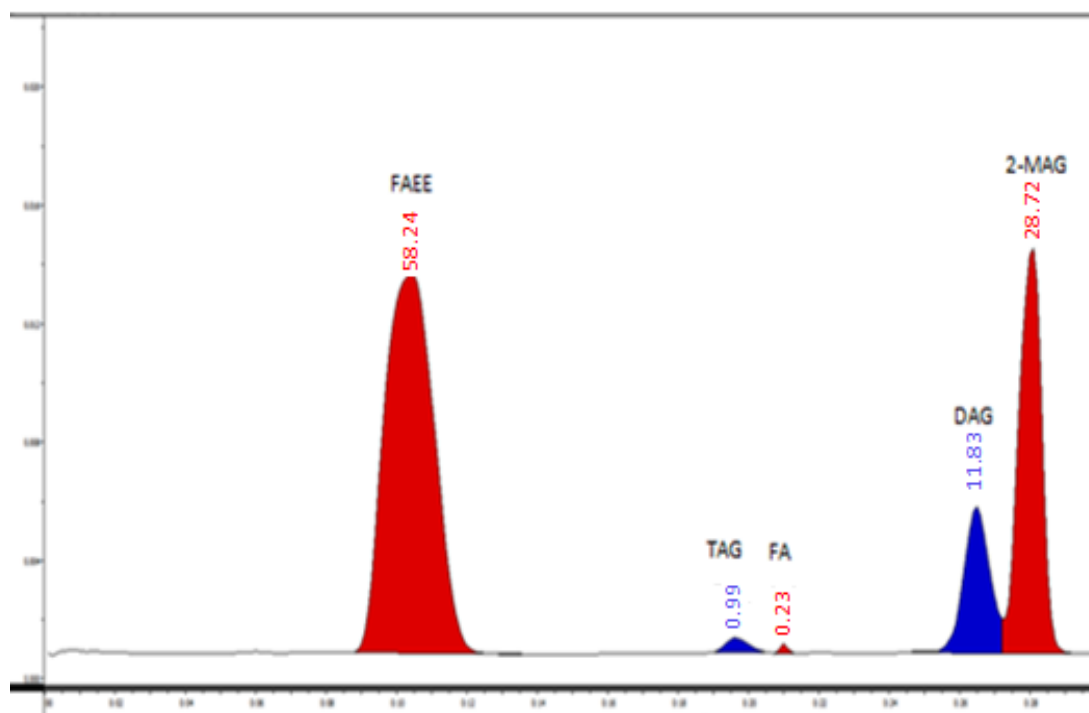


Figure 4.7 TLC-FID output of ethanolysis reaction products (2-MAG: 2-monoacylglycerols; FA: fatty acids; DAG: diacylglycerols; TAG: triacylglycerols and FAEE: fatty acid ethyl esters)

4.6.1 Solvent Extraction

Recovery of 2-MAGs by using solvent extraction was performed according to the experimental procedure described in section 3.3.4.1 (Figure 3.1). This method is based on the formation of biphasic system ethanol-water 90:10 (vol/vol) and hexane. MAGs remain in the hydroethanolic phase while esters and TAGs are extracted by the hexanic phase.

Lipid profile of initial reaction mixture, 2-MAG fraction and hexanic phase after extraction is shown in Table 4.5. After solvent extraction 2-MAG was obtained at 97% purity. Also MAG recovery yield was 87.7%. These results were similar to one obtained by Rodríguez et al. (2012). They obtained 2-MAG at 96.4% purity with the 83.9% recovery yield from the alcoholysis reaction mixture by using solvent extraction. Also ethanolysis reaction of fish oil to produce 2-monoacylglycerols had studied by Muñío et al. (2008). They reported that after solvent extraction of reaction products, 2-MAG was obtained at 89% purity with the 77% recovery yield.

