ENZYMATIC ESTERIFICATION OF OLIVE-POMACE OIL

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To My Mother and Father

ABSTRACT

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In this study, enzymatic esterification of free fatty acids of olive-pomace oil with glycerol was carried out in microaqueous conditions by using immobilized *Candida antarctica* lipase. The effects of reaction time, molecular sieve, enzyme concentration and reaction temperature on free fatty acid content, and the effect of glycerol amount on the change in glyceride composition of olive-pomace oil were investigated. The initial acidity of the olive-pomace oil (32 %) reduced to 2.36 % in the presence of 750 mg of molecular sieve in the reaction mixture. It was observed that the effectiveness of esterification reactions was related to the amount of molecular sieve present. As the amount of molecular sieve increased, the conversion of free fatty acids also increased at a defined time. In the absence of molecular sieve, the esterification reaction forced to reverse reaction that was the hydrolysis. The greater conversion of free fatty acids into glycerides was observed at an enzyme concentration of 27.2 mg/mL within 60 min. FFA content decreased sharply with increasing reaction time at all temperatures, and it was sharper at higher temperatures. FFA content reached into the equilibrium in almost 120 min at 60°C. However, the time to reach into the equilibrium was around 300 min at 40 and 50°C. ANOVA showed that the effects of temperature on free fatty acid content were significant ($p<0.05$). Results obtained from non-linear regression analysis indicated that reaction order was 1.3 for free fatty acid reduction in the olive-pomace oil. Calculated activation energy for free fatty acid reduction was 32.89 kJ mol^{-1}. In order to investigate the effect of glycerol content, esterification reactions were carried out in the absence and in the presence (glycerol to FFA molar ratio of 1/3 and

1/6) of glycerol. In the absence of glycerol, FFA concentration decreased from 32 to 21 % while triacylglycerol concentration increased from 33 to 40 % by the end of 8 hours of reaction time. The most significant decrease in FFA and increase in triacylglycerols was observed at the limiting concentration of glycerol (glycerol to FFA molar ratio of 1/3). The free fatty acid concentration decreased to a value of 2.5 % and triacylglycerol concentration increased up to 78 %. The change in both FFA and triacylglycerol concentrations was found to be significant ($p<0.05$) statistically.

Keywords: Enzymatic esterification, lipase, olive-pomace oil.

ÖZ

PİRİNA YAĞININ ENZİMATİK ESTERİFİKASYONU

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Bu çalışmada pirina yağındaki serbest yağ asitlerinin gliserolle enzimatik esterifikasyonu mikro miktarda suyun varlığında, tutuklanmış *Candida antarctica* lipaz enzimi kullanılarak gerçekleştirildi. Reaksiyon zamanı, moleküler elek, enzim konsantrasyonu ve sıcaklığın serbest yağ asidi miktarına ve gliserol miktarının pirina yağının gliserit kompozisyonu değişimine etkisi araştırıldı. Reaksiyon karışımında 750 mg moleküler elek varlığında pirina yağının asitliği (% 32), % 2.36' ya düştü. Esterifikasyon reaksiyonlarının etkinliğinin ortamdaki moleküler elek miktarına bağlı olduğu gözlendi. Moleküler elek miktarı arttıkça serbest yağ asitlerinin belirlenen zamanda dönüşümü de arttı. Moleküler eleğin yokluğunda esterifikasyon reaksiyonu tersi reaksiyona yani hidrolize yönlendi. Serbest yağ asitlerinin gliseritlere en fazla oranda dönüşümü 60 dakikada 27.2 mg/mL enzim konsantrasyonu ile gözlendi. Serbest yağ asidi miktarı tüm sıcaklıklarda, artan reaksiyon zamanı ile hızlı bir şekilde azaldı ve bu azalma yüksek sıcaklıklarda daha hızlı idi. Serbest yağ asidi miktarı 60°C' de ve 120 dakikada dengeye ulaştı. Fakat, 40 ve 50°C' de dengeye ulaşmak için gereken süre 300 dakika civarında idi. ANOVA, sıcaklığın serbest yağ asidi miktarına etkilerinin anlamlı oluğunu gösterdi ($p<0.05$). Lineer olmayan regresyon analizinden elde edilen sonuçlar, pirina yağının serbest yağ asitiliğindeki düşmenin reaksiyon derecesinin 1.3 olduğunu gösterdi. Serbest yağ asitliğindeki düşüşün aktivasyon enerjisi 32.89 kJ mol^{-1} olarak hesaplandı. Gliserol miktarının etkisini araştırmak için, esterifikasyon reaksiyonları gliserolün yokluğunda ve varlığında (gliserolün serbest yağ asitlerine molar oranı 1/3 ve 1/6) gerçekleştirildi. Gliserolün yokluğunda, 8 saatlik reaksiyon süresi sonunda, trigliserit konsantrasyonu

% 33' den % 40' a yükselirken, serbest yağ asidi konsantrasyonu % 32'den % 21'e düştü. Serbest yağ asitliğindeki en önemli düşüş ve trigliseritlerdeki en önemli artış, sınırlayıcı miktarda gliserolün (gliserolün serbest yağ asitlerine molar oranı 1/3) varlığında gerçekleşti. Serbest yağ asitliği % 2.5' e düştü ve trigliserit konsantrasyonu % 78' e yükseldi. Serbest yağ asitliğindeki ve trigliseritlerdeki değişim istatistiksel olarak anlamlı bulundu ($p<0.05$).

Anahtar kelimeler: Enzimatik esterifikasyon, lipaz, pirina yağı.

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LIST OF ABBREVIATIONS

µ	: Micron
µL	: Microliter
µm	: Micrometer
Å	: Angstrom
AOAC	: Association of Official Analytical Chemists
CO_2	: Carbondioxide
DAG	: Diacylglycerol
g	: Gram
Gly	: Glycerol
id	: Inner diameter
IOOC	: International Olive Oil Council
kg	: Kilogram
kj	: Kilojoule
MAG	: Monoacylglycerol
mg	: Miligram
min	: Minute
mL	: Mililiter
mm	: Milimeter
nm	: Nanometer
°C	: Degrees Celcius
PLU	: Propyl laurate units
ppm	: Parts per million
rpm	: Revolution per minute
RT	: Retention time
TAG	: Triacylglycerol
v/v	: Volume/Volume
w/v	: Weight/Volume
w/w	: Weight/Weight

CHAPTER I

INTRODUCTION

Olive pomace is the pulpy material remaining after removing most of the oil from the olive paste. The commercial value of the olive pomace depends mainly on its oil and water content which in turn depends on the process applied and on the operating conditions (Kiritsakis, 1998). Olive pomace contains about 8 % oil. The recovery of oil from the olive pomace is carried out by extraction with solvent (Di Giovacchino, 1996). The raw, unrefined oil extracted from the pomace is dark green color, has a medium to high acidity. The oil present in the olive pomace undergoes rapid deterioration due to the moisture content which favors triacylglycerol hydrolysis (Kiritsakis, 1998). Carola (1975) reported that the acidity of the olive-pomace oil may increase from 5 to 60 % in a short time. Olive-pomace oils with high acidity were mostly used for the production of household soaps, before detergents appeared on the market. Refined olive-pomace oil is an acceptable edible oil. Its chemical composition does not differ from refined olive oil (Kiritsakis, 1998). In the last few years olive-pomace oil has an increasing demand to be used as edible oils for frying or similar purposes. After refining, this oil may be used for edible purposes (Ferreira-Dias *et al.*, 2001). In order to make the oil edible, it has to be deacidified, bleached and deodorized (Di Giovacchino, 1996). However, its high free fatty acid content makes it very difficult to process by neutralization. The yield of the product is also very low and it increases the cost of unit product produced. So it is necessary to reduce the free fatty acid content before refining process (Fadıloğlu *et al.*, 2003). One way of reducing the free fatty acid content is to convert the free fatty acids to glycerides (Noureddini and Medikonduru, 1997).

Ester synthesis has a good potential for industrial applications. Nonenzymatic processes for glyceride production are based either on direct esterification of glycerol with fatty acids or on the interesterification of triacylglycerols with glycerol (glycerolysis) in the presence of inorganic catalysts at high temperatures such as 200-300°C. These reactions are nonselective and consume large amounts of energy

(Ferreira-Dias and Fonseca, 1993). Lipases are known to be useful catalysts for producing a number of commercially important esters. The application of lipase for esterification reactions in organic solvents has increased significantly during the last decade (Krishna and Karanth, 2001). The main reasons are the higher yields achieved and much milder reaction conditions, resulting in products of higher quality and less energy costs (Rosu *et al.*, 1997). In fact, although lipases were designed by nature to cleave ester bonds of triacylglycerols with the concomitant consumption of water molecules (hydrolysis), lipases are also able to catalyze the reverse reaction under microqueous conditions results in the formation of ester bonds between alcohol and carboxylic acid moieties (ester synthesis) (Balcao *et al.*, 1996). The substrates of enzymatic glycerolysis are triacylglycerol and glycerol in contrast to enzymatic ester synthesis where the substrates are fatty acids and glycerol (McNeill *et al.*, 1991). The potential use of vegetable oils as a substrate for the production of added-value compounds such as monoacylglycerol (MAG) and diacylglycerol (DAG) is an attractive way to valorize the olive oil industry (Ferreira-Dias *et al.*, 2001). Enzymatic esterification has been investigated for the production of MAG and/or DAG by several authors (Yamane *et al.*, 1994; Elfman-Börjesson and Harröd, 1999).