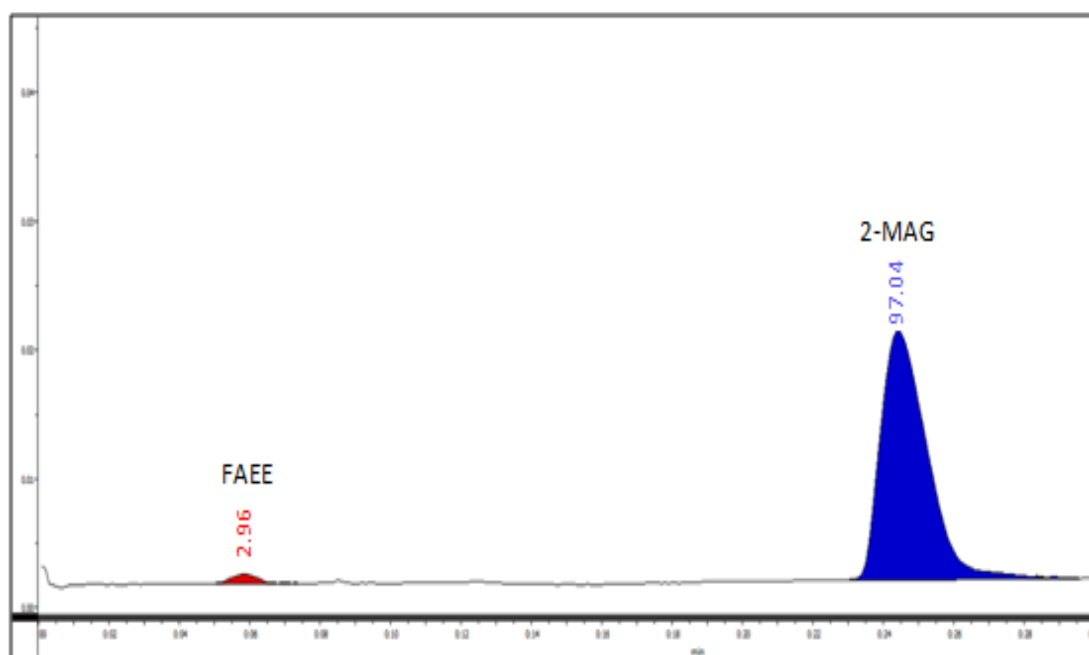


Figure 4.8 TLC-FID outputs of reaction products after solvent extraction (2-MAG: 2-monoacylglycerols and FAEE: fatty acid ethyl esters)

Table 4.5 Recovery of 2-MAG by solvent extraction

	Lipid profile (%)			
	FAEE + TAG	FFA	DAG	2-MAG
Initial mixture	76.59	-	-	23.41
2-MAG fraction after extraction	2.96	-	-	97.04
Hexanic phase after extraction	92.38	-	-	7.62

2-MAG: 2-monoacylglycerols; FFA: free fatty acids; DAG: diacylglycerols; TAG: triacylglycerols and FAEE: fatty acid ethyl esters

4.6.2 Low Temperature Crystallization

Purification of 2-MAGs by using low temperature crystallization was performed following the experimental procedure described in section 3.3.4.2. Low temperature crystallization is used successfully for purification of 2-MAGs after alcoholysis or hydrolysis of TAGs, when saturated or slightly unsaturated FAs are linked to the 2-position in MAGs (Cervera et al., 2013). The process of low temperature

crystallization includes a partial crystallization, followed by separation of the solids from the liquid portion.

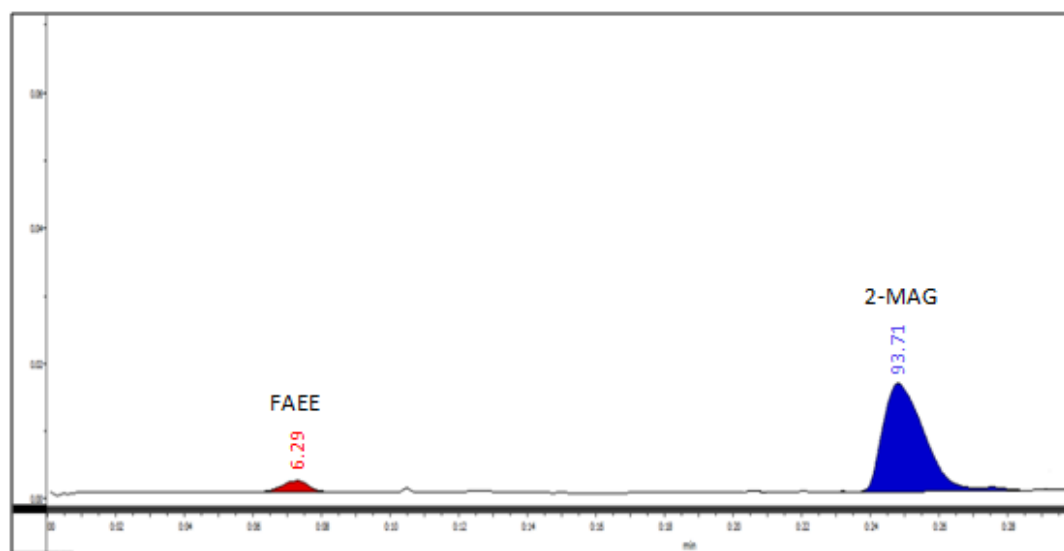


Figure 4.9 TLC-FID outputs of reaction products after low temperature crystallization (2-MAG: 2-monoacylglycerols and FAEE: fatty acid ethyl esters)

Lipid profile of initial reaction mixture, 2-MAG fraction and upper phase after low temperature crystallization is shown in Table 4.6. After crystallization 2-MAG was obtained at 93.7% purity with the 69.2% recovery yield. These results were similar to one obtained by Soumanou et al. (1998). They studied ethanolysis reaction of triolein and obtained 2-monoolein at high purity with the 71.8% recovery yield from the reaction products by low temperature crystallization.

Table 4.6 Recovery of 2-MAG by Low Temperature Crystallization

	Lipid profile (%)			
	FAEE + TAG	FFA	DAG	2-MAG
Initial mixture	75.15	-	-	24.85
2-MAG fraction after crystallization	6.29	-	-	93.71
Upper phase after crystallization	82.01	-	-	17.99

2-MAG: 2-monoacylglycerols; FFA: free fatty acids; DAG: diacylglycerols; TAG: triacylglycerols and FAEE: fatty acid ethyl esters

CONCLUSIONS

In this study, it was aimed to perform enzyme catalyzed alcoholysis reaction of olive pomace oil with ethanol to synthesize 2-monoacylglycerol. Immobilized lipase from *Candida antarctica* (Novozym 435) was used as an enzyme due to its very efficient catalytic activity in the ethanolysis reaction of the triglycerides to produce 2-MAG.

A five level, four factorial central composite rotatable design consist of 30 experiments with 6 centre point, 8 star point and 16 axial points was employed. The reaction temperature, reaction time, enzyme load and substrate mol ratio were selected as the main parameters. The evaluation of the effects of experimental factors on the response (2-MAG) was done by using response surface models that provide reducing the number of experimental trials, reducing the time and cost of the study. The best fitting quadratic model was determined by regression and backward elimination. This model was used to evaluate the effects of the main parameters and their interactions. The experimental results were in good agreement with the predicted mathematical model.

Optimization of ethanolysis reaction of olive pomace oil was successfully performed by response surface methodology for the maximal 2-MAG production. The 2-MAG can be obtained with a 87.8% yield at 15% enzyme load, 35 °C reaction temperature, 6 h reaction time and 65:1 (ethanol:oil) substrate mol ratio.

After the alcoholysis reaction, purification of 2-MAGs was performed by using two methods: solvent extraction and low temperature crystallization. Solvent extraction provided 97% purity and 87.7% recovery yield, while these values were 93.7% and 69.2%, respectively, after low temperature crystallization. The solvent extraction method provides higher purity and yield when compared to low temperature crystallization method. However, the amount of solvent used in low temperature crystallization is smaller than that used in solvent extraction process.

The results of this study showed that it is possible to produce 2-MAGs at high purities, predominantly containing oleic acid. Also the results of the study suggested

that the product produced in this study has the potential to add value to a by-product of olive oil industry and enzymatically produced 2-MAGs can be useful in the different industrial applications.

REFERENCES

- Adamczak, M. (2003). Synthesis, properties, and application of lipase from *Candida Antarctica* for high yield monoacylglycerol biosynthesis. Polish Journal of Food and Nutrition Sciences. **12/53(4)**, 3-8.
- Adamczak, M. and Bornscheuer, U. T. (2013). Application of medium engineering in the synthesis of structured triacylglycerols from evening primrose oil (*Oenothera biennis* L.). European Journal of Lipid Science and Technology. **115**, 405-412.
- Adlercreutz, P. (1994). Enzyme-catalysed lipid modification. Biotechnology and Genetic Engineering Reviews. **12(1)**, 231-254.
- Adlercreutz, P. (2013). Immobilisation and application of lipases in organic media. Chemical Society Reviews. **42**, 6406-6436.
- Akoh, C. C. and Min, D. B. (2002). Food lipids: Chemistry, nutrition, and biotechnology. C. C. Akoh (Ed.) New York: Marcel Dekker.
- American Oil Chemists' Society. AOCS Official Method Ce 5b-89. (1993). Official methods and recommended practices of the American Oil Chemists' Society, 4th edn., D. Firestone (Ed.), Champaigne.
- Amir, R. M., Shabbir, M. A., Khan, M. R. and Hussain, S. (2012). Interesterification of fats and oils- A Review. Pakistan Journal of Food Sciences. **22(3)**, 143-153.
- Aravindan, R., Anbumathi, P. and Viruthagiri, T. (2007). Lipase applications in food industry. Indian Journal of Biotechnology. **6**, 141-158.
- Arifin, N., Peng, K. S., Long, K., Ping, T. C., Yusoff, M. S. A. and Ming, L. O. (2012). Modelling and optimization of lipozyme RM IM-catalyzed esterification of medium- and long-chain triacylglycerols (MLCT) using response surface methodology. Food Bioprocess Technology. **5**, 216-225.
- Barros, M., Fleuri, L. F. and Macedo, G. A. (2010). Seed lipases: sources, applications and properties – A review. Brazilian Journal of Chemical Engineering. **27**, 15-29.
- Bektaş, I., Yucel, S., Ustun, G. and Aksoy, H. A. (2008). Production of reduced calorie structured lipid by acidolysis of tripalmitin with capric acid: optimisation by response surface methodology. Journal of the Science of Food and Agriculture. **88**, 1927-1931.
- Bergsson, G., Arnfinnsson, J., Karlsson, S. M., Steingrímsson, Ó. and Thormar, H. (1998). In vitro inactivation of *Chlamydia trachomatis* by fatty acids and monoglycerides. Antimicrobial Agents and Chemotherapy. **42**, 2290-2294.