The work reported here describes the application of enzymatic esterification of crude olive-pomace oil and glycerol. The aim of the study was to reduce the acidity of olive-pomace oil and to produce triacylglycerols by enzymatic esterification. The effect of molecular sieve, enzyme concentration and temperature on FFA reduction were investigated and the optimum conditions for a successful reaction were determined. The effects of glycerol contents on the change in FFA, MAG, DAG and TAG composition of olive-pomace oil were studied. The products were analyzed by using HPLC.

CHAPTER II

LITERATURE REVIEW

2.1. The Olive Fruit

Among the oldest known cultivated trees in the world, the olive tree has in fact been cultivated before recorded history. The sacredness with which the tree and its branches are associated indicates that the crop is almost as old as man himself. Modern historians consider the olive tree as a cultural marker and a compass to explore the development of cultures and civilizations (Boskou, 1996).

In ancient times the olive tree symbolized the friendship and peace among nations. The champions of Olympic games were crowned with a wreath of olive branches. The tree also symbolized the arrival of cultivated life, knowledge and divine enlightenment (Boskou, 1996).

The olive is a member of the botanical family *Oleaceae*, which contains 30 species such as jasmine, ash and privet. The only edible species is *Olea europaea* L., which is cultivated for its large, fleshy, oil-containing fruits (Luchetti, 2002). The fruit of the *Olea europaea* is an oval-shaped drupe consisting of a pericarp and endocarp (kernel, pit). The former has two parts: the epicarp (skin), and the mesocarp (flesh, pulp) which accounts for about 65-83 % of the total weight (Boskou, 1996). The mesocarp contains the bitter compound oleuropein, and has a high oil content (12-30 %) that varies from variety to variety and a low sugar content. The endocarp of the olive fruit is made up of tough, fibrous lignin. It is ovoid in shape and grooved to varying degrees, again depending on the variety. The endocarp encloses a kernel (olive seed) accounting for some 3 % of fruit weight and containing 2-4 % oil (Luchetti, 2002). The fruit contains water (up to 70 %) which is called "vegetation" water. The average chemical composition of the olive fruit is: water, 50 %; proteins, 1.6 %; oil, 22 %; carbohydrates, 19.1 %; cellulose, 5.8 %; minerals (ash), 1.5 % (Fedeli, 1977). Other important constituents are pectins, organic acids, pigments and glycosides of phenols (Boskou, 1996).

2.2. Importance of Olive Oil in the World Market

The three major olive-producing countries are Spain, Italy and Greece, followed by Tunisia, Syria and Turkey. Other countries with considerable production figures are Morocco, Algiers and Portugal, while France, Libya, Lebanon, Yugoslavia, Cyprus, Egypt, Israel and Jordan come far behind. Among the countries of the Western Hemisphere, Argentina has the highest level of production (Boskou, 1996). Figures 1 and 2 show the share of world countries in production and consumption of olive oil, respectively (www.r0.unctad.org).

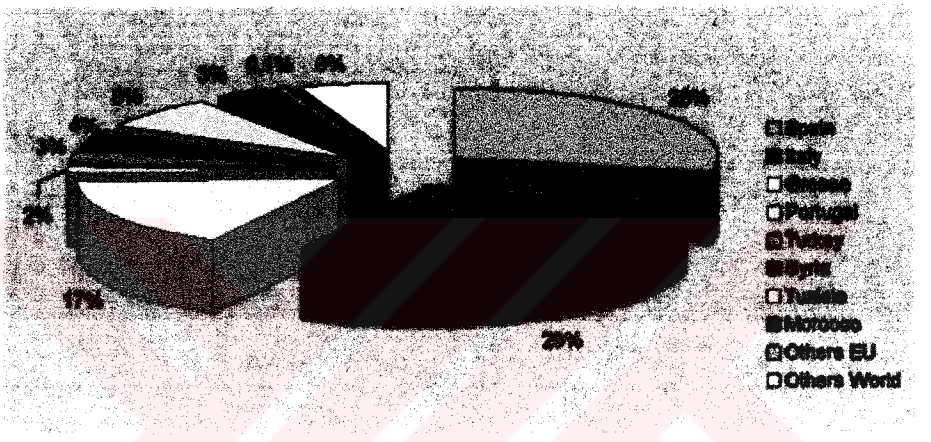

Figure 1. Olive oil producing countries, 1999.

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Olive oil is the Mediterranean region's principle source of oil. The Mediterranean diet is well-known internationally from the results of the seven countries' study in which the population of Crete was found to have the lowest coronary heart disease (CHD) mortality rate compared to other populations in the study. This low CHD mortality in Crete has been attributed to high olive oil consumption which was approximately 100 g per person per day (one-third of the total daily energy intake) (Kiritsakis, 1998).

Olive oil differs from all other vegetable oils in that can be consumed in the crude form, thus conserving its vitamins and other important natural compounds. Olive oil, a food staple and delight in the Mediterranean region for thousands of years, is becoming more popular than ever in the U.S. (Kiritsakis, 1998).

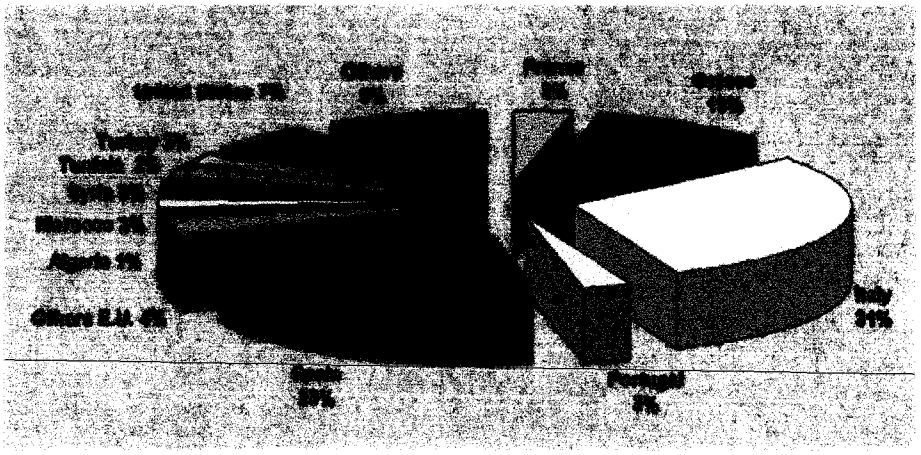

Figure 2. Olive oil consuming countries, 1999-2000.

2.3. Processing of Olive Fruit

Olive oil is present in the form of minute drops in the vacuoles of mesocarp cells in the olive fruit (Kiritsakis, 1998). It is also scattered to a lesser extent in the colloidal system of the cell's cytoplasm and to a lesser degree, in the epicarp and endosperm (IOOC, 1984). While it is possible for all of the oil in the vacuoles to be released during processing, it is hard to obtain oil dispersed in the cytoplasm. This oil generally remains in the olive pomace, a by product of olive fruit processing. The main processing steps needed to obtain olive oil include: feeding, washing, malaxation (mixing), separating the olive oil and centrifuging the oil (Kiritsakis, 1998).

2.3.1. Feeding

The fruit is put into a large feeding hopper attached to a moving belt. Removal of the leaves is necessary since they impart a bitter taste to the oil. Transfer of chlorophyll from the leaves to the oil is known to be promote olive oil deterioration in the presence of light (Kiritsakis, 1998).

2.3.2. Washing

Washing the fruit eliminates the presence of foreign material. A washing machine is a basic accessory of an olive oil mill (Kiritsakis, 1998).

2.3.3. Crushing

After washing, the olive fruit is transferred to the crushing unit. Crushing or milling is the first main step in olive fruit processing. The purpose of crushing is to tear the flesh cells to facilitate the release of the oil from the vacuoles. Thus, during crushing, microscopic drops coalesce to form larger drops of oil which can be separated from the other phases (Kiritsakis, 1998).

2.3.4. Mixing (Malaxation)

After the olive fruit has been crushed, the resulting paste is mixed. Malaxation entails stirring the olive mash slowly and constantly for about 30 minutes. The purpose of this operation is to increase the percentage of free oil. It also aids in the coalescence of small oil drops into larger ones, thereby facilitating separation of the oil and water phases It aids in breaking up the oil/water emulsion drops, as well (Kiritsakis, 1998).

2.3.5. Separation of Olive Oil From Olive Paste

The main constituents of olive paste are: olive oil, small pieces of kernel (pit), water and cellular debris of the crushed olives. Pressure, centrifugation or a selective filtration process may be applied for the separation of oil from the other constituents (Kiritsakis, 1998).

Pressure Process

The pressure process (pressure applied to separate the oily must from the solid phase) is the oldest and most widespread method for processing olive fruit to obtain olive oil. It has been used since the beginning of olive tree cultivation. The pressure process has been changed drastically over the years. Machinery used for this purpose has been improved and is more powerful and reliable. In very old mills, oil separation was achieved with pressure applied either by men or animals. The invention of hydraulic presses was a revolution for the operation of old olive oil mills and hydraulic presses are still used in improved traditional olive oil mills. Figure 3 shows the diagram of olive oil extraction by pressure process (Kiritsakis, 1998).