- Bergsson, G., Steingrímsson, Ó. and Thoramr, H. (2002). Bactericidal effects of fatty acids and monoglycerides on *Helicobacter pylori*. *International Journal of Antimicrobial Agents*. **20**, 258 - 262.
- Bergsson, G., Steingrímsson, Ó. and Thormar, H. (1999). In vitro susceptibilities of *Neisseria gonorrhoeae* to fatty acids and monoglycerides. *Antimicrobial Agents and Chemotherapy*. **43**, 2790-2792.
- Bezerra, M. A., Santelli, R. E., Oliveira, E. P., Villar, L. S. and Escaleira, L. A. (2008). Response surface methodology (RSM) as a tool for optimization in analytical chemistry. *Talanta*. **76**, 965–977.
- Bornscheuer, U. T. (1995). Lipase-catalyzed syntheses of monoacylglycerols. *Enzyme and Microbial Technology*. **17**, 578-586.
- Boskou, D. (1996). *Olive Oil: chemistry and technology*. Champaign, Illinois: American Oil Chemists' Society (AOCS) Press.
- Boyle, E. (1997). Monoglycerides in food systems: current and future uses. *Food Technology*. **51**, 52-59.
- Brockerhoff, H. (1965). A stereospecific analysis of triglycerides. *Journal of Lipid Research*. **6**, 10-15.
- Can, A. and Özçelik, B. (2005). Enrichment of hazelnut oil with long-chain n-3 PUFA by lipase-catalyzed acidolysis: Optimization by response surface methodology. *Journal of American Oil Chemists' Society*. **82(1)**, 27-32.
- Carola, C. (1975). *Olive Oil Technology, By products*, Moreno Martinez, J. M. (Ed.). Rome: FAO.
- Cervera, M. Á. R., Venegas, E. V., Bueno, R. R., García, I. R. and Guil-Guerrero, J. L. (2013). Acyl migration evaluation in monoacylglycerols from *Echium plantagineum* seed oil and marinol. *Journal of Bioscience and Bioengineering*. **115(5)**, 518-522.
- Chang, C. M. and Bodmeier, R. (1997). Binding of drugs to monoglyceride-based drug delivery systems. *International Journal of Pharmaceutics*. **147**, 135-142.
- Cheong, L. Z., Tan, C. P., Long, K., Yusoff, M. S. A., Arifin, N., Lo, S. K. and Lai, O. M. (2007). Production of a diacylglycerol-enriched palm olein using lipase-catalyzed partial hydrolysis: Optimization using response surface methodology. *Food Chemistry*. **105(4)**, 1614-1622.
- Cho, S. W. and Rhee, J. S. (1993). Immobilization of lipase for effective interesterification of fats and oils in organic solvent. *Biotechnology and Bioengineering*. **41(2)**, 204-210.
- Çifçi, O. N. and Fadiloğlu, S. (2010). *Enzymes in food processing: fundamentals and potential applications*. Panesar, P. S., Marwaha, S. S. and Chopra, H. K. (Eds.) India: International Publishing House.

Çiftçi O. N., Fadiloğlu S. and Göğüş F. (2009). Conversion of olive pomace oil to cocoa butter-like fat in a packed-bed enzyme reactor. *Bioresource Technology*. **100**, 324-329.

Damstrup, M.L., Jensen, T., Sparsø, F.V., Kiil, S.Z., Jensen, A.D. and Xu, X. (2005). Solvent optimization for efficient enzymatic monoacylglycerol production based on a glycerolysis reaction. *Journal of the American Oil Chemists' Society*. **82**, 559-564.

Domingos, A.K., Saad, E.B., Wilhelm, H.M. and Ramos, L.P. (2008). Optimization of the ethanolysis of *Raphanus sativus* crude oil applying the response surface methodology. *Bioresource Technology*. **6**, 1837-1845.

Fadiloğlu, S., Çiftçi, O. N. and Göğüş, F. (2003). Reduction of free fatty acid content of olive pomace oil by enzymatic glycerolysis. *Food Science and Technology International*. **9 (1)**, 11-15

Fathabad, E. G. (2011). Biosurfactants in pharmaceutical industry: A Mini-Review. *American Journal of Drug Discovery and Development*. **1 (1)**, 58-69.

Feltes, M. M. C., Oliveira, D., Block, J. M. and Ninow, J. L. (2013). The production, benefits, and applications of monoacylglycerols and diacylglycerols of nutritional interest. *Food Bioprocess Technology*. **6**, 17-35.

Ferreira-Dias, S., Sandoval, G., Plou, F. and Valero, F. (2013). *Electronic Journal of Biotechnology*. **16(3)**, 1-28.

Firestone, D. (2005). Olive oil. *Bailey's industrial oil and fat products, Volume 2: Edible oil and fat products: Edible oils*, 6th Edition, Shadidi, F. (Ed.), USA: John Wiley and Sons, Inc.

Fomuso, L. B. and Akoh, C. C. (1997). Enzymatic modification of triolein: Incorporation of caproic and butyric acids to produce reduced-calorie structured lipids. *Journal of the American Oil Chemists' Society*. **74(3)**, 269-272.

Fregolente, L. V., Batistella, C. B., Filho, R. M. and Maciel M. R. W. (2006). Optimization of distilled monoglycerides production. *Applied Biochemistry and Biotechnology*. **129-132**, 680-693.

Gandhi, N. N. (1997). Applications of lipase. *Journal of American Oil Chemists' Society*. **74 (6)**, 621-634.

Ganem-Quintanar, A., Quintanar- Guerrero, D. and Buri, P. (2000). Monoolein: A review of the pharmaceutical applications. *Drug Development and Industrial Pharmacy*. **26(8)**, 809-820.

Gecol, H. (2006). The basic theory, chemistry and technology of surfactants. Farn, R. J. (Ed.) UK: Blackwell Publishing.

- Ghattas, N., Abidi, F., Galai, S., Marzouki, M. N. and Salah, A. B. (2014). Monoolein production by triglycerides hydrolysis using immobilized *Rhizopus oryzae* lipase. *International Journal of Biological Macromolecules*. **68**, 1-6.
- Ghosh, P. K., Saxena, R. K., Gupta, R., Yadav, R. P. and Davidson, S. (1996). Microbial lipases: production and applications. *Science Progress*. **79** (2), 119-157.
- Giese, J. (1996). Fats, oils, and fat replacers. *Food Technology*. **50**(4), 78-84.
- Göğüş, F., Fadiloğlu, S. and Çiftçi, O. N. (2004). Change in glyceride composition of olive pomace oil during enzymatic esterification. *Nahrung/Food*. **48**(3), 205-208.
- Göğüş, F., Özkaya, M. T. and Ötleş, S. (2009). *Zeytinyağı*. Ankara: Eflatun Yayın Evi.
- Gomes, T. and Caponio, F. (2000). Possibility of improving the quality characteristics of olive-pomace oil and enhancing its differentiation from refined olive-pomace oil. *Journal of the Science of food and Agriculture*. **81**, 62-67.
- Gunstone, F. D. (1999). Enzymes as biocatalysts in the modification of natural lipids. *Journal of the Science of Food and Agriculture*. **79**, 1535-1549.
- Gupta, R., Gupta, N. and Rath, P. (2004). Bacterial lipases: an overview of production, purification and biochemical properties. *Applied Microbiology and Biotechnology*. **64**, 763-781.
- Hasan, F., Shah, A. A. and Hameed, A. (2006). Industrial applications of microbial lipases. *Enzyme and Microbial Technology*. **39**, 235-251.
- Hasenhuettl, G.L. and Hartel R.W. (2008). *Food emulsifiers and their applications*. 2nd edition. New York: Springer Science + Business Media.
- Hou, C. T. And Shimada, Y. (2009). Lipases, *Encyclopedia of Microbiology*, Volume 1, 3rd edition, Schaechter, M. (Ed.). Oxford : Academic Press.
- Houde, A., Kademi, A. and Leblanc, D. (2004). Lipases and their industrial applications. *Applied Biochemistry and Biotechnology*. **118**, 155-170.
- Hübscher, G. (1970). Lipid metabolism. S. J. Wakil (Ed.) London: Academic Press.
- Iqbal, J. and Hussain, M. M. (2009). Intestinal lipid absorption. *American Journal of Physiology Endocrinology and Metabolism*. **296**(6), 1183-1194.
- Irimescu R., Iwasaki Y. and Hou C. T. (2002). Study of TAG ethanolysis to 2-MAG by immobilized *Candida antarctica* lipase and synthesis of symmetrically structured TAG. *Journal of the American Oil Chemists' Society*. **79**(9), 879-883.
- Isaacs, C. E., Litov, R. E. and Thormar, H. (1995). Antimicrobial activity of lipids added to human milk, infant formula, and bovine milk. *Journal of Nutritional Biochemistry*. **6**(7), 362-366.

Jaeger, K-E., Dijkstra, B. W. and Reetz, M. T. (1999). Bacterial Biocatalysts: Molecular biology, three-dimensional structures, and biotechnological applications of lipases. *Annual Review of Microbiology*. **53**, 315-351.

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

Kanerva, L. T., Vihanto, J., Halme, M. H., Lojonen, J. M. and Euranto, E. K. (1990). Solvent effects in lipase-catalysed transesterification reactions. *Acta Chemica Scandinavica*. **44**, 1032-1035.

Karsa, D. R. (2006). What are surfactants? Chemistry and Technology of Surfactants. Farn, R. J. (Ed.) UK: Blackwell Publishing.

Kiritsakis, A. P. K., Lenart, E. B., Willet, W. C. and Hernandez, R. J. (1998). Olive oil from the tree to the table. Connecticut: Food and Nutrition Press, Inc.

Kiritsakis, K., Kiritsakis, A. P., Manousaki-Karacosta, E. and Genigeorgis, F. (2010). Table olives and olive oil: Production, processing, composition and nutritional qualities. *Handbook of Vegetables and Vegetable Processing*. Sinha, N., Hui, Y. H., Evranuz, E. Ö., Siddiq, M. and Ahmed, J. (Eds), 1st edition. USA: Blackwell Publishing.

Klekner, V. and Kosaric, N. (1993). Biosurfactants for cosmetics. Surfactant science series, volume 48, Biosurfactants: production, properties, applications, Kosaric, N. (Ed.) USA: Marcel Dekker, Inc.

Knežević, Z. D., Šiler-Marinković, S. S. and Mojović, L. V. (2004). Immobilized lipases as practical catalysts. *Acta Periodica Technologica*. **35**, 151-164.