The olive paste obtained after crushing and malaxation is placed in oil diaphragms by a loading unit with a pierced cylinder-guide (trolley fitted with central shaft) to provide even arrangement of the diaphragms and support so as to avoid any movement when the system is subjected to hydraulic press. A metal tray and a cloth

without paste are placed after every 3 or 4 full diaphragms to obtain uniform application and a more stable load. The moving unit along with its load is placed under a hydraulic pressure unit. When applying pressure, the liquid phases (oil and water) run through the olive cake (Kiritsakis, 1998).

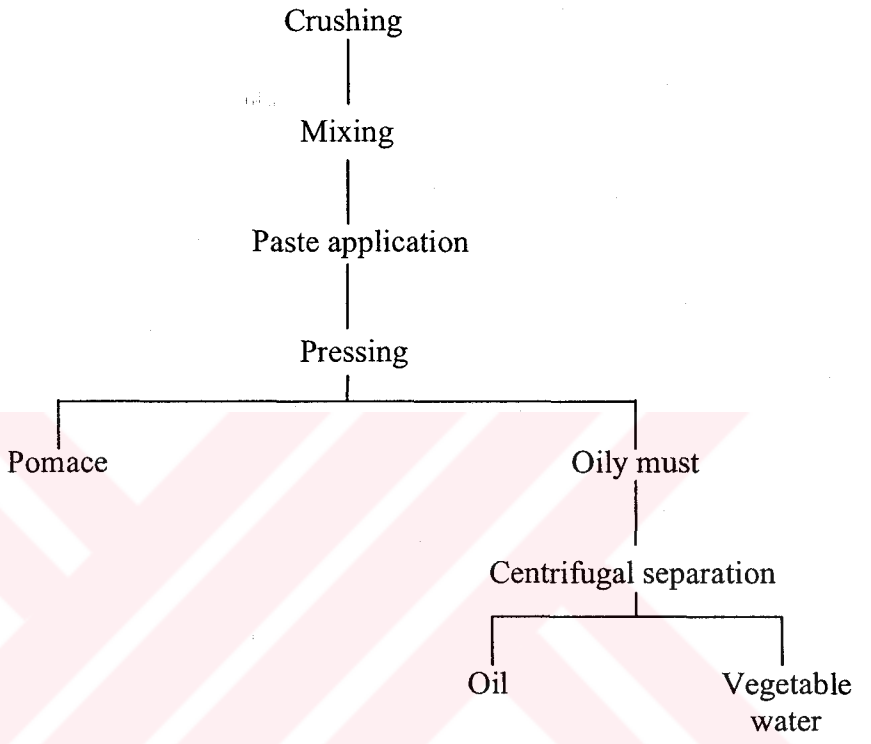

Figure 3. Diagram of olive oil extraction by pressure (single pressing)

The Centrifugation Process

Centrifugation is a relatively new process for separation of oil from olive paste. It is based on the differences in density of the olive paste constituents (olive oil, water and insoluble solids). Separation is accomplished through a horizontal centrifuge (decanter). After crushing and malaxation, the olive oil is either completely free or in the form of small droplets inside microgels, or emulsified in the aqueous phase. The separation of the oil by centrifugation is much easier when most of the oil is free. Free olive oil can be separated by applying centrifugation, while the oil locked in the microgels is released by adding water. Figure 4 shows the diagram of olive oil extraction by direct centrifugation (Kiritsakis, 1998).

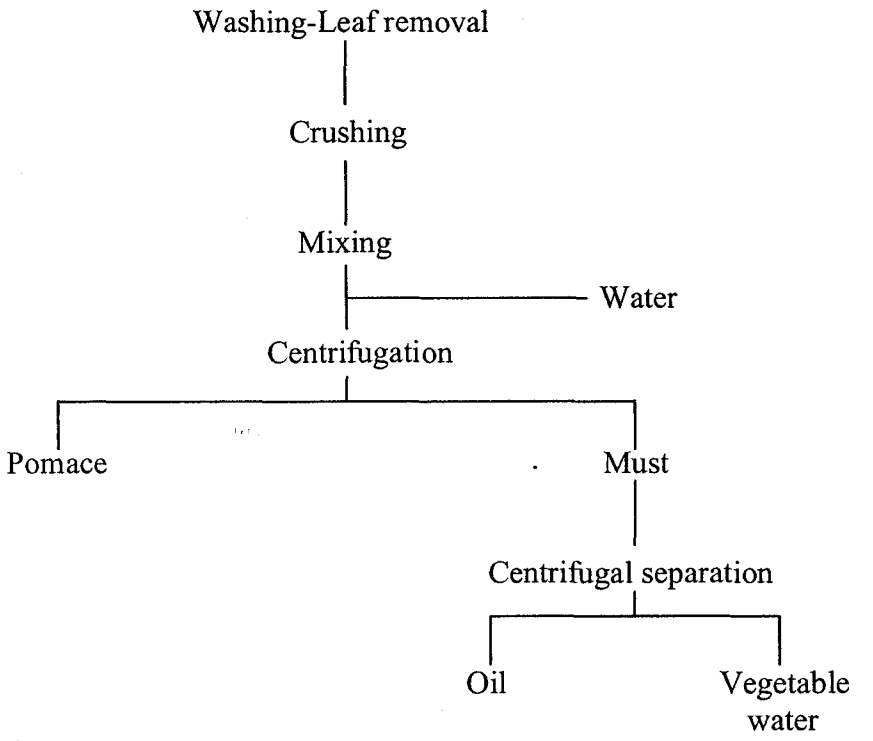

Figure 4. Diagram of olive oil extraction by direct centrifugation

Horizontal centrifuges (decanters) consist of a cylinder-conical bowl. Inside there is a hollow, similarly shaped component with helical blades. A slight difference between the speed at which the bowl rotates and that which the inner screw gyrates results in the movement of the pomace to one and of the centrifuge, while the two other constituents of the olive paste (oil and water) are pushed to the other end. The oily must (oil with a small amount of water and water containing a small amount of oil) passes through vertical centrifuges which revolve at 6000 to 7000 rpm for the final separation of the oil. The olive pomace runs out through a pipe (Kiritsakis, 1998).

Selective Filtration (Sinolea) Process

Besides pressure and centrifugation, selective filtration combined with centrifugation is used for the separation of olive oil from the olive paste. Selective filtration (or percolation) is based on the different interfacial tension of oil and water coming in contact with a steel plate. The steel plate will be coated with oil when plunged into olive paste since the interfacial tension of the oil is less than that of the water. Figure 5 shows the diagram of olive oil extraction by selective filtration (Kiritsakis, 1998).

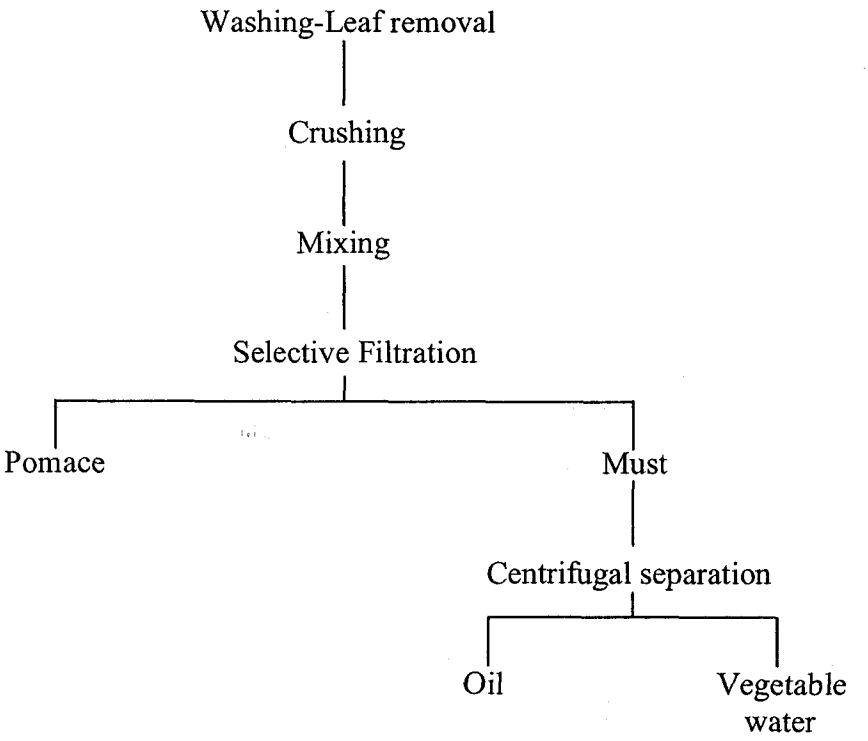

Figure 5. Diagram of olive oil extraction by selective filtration.

The most common selective filtration system is the Sinolea unit. It is made up of one or more units that can handle about 350-750 kg of olive paste, each of which has a stainless steel grating unit at the bottom. Five to seven thousand moving sheets are slotted through the slits in the grating unit and slowly penetrate the paste in reciprocating motion. When the steel plate is moved into the paste, it will preferably be coated with oil which then drips off, creating a flow of oily must consisting almost solely of oil. When this process is repeated many times, most of the oil from the paste is recovered (Kiritsakis, 1998).

2.3.6. Final Centrifugation of Olive Oil

A final centrifugation of the oil is needed regardless of the process applied (pressure, centrifugation or selective filtration) for its separation from the other constituents of the fruit. The centrifuge unit consists of a stable and a mobile part which turn very quickly. A large number of cone-shaped disks are attached to the centrifugal unit. The liquid phase is distributed on the total surface in thin layers and the centrifugation in the vertical centrifuge results in a final separation of the oil from the water and other substances (Kiritsakis, 1998).

2.4. Composition of Olive Oil

Olive oil is composed of triacylglycerols and contains small quantities of free fatty acids, (FFA), glycerol, phosphatides, pigments, flavor compounds, sterols, unidentified resinous substances and other constituents (Kiritsakis, 1998).

2.4.1. The Fatty Acids of Olive Oil

The major fatty acids present as glycerides in olive oil are oleic (C18:1), linoleic (C18:2), palmitic (C16:0), and stearic acid (C18:0) (Codex Alimen. Comm. (CAC), 1970; IOOC, 1995; Europian Union (EU) Regulation No. 2568/1991). Oleic acid is present in higher concentrations (55-83 %) than other acids. Table 1 shows the fatty acid composition of olive oil (IOOC, 1995).

Table 1. Fatty acids of olive oil.

Acids	Content (%)
Oleic	55.0 - 83.0
Palmitic	7.5 – 20.0
Linoleic	3.5 – 21.0
Stearic	0.5 – 5.0
Palmitoleic	0.3 – 3.5
Linolenic	≤ 0.9
Myristic	≤ 0.1
Arachidic	≤ 0.6
Behenic	≤ 0.2*
Lignoceric	≤ 0.2
Heptadecanoic	≤ 0.3
Heptadecenoic	≤ 0.3
Eicosenoic	≤ 0.4

(* Limit is ≤ 0.3 for olive-pomace oils)

2.4.2. Triacylglycerols of Olive Oil

Most of the fatty acids of olive oil are present as triacylglycerols (Kiritsakis, 1998). Theoretically, based on the fatty acid composition, more than 70 different

triacylglycerols should be present in olive oil. However, the number of triacylglycerols actually encountered is much smaller because some triacylglycerols are constantly absent and others occur in negligible amounts (Boskou, 1996). When diacylglycerols are present, olive oil is of low quality (Kiritsakis, 1998). Fedeli (1977) reported the major triacylglycerols as: POO (18.4 %), SOO (5.1 %), POL (5.9 %), OOO (43.5 %), OOL (6.8 %), (P: palmitic, O: oleic, S: stearic and L: linoleic acids). The three major triacylglycerols of olive oil are OOL, OOO and POO. Table 2 shows the percentage distribution of individual triacylglycerols of olive oil (Kiritsakis, 1998).

2.4.3. Mono and Diacylglycerols

The presence of partial glycerides in olive oil is due to either incomplete triacylglycerol biosynthesis or hydrolytic reactions. In virgin olive oil, concentrations of diacylglycerols may range from 1 % to 2.8 % (Kiritsakis, 1998; Boskou, 1996). Monoacylglycerols are present in much smaller quantities (less than 0.25 %) (Boskou, 1996).

In the diacylglycerol fraction C-34 and C-36 prevail (Boskou, 1996). Lampante and extracted olive oils have relatively higher amounts of C-36 (Kiritsakis, 1998). In the monoacylglycerol fraction, glycerol oleate, glycerol linoleate and glycerol palmitate are the major constituents. Other monoacylglycerols found are; glycerol stearate, glycerol palmitoleate, glycerol linolenate and glycerol laurate (Boskou, 1996). Table 2 shows the fatty acid distribution among the chief triacylglycerols of olive oil (Kiritsakis, 1998).

2.4.4. Minor Nonglyceride Constituents of Olive Oil

Several minor nonglyceride (unsaponifiable) constituents are present in olive oil. The nonglyceride fraction of olive oil contains nonglyceride fatty acid esters, hydrocarbons, sterols, triterpene alcohols, tocopherols, phenols, chlorophylls, flavor compounds and polar phenolic compounds such as hydroxytyrosol. Table 3 shows the minor nonglyceride constituents determined in virgin and refined olive oil (Kiritsakis, 1998).

Table 2. Fatty acid distribution among the chief triacylglycerols of olive oil

Number of double bonds per triacylglycerols	Fatty acid distribution	Content %
0	NA	0.0
1	POP	2.9
	PPO	0.6
	POS	0.5
	PSO	0.3
	SOS	0.2
	N.D	0.2
2	PPL	1.2
	POO	18.4
	POS	2.3
	PLS	0.1
	LPS	0.7
	SOO	5.1
	N.D	2.2
	PLO	0.2
3	POL	5.9
	OPL	0.9
	SLO	2.4
	LSO	0.7
	SOL	1.3
	OOO	43.5
	N.D	0.6
4	PLL	0.4
	LPL	0.2
	OOL	6.8
	OLO	3.5
	SLL	0.2
	LSL	0.3
	N.D	0.1

(P: palmitic; O: oleic; S: stearic; L: linoleic; N.D: non determined acid)

Table 3. Nonglyceride constituents of virgin and refined olive oil (ppm)

Nonglyceride constituents	Virgin olive oil	Refined olive oil
Hydrocarbons	2000	120
Squalene	1500	150
β-Carotene	300	120
Tocopherols	150	100
Phenols and related substances	350	80
Esters	100	30
Aldehydes and ketones	40	10
Fatty alcohol	200	100
Terpene alcohols	3500	2500
Sterols	2500	1500

2.5. Classification of Olive Oil

The IOOC (1985, 1995, 1997) proposed the following designations and definitions for olive oil and olive-pomace oil, which are in general agreement with those of European Union (EU Commission Regulations, 1991, 1995).

Virgin Olive Oil, is the oil obtained from the fruit of the olive tree only by mechanical or other physical means under conditions, mainly thermal, that do not lead to alterations in the oil and which has not undergone treatment other than washing, decantation, centrifugation and filtration. The virgin olive oil which can be consumed as it is and referred to as natural, includes:

Extra Virgin Olive Oil, which has a maximum acidity, expressed as oleic acid, of no more than 1.0 g/100 g and meets the requirements for the sensory (organoleptic) characteristics and other quality criteria of this oil category.

Virgin Olive Oil, (the qualifier "fine" may be used at the production and the wholesale stage). Virgin olive oil which has a maximum acidity, expressed as oleic acid, of no more than 2.0 g/100 g meets the requirements for the sensory (organoleptic) characteristics and other quality criteria of this oil category.

Ordinary Virgin Olive Oil, which has a maximum acidity, expressed as oleic acid, of no more than 3.3 g/100 g meets the requirements for the sensory (organoleptic) characteristics and other quality criteria of this oil category.

Lampante Virgin Olive Oil, which has acidity expressed as oleic acid of no more than 3.3 g/100 g and/or sensory (organoleptic) characteristics and other quality criteria corresponding to this oil category. This oil can not be used for consumption as it is. It should undergo refining or must be used for technical purposes.

Refined Olive Oil, obtained from virgin olive oil by refining process, which does not lead to alterations in the initial glycerols structure.

Olive Oil, consisting of a blend of virgin olive oil (except lampante) and refined olive oil.

Olive-Pomace Oil, extracted from olive pomace using solvent, to the exclusion of oils obtained by re-esterification processes and any mixture of other oils. 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 for technical purposes as it is.

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

Olive-pomace oil. A mixture of refined olive-pomace oil and virgin olive oil (except lampante). This blend should not be called "olive oil".

2.6. Olive-Pomace Oil

Olive pomace is the by-product of olive fruit processing. Olive pomace is the pulpy material remaining after removing most of the oil from the olive paste (Kiritsakis, 1998). Olive-pomace is of varying importance, mainly to 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 which in turn depends on the process applied (pressure, centrifugation, selective filtration) and on the operating conditions (Kiritsakis, 1998).

2.6.1. Olive Pomace Composition

Olive pomace contains fragments of skin, pulp, pieces of kernels and some oil. The main constituents are cellulose, proteins and water. The moisture of olive pomace obtained by the pressure process is low (Kiritsakis, 1998), while the centrifugation process results in high moisture content (Carola, 1975). Olive pomace also contains polyphenols and other constituents. Table 4 shows the composition of olive pomace obtained by pressure process (Kiritsakis, 1998).

Table 4. Composition of olive pomace obtained by a pressure-type olive oil mill.

Costituents	Content
Water	25.0
Nitrogen compounds	4.4
Non-nitrogen extractable compounds	20.0
Cellulose	40.0
Ash	6.6
Oil	4.0

T.C. YÜKSEKÖĞRETİM KURULU
DOKÜMANTASYON MERKEZİ

2.6.2. Extraction of Olive-Pomace Oil

In most areas, olive pomace is further processed to extract olive-pomace oil and other products. Furthermore, extraction of the oil from olive pomace reduces environmental contamination (IOOC, 1989). Figure 6 shows schematically the treatments and products obtained from olive pomace processing (Kiritsakis, 1998).

Pomace must be dried to a 10 % moisture content for extraction of olive-pomace oil. Higher moisture content results in poorer recoveries. The driers consist of 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. The olive pomace, hot and dry, is taken to the extractors where it is mixed with an ample amount of solvent. The extractors are arranged in a battery form. Thus, the solvent passes from one extractor to the next. The solvent/oil mixture is continuously

collected at the outlet of the extractor. Once the oil in one extractor has been recovered, steam is injected to eliminate the solvent residue. The solvent passes through the load many times. Today, continuous extractors are available. The solvent used in the past for extraction of olive-pomace oil was carbon disulfide. Most of the old plants had their own facilities for the production of carbon disulfide from sulfur and charcoal. The solvent carbon disulfide is not very selective. It pulls along resins and gums into the oil. Trichloroethylene was used as substitute for carbon disulfide. However, the cost of this solvent was high and the oil was inferior quality, so trichloroethylene was replaced with hexane, which is mainly used today. The amount of hexane used affects the quantity of oil recovered. Supercritical CO_2 can be used to extract olive-pomace oil. It is a rapid and simple process. Supercritical CO_2 is inexpensive, nontoxic and does not contaminate the environment (Kiritsakis, 1998).