Knezevic, Z., Milosavic, N., Bezbradica, D., Jakovljevic, Z. and Prodanovic, R. (2006). Immobilization of lipase from *Candida rugosa* on Eupergit® C supports by covalent attachment. *Biochemical Engineering Journal*. **30(3)**, 269-278.

Koh, S. P., Tan, C. P., Lai, O. M., Arifin, N., Yusoff, M. S. A. and Long, K. (2010). Enzymatic synthesis of medium- and long-chain triacylglycerols (MLCT). *Food Bioprocess Technology*. **3**, 288-299.

Köse, Ö., Tüter, M. and Aksoy, A. H. (2002). Immobilized *Candida antarctica* lipase-catalyzed alcoholysis of cotton seed oil in a solvent-free medium. *Bioresource Technology*. **83**, 125-129.

Kristmundsdóttir, T., Árnadóttir, S. G., Bergsson, G. and Thormar, H. (1999). Development and evaluation of microbicidal hydrogels containing monoglyceride as the active ingredient. *Journal of Pharmaceutical Sciences*. **88**, 1011-1015.

Krog, N. J. and Sparso, F. V. (2004). Food emulsions, food emulsifiers: their chemical and physical properties. Friberg, S. E., Larsson, K. and Sjöblom, J. (Eds.) New York: Marcel Dekker.

Kuo, T. M. and Gardner, H. W. (Eds.) (2002). Lipid Biotechnology. New York: Marcel Dekker.

Langone, M. A. P., De Abreu, M. E., Rezende, M. J. C. and Sant'anna, G. L. (2002). Enzymatic synthesis of medium chain monoglycerides in a solvent-free System. Applied Biochemistry and Biotechnology. **98-100**, 987-996.

Lee, G., Wang, D., Ho, Y. and Shaw, J. (2004). Lipase-catalyzed alcoholysis of triglycerides for short-chain monoglyceride production. Journal of the American Oil Chemists' Society. **81**, 533-536.

Lin, S. W. and Yoo, C. K. (2009). Short- path distillation of palm olein and characterization of products. European Journal of Lipid Science and Technology. **111**, 142-147.

Macrae, A. R. and Hammond, R. C. (1985). Present and future applications of lipases. Biotechnology and Genetic Engineering Reviews. **3:1**, 193-218.

Maneerat, S. (2005). Production of biosurfactants using substrates from renewable-resources. Songklanakarin J. Sci. Technol. **27(3)**, 675-683.

Marangoni, A. G. (2002). Lipid Biotechnology. Kuo, T. M. and Gardner, H. W. (Eds.) USA: Marcel Dekker, Inc.

McNeill, G. P. and Yamane, T. (1991). Further improvements in the yield of monoglycerides during enzymatic glycerolysis of fats and oils. Journal of the American Oil Chemists' Society. **68(1)**, 6-10.

McNeill, G. P., Shimizu, S. and Yamane, T. (1990). Solid phase enzymatic glycerolysis of beef tallow resulting in a high yield of monoglyceride. Journal of the American Oil Chemists' Society. **67(11)**, 779-783.

Mendes, A. A., Freitas, L., F. De Carvalho, A. K., de Oliveira P. C. and de Castro, H. F. (2011). Immobilization of a commercial lipase from *Penicillium camembertii* (Lipase G) by different strategies. Enzyme Research. **2011**, 1-8.

Meziane, S. (2011). Drying kinetics of olive pomace in a fluidized bed dryer. Energy Conversion and Management. **52(3)**, 1644-1649.

Meziane, S. (2013). Optimization of oil extraction from olive pomace using response surface methodology. Food Science and Technology International. **19(4)**, 315-322.

Mishra, M., Muthuprasanna, P., Prabha, K. S., Rani, P. S., Babu, I. A. S., Chandiran, I. S., Arunachalam, G. and Shalini, S. (2009). Basics and potential application of surfactants- A review. International Journal of Pharm Tech Research. **1(4)**, 1354-1365.

Montoya, J., Benjumea, P., and Pashova, V. (2011). Optimization of the basic ethanolysis of ricin oil using the response surface methodology. Dyna. **78 (168)**, 90-97.

Moonen, H. and Bas, H. (2004). Emulsifiers in food technology, Mono- and diglycerides. Whitehurst, R. J. (Ed.) Oxford: Blackwell.

Mouloungui, Z., Rakotondrazafy, V., Peyrou, G., Gachen, C. and Eychenne, V. (1998). Pure α -monoglycerides for industrial applications. *Agro Food Industry Hi-Tech*. **9**, 10-14.

Muño, M. M., Esteban, L., Robles, A., Hita, E., Jiménez, M., González, P. A., Camacho, B. and Molina, E. (2008). Synthesis of 2-monoacylglycerols rich in polyunsaturated fatty acids by ethanolysis of fish oil catalyzed by 1,3 specific lipases. *Process Biochemistry*. **43**, 1033-1039.

Myers, D. (2006). *Surfactant Science and Technology*. 3rd edition. USA: John Wiley & Sons, Inc., Publication.

Myers, R. H. and Montgomery, D. C. (2002). *Response surface methodology: process and product optimization using designed experiments*. 2nd edition. New York: Wiley.