The extraction product (a mixture of oil and solvent) is called miscella. 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. The oil obtained is crude olive-pomace oil. It contains large amounts of FFA which increase its acidity and lower its quality. It also contains pigments (e.g. chlorophylls, anthocyanins) as well as other constituents contributing a characteristic flavor and sweet taste. Olive-pomace oil has a fatty acid composition similar to virgin olive oil, but of inferior quality (Kiritsakis, 1998).

2.6.3. Deterioration of Olive-Pomace Oil

The oil present in olive pomace undergoes rapid deterioration due to the moisture content which favors triacylglycerol hydrolysis (Kiritsakis, 1998). Carola (1975) reported that the acidity of the 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 (*Gliomastix chartarum, Cephalosporium Sp., Aspergillus glaucus,* etc.) growing on the olive pomace. These microorganisms are favored by high moisture and temperature during storage. Besides the increase in the acidity, oxidation products (aldehydes and ketones) are also formed during storage of the olive (Kiritsakis, 1998). These products significantly affect the quality of the pomace oil (Carola 1975). In order to minimize the deterioration of the olive-pomace oil, the olive pomace should be processed as soon as possible (Kiritsakis, 1998).

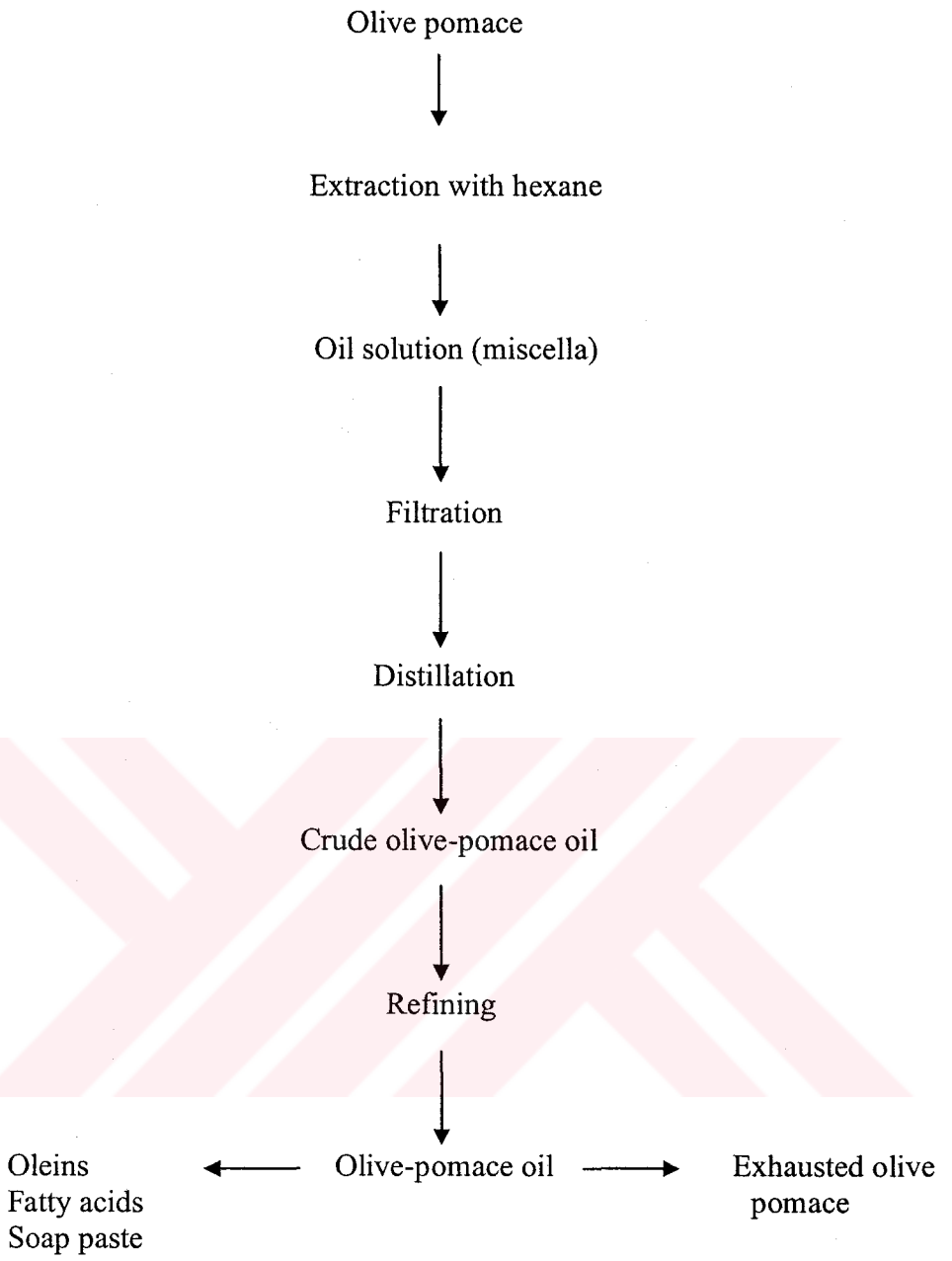

Figure 6. Treatments and by-products of olive pomace processing

2.6.4. Refining of Olive-Pomace Oil

Refined olive-pomace oil is produced by refining crude pomace oil, which is extracted by solvents from the olive-pomace residue of olive oil processing (Gomes and Caponio, 2000). This includes neutralization, deodorization and decolorization. The products obtained from refining pomace oil are refined olive-pomace oil, FFA, soap paste and exhausted olive pomace (Kiritsakis, 1998).

Refined olive-pomace oil is an acceptable edible oil. Its chemical composition does not differ from refined olive oil (Kiritsakis, 1998). Refined olive-pomace oil is eligible for sale for human consumption only it is mixed with a proper amount of virgin olive oils so that it may be classified as olive-pomace oil according to European Community (EC) regulation 356/92 (Off.J. Eur Communities, 1992). The addition of virgin olive oils to refined olive-pomace oil has several effects: it is partially responsible for the characteristic aroma of olive oil that the product acquires; the quality is enhanced, since the added oil is of greater market value; the refined olive-pomace oil is provided with a series of natural antioxidants, such as phenolic substances and tocopherol, which are lost through oxidation of the oil during transfer of the pomace from the oil mill to the pomace-processing plant and during subsequent processing procedures. All this helps to greatly improve the shelf life of these oils as marketed (Gomes and Caponio, 2000).

But, the quality of the oil recovered from the olive pomace is mainly depends on the process conditions. Long storage periods and high temperature drying applications makes the oil inferior grade (Fadıloğlu *et al.*, 2003). High acidity olive-pomace oils were mostly used for the production of household soaps, before detergents appeared on the market (Kiritsakis, 1998). In the last few years olive-pomace oil has an increasing demand to be used as edible oils for frying purposes. Crude olive-pomace oil is often very acid, colored and oxidized. Intensive refining is thus required to make it suitable for human consumption (Gomes and Caponio, 1998). However, its high free fatty acid content makes it very difficult to process by neutralization. The yield of product is also very low and it increases the cost of unit product produced. So it is necessary to reduce the free fatty acid content before refining process (Fadıloğlu *et al.*, 2003).

2.7. Lipases

Lipases (EC 3.1.1.3) are distributed throughout the living organisms which form two primary divisions of the phylogenic tree, namely the bacteria and a second division branching into both the eukorya, including animals, plants and fungi, and the archaea, with the former archaebacteria (Olson, 1994). Lipases are ubiquitous enzymes that catalyze the breakdown of fats and oils with subsequent release of free fatty acids, diacylglycerols, monoacylglycerols and glycerol (Villeneuve *et al.*, 2000). Their major substrates are long chain triacylglycerols, and this property is the

basis of an old definition of lipases as 'long-chain fatty acid ester hydrolases' or 'esterases capable of hydrolyzing esters of oleic acid' (Jaeger, 1994). These enzymes are fulfill a key role in the biological turnover of lipids. They are required as digestive enzymes to facilitate not only the transfer of lipids from one organism to another, but also the deposition and the mobilization of fat that is used as an energy reservior within the organism. They are also involved in the metabolism of intracellular lipids and therefore in the functionating of biological and physiological properties, and lately for their industrial applications (Villeneuve *et al.*, 2000).

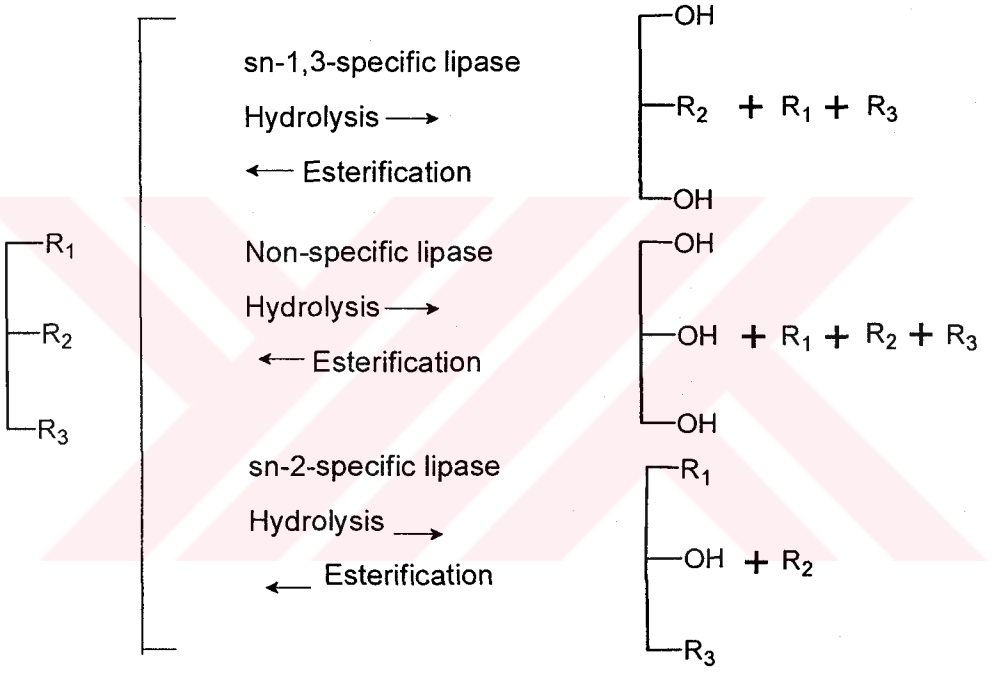

Figure 7. Specificity of triacylglycerol lipases in hdrolysis and esterification: R_1, R_2, R_3, fatty acids/acyl moieties.

The triacylglycerol lipases have widely varying substrate specificities, preferring substrates with long and medium chain fatty acids over the short chain ones, and vice versa. Morover, specifity of lipases for the fatty acids esterified at the sn-1, sn-2 and sn-3 positions of the glycerol backbone varies widely, ranging from nonspecificity for either of the three sn-1, sn-2 and sn-3 positions to strong sn-1,3 or sn-3 specificity. In general, the substrate specificity and the positional specificity of

triacylglycerol lipases observed in the hydrolysis reactions are also maintained in the reverse reaction of hydrolysis (i.e., esterification: Fig. 14) or in interesterification and transesterification reactions. The foregoing preperties of triacylglycerol lipases permit their use as biocatalysts for the preparation of specific lipid products of definite composition and structure that often can not be obtained by reactions carried out using chemical catalysts. Figure 7 shows the specificity of triacylglycerol lipases in hydrolysis and esterification (Mukherjee, 1998).

The increasing interest in lipase research over the past decades has likely occured for three reasons (Kazlauskas and Bornscheuer, 1998). The first is related to the molecular basis of the enzyme catalytic function or the lipase paradigm. Indeed, lipases, though water soluble, catalyze reactions involving insoluble lipid substrates at the lipid-water interface. This capability is due to the unique structural characteristic of lipases. These latter indeed contain a helical oligopeptide unit that covers the entrance to the active site. This so-called lid only moves upon access to a hydrophobic interface such as a lipid droplet. Thus, it is not surprising that lipases as well as phospholipases have, for many years, served as models for studying the regulation of interfacial, enzyme catalyzed reactions. The second reason is linked to the enzyme's medical relevance, particularly to atherosclerosis and hyperlipidemia, and its importance in regulation and metabolism, since products of lipolysis such as free fatty acids and diacylglycerols play many critical roles, especially as mediators in cell activation and signal transduction. Lastly, it was discovered that lipases are powerful tools for catalyzing not only hydrolysis, but also reverse reactions, such as esterification, transesterification and aminolysis in organic solvents (Villeneuve *et al.*, 2000).

Recently, lipase-catalyzed glycerolysis and direct esterification for the biosynthesis of partial glycerides are increasingly being studied as possible alternatives to the classical method (Rosu, 1997). Lipases present some important advantages over classical catalysts. Indeed, their specifity, regioselectivity and enantioselectivity allow them to catalyze reactions with reduced side products, lowered waste costs and under conditions of mild temperature and pressure (Villeneuve, 2000). Moreover, the products from enzymatic synthesis may satisfy consumer's demand for medical and nutritional products (Babayan, 1987). Accordingly, considerable attention has been lately to the commercial use of lipases (Villeneuve *et al.*, 2000). However, the

current price of lipases is about an order magnitude higher than the necessary to match the energy costs associated with standard processes (Brady *et al.*, 1988). Due to economical considerations their application on an industrial scale requires their immobilization and thus re-usability (Ivanov, 1997). Immobilized lipases are considered as lipases which are localized in a defined region of space, which is enclosed by an imaginary or material barrier which allows for physical separation of the enzyme from the bulk reaction medium, and which is at the same time permeable to reactant and product molecules (Balcao *et al.*, 1996). An immobilized lipase preparation with high operational stability will reduce costs, making lipase-catalyzed reactions a feasible process option (Ferreria-Dias and Fonseca, 1995b), from an industrial point of view, immobilized lipases generally offer the economic incentives of enhanced thermal and chemical stability, and ease of handling, recovery, and reuse relative to nonimmobilized forms (Malcata *et al.*, 1990; Yahya *et al.*, 1998). Immobilization also facilitates dispersal of enzyme on a solid surface to provide for greater interfacial area and accessibility of substrates relative to the use of enzyme powders in low water reaction media (Bell *et al.*, 1995).

Various features of reaction selectivity of lipases are modulated by exogenous factors such as the type of organic solvent, choice of cosubstrates, water activity, pH and immobilization although this is not always the case (Lee, 2001).

2.7.1. Lipases as Applied Catalysts

The biological function of lipases is to catalyze the hydrolysis of esters, especially long-chain triacylglycerols, to yield free fatty acids, di- and monoacylglycerols and glycerol. Lipases are also capable of catalyzing the reverse reaction, achieving esterification, interesterification (acidolysis, transesterification, alcoholysis), aminolysis, oximolysis and thiotransesterification in anhydrous organic solvents, biphasic systems and in micellar solution with chiral specifity. Most industrial applications are found in racemic mixtures, textile detergency, pharmaceuticals synthesis or in oils and fats bioconversion. Table 5 shows examples of industrial applications of lipases (Villeneuve *et al.*, 2000).

Table 5. Examples of industrial applications of lipases

Field of industry	Application	Product
Hydrolysis		
Food (dairy)	Hydrolysis of milk fat	Flavoring agents for dairy products
Chemical (oil processing)	Hydrolysis of oils and fats	Fatty acids, diacylglycerols and monoacylglycerols
	Analysis of fatty acid distribution in triacylglycerols	Reagents for lipid analysis
Chemical (detergent)	Removal of oil strains, spots and lipids	Detergents for laundry and household uses
Medical	Blood triacylglycerol assay	Diagnostic kits
Esterification		
Chemical (fine chemical)	Synthesis of esters	Chiral intermediates Esters, emulsifiers
Food (chemical and pharmaceutical)	Interesterification of natural oils	Oils and fats (e.g., cocoa butter equivalent

2.7.2. Hydrolysis versus Esterification

The hydrolysis of fats and oils (triacylglycerols) is an equilibrium reaction and therefore it is possible to change the direction of the reaction to ester synthesis by modifying the reaction conditions (Figure 8) (Jaeger *et al.*, 1994).

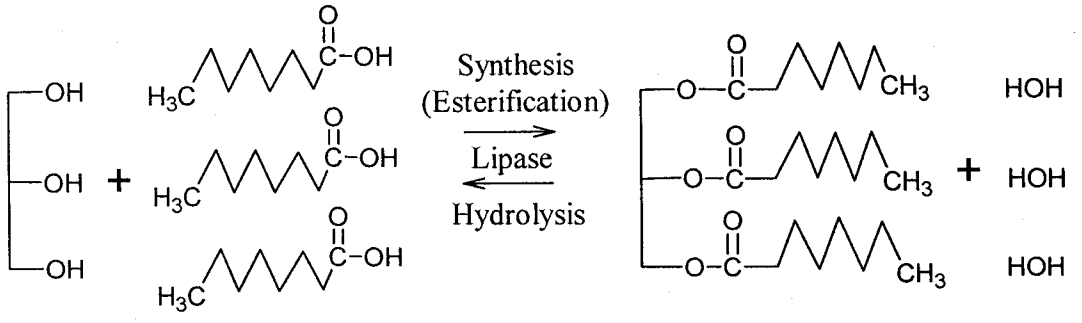

Figure 8. Enzymatic reaction of a lipase catalyzing hydrolysis or synthesis of a triacylglycerol substrate.

Lipases normally catalyze the hydrolysis of glycerides and the equilibrium of this process is far to the direction of hydrolysis products since water present is excess. When the concentration of water in the reaction medium is reduced, e.g., by working in an organic solvent containing a limited amount of water, a drastic shift of the chemical equilibrium can be observed, so that reagents and products are present in comparable concentrations. Mechanistically, this means that the reverse reaction, the esterification, takes place at a rate comparable to that of the hydrolysis reaction (Goderis *et al.*, 1987). In esterification reactions, it is desirable to shift the position of thermodynamic equilibrium by removal of the water produced by the reaction (Arcos *et al.*, 2000). As the amount of water present in the system increases, more triacylglycerols are hydrolyzed without subsequent reesterification and equilibrium is more to the hydrolysis products. From this point of view, maximization of final triacylglycerol concentration is obtained by working under circumstances as dry as possible (Goderis *et al.*, 1987). The equilibrium between forward and reverse reactions in this case is controlled by the water content of the reaction mixture, so that in a non-aqueous environment lipases catalyze ester synthesis reactions (Jaeger *et al.*, 1994). Different types of synthesis reactions can be distinguished: common ester synthesis from glycerol and fatty acids and the biotechnologically more important interesterification reactions, is the exchange of acyl groups, between an ester and an acid (acidolysis), an ester and an alcohol (alcoholysis), or an ester and an ester (transesterification) (Malcata *et al.*, 1990).