Niaounakis, M. and Halvadakis, C. P. (2006). *Characterization of olive processing waste, olive processing waste management, literature review and patent survey*. 2nd edition. Italy: Elsevier.

Nichols, D. S. and Sanderson, K. (2003). *The nomenclature, structure, and properties of food lipids, chemical and functional properties of food lipids*. Sikorski, Z. E. and Kolakowska, A. (Eds.) USA: CRC Press.

Orthoefer, F. (2008). *Food emulsifiers and their applications, Applications of emulsifiers in baked foods*. Hasenhuettl, G. L. and Hartel, R. W. (Eds), 2nd edition. New York: Springer Science+Business Media.

Pagliaro, M. and Rossi, M. (2008). *The future of glycerol*. 2nd Edition. Cambridge: RSC Publishing.

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

Petschow, B. W., Batema, R. P. and Ford, L. L. (1996). Susceptibility of *helicobacter pylori* to bactericidal properties of medium-chain monoglycerides and free fatty acids. *Antimicrobial Agents and Chemotherapy*. **40**, 302-306.

Pinyaphong, P., Sriburi, P. and Phutrakul, S. (2012). Synthesis of monoacylglycerol from glycerolysis of crude glycerol with coconut oil catalyzed by carica papaya lipase. *World Academy of Science, Engineering and Technology*. **(6)**, 487-492.

Powers, J. R. (2003). *Food analysis, application of enzymes in food analysis*. Nielsen, S. S. (Ed.), 3rd edition. USA: Springer.

Preffer, J., Freund, A., Bel-Rhliid, R., Hansen, C. E., Reuss, M., Schmid, R. D. and Maurer, S. C. (2007). Highly efficient enzymatic synthesis of 2-Monoacylglycerides

and structured lipids and their production on a technical scale. *Lipids*. **42(10)**, 947-953.

Prietro-Blanco, M. C., Lopez-Mahia, P., Muniategui-Lorenzo, S. and Rodriguez, D. P. (2007). *Surfactants in cosmetics, analytical methods, analysis of cosmetic products*. Salvador, A. and Chisvert, A. (Eds), 1st edition, Italy: Elsevier.

Qin, H. And Ruan, Z. (2014). The role of monoacylglycerol lipase (MAGL) in the cancer progress. *Cell Biochemistry and Biophysics*. **70(1)**, 33-36.

Ravi, R., Manohar, R. S. and Rao, P. H. (2000). Influence of additives on the reological characteristics and baking quality of wheat flours. *European Food Research and Technology*. **210**, 202-208.

Ray, A. (2012). Application of lipase in industry. *Asian Journal of Pharmacy and Technology*. **2(2)**, 33-37.

Ribeiro, B. D., de Castro, A. M., Coelho, M. A. Z. and Freire, D. M. G. (2011). Production and use of lipases in bioenergy: A review from the feedstocks to biodiesel production. *Enzyme Research*. **2011**, 1-16.

Rodrigues, R. C., Volpato, G., Ayub, M. A. Z. and Wada, K. (2008). Lipase-catalyzed ethanolysis of soybean oil in a solvent free system using central composite design and response surface methodology. *Journal of Chemical Technology and Biotechnology*. **83**, 849-854.

Rodriguez A., Esteban L., Martin L., Jimenez M. J., Hita E., Castillo B., Gonzalez P. A. and Robles A. (2012). Synthesis of 2-monoacylglycerols and structured triacylglycerols rich in polyunsaturated fatty acids by enzyme catalyzed Reactions. *Enzyme and Microbial Technology*. **51**, 148-155.

Rosen, M. J. and Kunjappu, J. T. (2012). *Surfactants and interfacial phenomena*. 4th Edition. US: John Willey & Sons, Inc.

Sagalowicz, L., Leser, M. E., Watzke, H. J. and Michel, M. (2006). Monoglyceride self-assembly structures as delivery vehicles. *Trends in Food Science & Technology*. **17(5)**, 204-214.

Schramm, L. L. (2000). *Surfactants: Fundamentals and applications in the petroleum industry*. 1st edition. Cambridge: Cambridge University Press.

Schreier, S., Malheiros, S. V. P. and de Paula, E. (2000). Surface active drugs: self-association and interaction with membranes and surfactants, physicochemical and biological aspects. *Biochimica et Biophysica Acta*. **1508**, 210-234.

Schröder, U., Svenson, S. B. (1999). Nasal and parenteral immunizations with diphtheria toxoid using monoglyceride/fatty acid lipid suspensions as adjuvants. *Vaccine*. **17**, 2096 - 2103.

Schuchardt, U., Sercheli, R. and Vargas, R. M. (1998). Transesterification of vegetable oils: a review. *Journal of the Brazilian Chemical Society*. **9(1)**, 199-210.

Senanayake, N. and Shahidi, F. (2005). Modification of fats and oils via chemical and enzymatic methods. Bailey's Industrial Oil and Fat Products. F. Shahidi (Ed.) Hoboken: John Wiley & Sons, Inc.

Shahani, K.M. (1975). Enzymes in food processing, lipases and esterases. G. Reed (Ed.) New York: Academic Press.