Interesterifications require a small amount of water, in addition to the amount needed for the enzyme to maintain an active hydrated state. As the presence of (too much) water will decrease the amount of ester synthesis products, the water content should be carefully adjusted to achieve accumulation of desired reaction products (Jaeger *et al.*, 1994).

2.7.3. Lipase Reactions in the Processing of Fats and Oils

2.7.3.1. Hydrolysis

Total hydrolysis of ester bonds in triacylglycerols may be accomplished at high temperatures and pressure in the presence of steam. Fatty acids can alternatively be produced by ambient pressure saponification or chemically catalyzed hydrolysis. The conventional chemical fat-splitting processes require rather harsch conditions with

respect to temperature (240-260°C) and pressure (60 bar). This inevitably produces undesirable side effects, like product discoloration and degradation of some fatty acids. However, the use of lipases for enzymatic splitting of fats in the presence of excess water (Figure 8) is more appealing since the reaction proceeds under mild conditions of pressure and temperature with specifity and reduced waste (Malcata *et al.*, 1990). This technology is currently employed in the production of fatty acids, diacylglycerols, monoacylglycerols, flavoring agents for dairy products and detergents for laundry and household uses (Table 5) (Villeneuve *et al.*, 2000).

2.7.3.2. Esterification

Glycerides can also be obtained by direct esterification of free fatty acids to glycerol (Jaeger *et al.*, 1994). Esterification reactions between polyhydric alcohols and free fatty acids are, in essence, the reverse of the hydrolysis reaction of the corresponding glyceride (Figure 8) (Villeneuve *et al.*, 2000). In esterification reactions, it is desirable to shift the position of thermodynamic equilibrium by removal of water produced by the reaction (Arcos *et al.*, 2000). The equilibrium between forward and the reverse reactions is usually controlled by the water content of the reaction mixture (Malcata *et al.*, 1990). Some studies reported that the water thermodynamic activity (a_w) is conditioning the lipase spatial structure and thus its enzymatic activity either toward synthesis or hydrolysis (Chandler *et al.*, 1998; Schmid et al., 1998; Soumanou et al., 1998; Svensson et al., 1994; Valivety *et al.*, 1994; Villeneuve *et al.*, 1997; Lee and Foglia, 2000). For synthesis reactions performed with water-free substrates and in a solvent-free system, the aqueous phase is limited to the one coming from the enzyme preparation. For each lipase catalyzed synthesis reaction performed in these conditions, an initial hydration level ruled by the initial a_w and initial water content of the biocatalyst, for which the best synthesis performance is obtained, does exist (Caro *et al.*, 2002). However, esterification catalyzed by various microbial lipases always resulted in mixtures of glycerides, with yield and composition of the mixture depending on the source of lipase (Osada *et al.*, 1990). A process resulting in regioisomerically pure glycerides has been developed comprising as an essential step the adsorption of glycerol onto a solid support. Lipase-catalyzed glyceride synthesis with the immobilized glycerol and various acyl donors (e.g. free fatty acids, fatty acid alkylesters, natural fats and oils) yielded multigram quantities of regioisomerically pure di- (Berger *et al.*, 1992) and monoacylglycerols (Berger

and Schneider, 1992). *C. viscosum* was one of the 1,3-selective lipases producing the desired glycerides with high yield. Monoacylglycerols were separated from the other reactants in a separate vessel and the undesired products were fed back to the reactor (Berger and Schneider, 1992).

2.7.3.3. Interesterification

The development of methods to improve the nutritional and functional properties of fats and oils is great interest to food processors. The molecular weight, unsaturation and positional distribution of fatty acid residues on the glycerol backbone of triacylglycerols are the principal factors determining the physical properties of fats and oils (Goderis *et al.*, 1987). Chemical interesterification produces a complete positional randomization of acyl groups in triacylglycerols. It is used in the manufacture of shortenings, margarines and spreads to improve their textural properties, modify melting behavior and enhance stability (Willis and Marangoni, 2002). Interesterification, whether chemical or enzymatic, is the exchange of acyl groups, between an ester and an acid (acidolysis), an ester and alcohol (alcoholysis) or an ester and an ester (transesterification) (Malcata *et al.*, 1990).

The physical properties of various fats and oils are different because of the structure and distribution of fatty acids in the triacylglycerols. In natural fats, acyl groups are distributed in a nonrandom fashion. During chemical or enzymatic interesterification, acyl groups are redistributed first intramolecularly, then intermolecularly, until a random distribution is achieved. With enzymatic interesterification, more control of final product composition is possible and glyceride mixtures that can not be obtained using chemical interesterification can be produced (Willis and Marangoni, 2002).

a) Transesterification

Transesterification, the exchange of acyl groups between two esters, namely two triacylglycerols (Figure 9), is used predominantly to alter the physical properties of individual fats and oils or fat-oil blends by altering the positional distribution of fatty acids in the triacylglycerols (Willis and Marangoni, 2002)

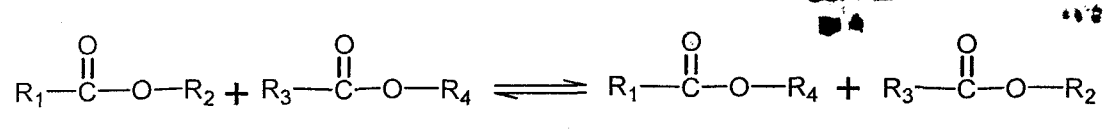

Figure 9. Lipase-catalyzed transesterification between two different triacylglycerols.

Transesterification of butter using a nonspecific lipase has been reported to improve the plasticity of the fat. Willis and Marangoni (2002) reported that transesterification of butterfat with a positionally nonspecific lipase at 40°C increased the level of saturated C48 to C54 triacylglycerols, monoene C38 and C46 to C52 triacylglycerols and diene C40 to C54 triacylglycerols. These authors reported that the diacylglycerol content increased by 45 % while the free fatty acid content doubled. Overall, lipase-catayzed transesterification of butterfat at 40°C produced an increase in the solid fat content below 15°C and a decrease in the solid fat content above 15°C.

b) Acidolysis

Acidolysis, the transfer of an acyl group between an acid and an ester, is an effective means of incorporating novel fatty acids into triacylglycerols (Figure 10).

Figure 10. Lipase-catalyzed acidolysis reaction between an acylglycerol and an acid.

Acidolysis has been used to incorporate free acid or ethyl ester forms of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) into vegetable and fish oils to improve their nutritional properties. Immobilized lipase from *Mucor miehei* was used to increase the EPA content in the oil from 8.6 % to 25 % and the DHA content from 12.7 % to 40 %. EPA and DHA have also been used to enrich the content of these fatty acids in fish oils. Acidolysis has also been used to incorporate oleic acid into milk fat. Acidolysis is also a common method for production of cocoa butter substitutes. The most common method is acidolysis of palm oil midfraction, which contains predominantly 1,3-dipalmitoyl-oleoyl-glycerol with stearic acid to increase the level of stearate in the lipid. Stearic acid was incorporated into palmolein to produce 25 % cocoa butter-like triacylglycerols (Willis and Marangoni, 2002).

c) Alcoholysis

Alcoholysis, the esterification reaction between an alcohol and an ester (Figure 11), has been used in the production of methyl esters from esterification of

triacylglycerols and methanol with yields of up to 53 %. During alcoholysis, hydrolysis of triacylglycerols to produce diacylglycerols and monoacylglycerols can occur, although the presence of small amounts of alcohol can inhibit hydrolysis.

Figure 11. Lipase-catalyzed alcoholysis reaction between an acylglycerol and an alcohol.

The main use of alcoholysis is in the performance of glycerolysis reactions. Glycerolysis is the exchange of acyl groups between glycerol and a triacylglycerol to produce monoacylglycerols, diacylglycerols and triacylglycerols. Monoacylglycerols can be produced by ester exchange between triacylglycerols and glycerols, or by free fatty acids and glycerol, although only the former reaction is termed glycerolysis (Figure 12) (Willis and Marangoni, 2002). Glycerolysis of fats and oils produces industrially important MAG and DAG. Fatty acid MAG and their derivatives have many applications as surfactants and emulsifiers in a wide range of food, cosmetics and pharmaceutical products (Meffert, 1984). Lipase-catalyzed glycerolysis experiments, aimed at the production of MAG and /or DAG, have been carried out with free (McNeill *et al.*, 1991) or immobilized lipases (Ferreira-Dias and Fonseca, 1993; Stevenson *et al.*, 1993; Ferreira-Dias and Fonseca, 1995a), with lipases in reversed micelle systems (Chang and Rhee, 1990) or on liposomes in reversed micelles (Chang *et al.*, 1991).