Shamel, M. M., Ramachandran, K. B., Hasan, M. and Al-Zuhair, S. (2007). Hydrolysis of palm and olive oils by immobilised lipase using hallow fibre reactor. Biochemical Engineering Journal. **34**, 228-235.

Sharma, R., Chisti, Y. and Banerjee, U. C. (2001). Production, purification, characterization, and applications of lipases. Biotechnology Advances. **19**, 627-662.

Shimada, Y., Ogawa, J., Watanabe, Y., Toshihiro, N., Kawashima, A., Kobayashi, T. and Shimizu, S. (2003). Regiospecific analysis by ethanolysis of oil with immobilized candida antarctica lipase. Lipids. **38(12)**, 1281-1286.

Silva, J. A., Macedo, G. P., Rodrigues, D. S., Giordano, R. L. C. and Gonçalves, L. R. B. (2012). Immobilization of *Candida antarctica* lipase B by covalent attachment on chitosan-based hydrogels using different support activation strategies. Biochemical Engineering Journal. **60**, 16-24.

Singh, A., Van Hamme, J. D., and Ward, O. P. (2007). Surfactants in microbiology and biotechnology: Part 2. Application aspects, Biotechnology Advances. **25**, 99-121.

Song, X., Qi, X., Hao, B. and Qu, Y. (2008). Studies of substrate specificities of lipases from different sources. The European Journal of Lipid Science and Technology. **110**, 1095-1101.

Szymański, A. (2008). Determination of sulfonamide residues in food by micellar liquid chromatography. Toxicology Mechanisms and Methods. **18(6)**, 473-481.

Tadros, T. F. (1995). Surfactants in agrochemicals, surfactant science series, volume 54. USA: Marcel Dekker, Inc.

Tadros, T. F. (2005). Applied Surfactants, principles and applications. Weinheim: WILEY-VCH Verlag GmbH & Co. KGaA,.

The American Oil Chemists' Society Lipid Library, Monoacylglycerols (Including 2-Arachidonoylglycerol), Structure, composition, function and analysis. <http://lipidlibrary.aocs.org/Lipids/mg/index.htm>, 05.08.2014.

Thorgerisdóttir, T. O., Thormar, H. and Kristmundsdóttir, J. (2005). The influence of formulation variables on stability and microbicidal activity of monoglyceride monocaprin. Journal of Drug Delivery Science and Technology. **15**, 233 - 236.

Valério, A., Rovani, S., Treichel, H., Oliveira, D. and Oliveira J. V. (2010). Optimization of mono and diacylglycerols production from enzymatic glycerolysis in solvent-free systems. Bioprocess and Biosystems Engineering. **33**, 805-812.

Villeneuve, P., Muderhwa, J. M., Graille, J. and Haas, M. (2000). Customizing lipases for biocatalysis: a survey of chemical, physical and molecular biological approaches. *Journal of Molecular Catalysis B: Enzymatic*. **9**, 113-148.

Wanasundara, U. N. and Shahidi, F. (1998). Concentration of ω -3 polyunsaturated fatty acids of marine oils using *Candida cylindracea* lipase: Optimization of reaction conditions. *Journal of American Oil Chemists' Society*. **75 (12)**, 1767-1774.

Willis, M. W. and Marangoni, A. G. (2002). Food lipids chemistry, nutrition, and biotechnology, Enzymatic interesterification. C. C. Akoh and D. B. Min (Eds.) New York: Marcel Dekker.

Xu, X. (2000). Production of specific-structured triacylglycerols by lipase-catalyzed reactions: a review. *European Journal of Lipid Science and Technology*. **102**, 287-303.

Xu, X., Guo, Z., Zhang, H., Vikbjerg, A. F. and Damstrup, M. L. (2006). Modifying lipids for use in food. Gunstone, F. D. (Ed.) USA: Blackwell CRC Press.

Yang, T., Rebsdorf, M., Engelrud, U. and Xu, X. (2005). Monoacylglycerol synthesis via enzymatic glycerolysis using a simple and efficient reaction system. *Journal of Food Lipids*. **12**, 299–312.

Yangxin, Y., Jin, Z. and Bayly, A. E. (2008). Development of surfactants and builders in detergent formulations. *Chinese Journal of Chemical Engineering*. **16(4)**, 517-527.

Zambiasi, R. C., Przybylski, R., Zambiasi, M. W. and Mendonça, C. B. (2007). Fatty acid composition of vegetable oils and fats. *B.CEPPA, Curitiba*. **25(1)**, 111-120.

Zhong, N., Cheong, L. and Xu, X. (2014). Strategies to obtain high content of monoacylglycerols. *European Journal of Lipid Science and Technology*. **116**, 97-107.

Zhong, N., Li, L., Xu, X., Cheong, L., Li, B., Hu, S. and Zhao, X. (2009). An efficient binary solvent mixture for monoacylglycerol synthesis by enzymatic glycerolysis. *Journal of the American Oil Chemists' Society*. **86**, 783–789.

Zocchi, G. (2009). Handbook of cosmetic science and technology, skin feel agents. Barel, A. O., Paye, M. and Maibach, H. I. (Eds) 4rd Edition. New York: CRC Press.