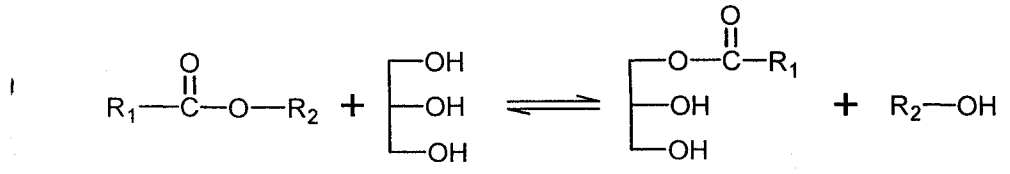

Figure 12. Lipase-catalyzed glycerolysis reaction between glycerol and a triacylglycerol to produce monoacylglycerols.

From a wide range of microbial lipases, which were screened for glycerolysis, bacterial lipases were found to be the most suitable for this process (Bornscheuer and

Yamane, 1994). The esterification of glycerol with free fatty acids (FFA) by lipases to produce primarily MAG was reported by several authors (Akoh *et al.*, 1992; Berger and Schneider, 1992; Akoh, 1993).

CHAPTER III

MATERIALS AND METHODS

3.1. Materials

Immobilized lipase from *Candida antarctica* (designated Novozym 435, activity 7000 PLU/g) was kindly obtained from Novo Nordisk A/S (Bagsvaerd, Denmark). Olive-pomace oil was kindly supplied from Güvenal Sabun Pirina Yağ San. ve Tic. A.Ş. (Gaziantep, Turkey), glycerol and molecular sieve (particle size of 5 Å) were purchased from Sigma Chemical Co. (St. Louis, MO). Hexane, acetone and acetonitrile were High Performance Liquid Chromatography (HPLC) grade and were purchased from Merck Chemical Co. (Darmstadt, Germany). Standards for fatty acids (stearic, palmitic, oleic) and triacylglycerols were also supplied from Sigma Chemical Co. (St. Louis, MO). Ethanol, diethyl ether, sodium hydroxide and phenolphthalein used for the determination of free fatty acid content were all analytical grade and purchased from Merck Chemical Co. (Darmstadt, Germany).

3.2. Chromatographic Equipment

The HPLC system consisted of quadratic pump (model LC-10ADVP; Shimadzu, Japan) equipped with a column (Sphereclone 5µ ODS (2), 250 x 4.6 mm; Phenomenex, USA) with an accompanying guard column (40 x 3 mm id) of the same phase, ultra violet (UV) detector (Hewlett Packard Series 1100) and refractive index (RI) detector (Jasco 830 RI).

3.3. Methods

3.3.1. Esterification Reactions

3.3.1.1. The Effect of Molecular Sieve

The reaction mixtures consisted of 2.5 mL olive-pomace oil, 0.5 g glycerol, 10 µL water, 7.5 mL hexane, 18.1 mg/mL lipase and molecular sieve were prepared in 100 mL flasks. Four sets of reaction mixtures were prepared. One of the sets did not contain molecular sieve and other three sets contained 350, 500 and 750 mg

molecular sieve, separately. The enzymatic reactions were carried out in a thermoconstanter shaker water bath (Nüve, İstanbul, Turkey) with shaking at 120 strokes/min at 50°C and for 30, 60, 90, 120, 150, 180, 300 and 420 minutes. All experimental runs were conducted in duplicate.

3.3.1.2. The Effect of Enzyme Concentration

The reaction mixtures consisted of 2.5 mL olive-pomace oil, 0.5 g glycerol, 10 µL water, 7.5 mL hexane, 750 mg molecular sieve and lipase were prepared in 100 mL flasks. Each reaction mixture contained 4.5, 9.0, 18.1, 27.2, 36.3 and 54.5 mg/mL lipase, separately. The enzymatic reactions were carried out in a thermoconstanter shaker water bath (Nüve, İstanbul, Turkey) with shaking at 120 strokes/min at 50°C for 60 minutes. All experimental runs were conducted in duplicate.

3.3.1.3. The Effect of Temperature

The reaction mixtures consisted of 2.5 mL olive-pomace oil, 0.5 g glycerol, 10 µL water, 7.5 mL hexane, 18.1 mg/mL lipase and 750 mg molecular sieve were prepared in 100 mL flasks. The reaction mixtures were introduced the reaction at three different temperatures (40, 50 and 60°C) separately. The enzymatic reactions were carried out in a thermoconstanter shaker water bath (Nüve, İstanbul, Turkey) with shaking at 120 strokes/min at 50°C for 30, 60, 90, 120, 150, 180, 300 and 420 minutes. All experimental runs were conducted in duplicate.

3.3.1.4. The Effect of Glycerol Content

Esterification reactions were carried out in the absence and presence of different amounts of glycerol. Limiting amount of glycerol (glycerol to FFA molar ratio of 1/3) and half of the limiting amount of glycerol (glycerol to FFA molar ratio of 1/6) were used in esterification reactions. The limiting glycerol content was calculated on the basis of initial amount of FFA (32 %) in olive-pomace oil. A mixture of 2.5 mL olive-pomace oil, 10 µL water, 7.5 mL hexane, 18.1 mg/mL of lipase, 750 mg molecular sieve and defined amount of glycerol was prepared in 100 mL flasks. The enzymatic reactions were carried out in a thermoconstanter shaker water bath (Nüve, İstanbul, Turkey) with shaking at 120 strokes/min at 50°C for 30, 60, 120, 180, 300, 420 and 480 minutes.

3.3.2. Methods of Analysis

3.3.2.1. Determination of Free Fatty Acid (FFA) Content

Effects of molecular sieve, enzyme concentration and temperature on esterification reaction were investigated by determining the free fatty acid contents of each reaction mixture. At the end of each reaction period, the reaction mixtures were centrifuged at 6000 rpm for 10 minutes to separate the lipase and molecular sieve, and the supernatants were used to determine the free fatty acid (FFA) content of the olive-pomace oil.

FFA content was determined by titration (AOAC, 1975). 20 mL of alcohol, 20 mL of diethyl ether and 1 mL of phenolphthalein mixture was neutralized and added to the supernatant (olive-pomace oil, glycerol, hexane and the free fatty acids). The mixture was titrated with 0.1 N NaOH. The result was defined in percentage free fatty acids. The FFA content determinations were made in duplicate and are reproducible within ± 10 %.

3.3.2.2. HPLC Analysis

In order to investigate the effect of glycerol content on the change in glyceride composition of olive-pomace oil, the reaction mixtures were centrifuged at 6000 rpm for 10 minutes to separate the lipase and molecular sieve at the end of each reaction period. Then, the hexane in the supernatants was evaporated in a vacuum oven at 25 °C for 7 hours. The FFA, MAG, DAG and TAG composition of the reaction mixture was determined by using HPLC. 0.05 g of the oil samples obtained from the reactions were diluted in 2 mL of acetone and filtered with syringe filter (0.2 µm, Minisart, Sartorius) prior to injection. Injection volume was 20 µL. The column was thermostated at 35°C. The mobile phase used was acetone/acetonitrile (50:50, v/v). A flow gradient was used: 0.4 mL/min for 12 minutes, increased in 4 minute up to 3 mL/min and held for another 10 minutes. The UV detector was run at 206 nm. The FFA composition was analysed by the same HPLC equipment and procedure but with a RI detector. An integration system (Borvin V 1.21) was used for data collection and integration. The percent FFA was found by using the calibration curve of oleic acid concentration vs peak area that was obtained from the HPLC chromatogram. Different concentrations of oleic acid in acetone solutions (0.8, 0.4, 0.2, 0.1, 0.05 and 0.025 % oleic acid solutions correspond to 32, 16, 8, 4, 2 and 1 %

acidity, respectively) were prepared and injected to HPLC by using RI detector. The FFA content of each reaction product were found by using the areas obtained from the HPLC chromatograms. Each area were investigated for the equivalent FFA content by using the oleic acid calibration curve. The percentages of MAG, DAG and TAG of each reaction run was found by calculating the area fraction of each glyceride within MAG, DAG and TAG obtained from HPLC runs achieved by using UV detector.

3.3.3. Statistical Analysis

Analysis of variance (ANOVA) was carried out using a general linear model (GLM) procedure of SPSS for Windows Release 8.0 (SPSS Inc., Chicago, USA). The least significant difference (LSD) test was used for multiple comparision at 5 % significance level.

CHAPTER IV

RESULTS AND DISCUSSION

4.1. The Effect of Molecular Sieve

Enzymatic esterification of free fatty acids (FFA) present in olive-pomace oil was carried out in the absence and presence of molecular sieves in order to compare the conversion rate of esterification that can be favored by adsorption of water produced during the reaction. A certain amount of water in the reaction mixture is necessary for a good catalytic activity, but this content should keep the hydrolysis at a low value (Rosu *et al.*, 1998). Several different techniques have been reported for the elimination the water produced during the reaction, e.g., inclusion of silica gel with the immobilized lipase (Mukesh *et al.*, 1993) and synthesis under reduced pressure at high temperature (90°C) in a solvent with a high boiling point (Ducret *et al.*, 1995), evaporation (Ergan *et al.*, 1990) and the use of molecular sieves (Schmid *et al.*, 1999). Schmid *et al.* (1999) used molecular sieves to remove water produced during the esterification of 2-MP with oleic acid to produce OPO, and also Omar *et al.* (1988) used immobilized lipase packed-bed reactor installed with a molecular sieve column during the esterification to study the role of water on the equilibrium. Adsorbents such as alumina, silica gel and zeolites are effective in removing water from organic solvents. However, the molecular sieves are superior in their drying ability because they can not co-adsorb large hydrocarbon molecules as do common silica and alumina adsorbents.

Figure 13 shows the decrease in free fatty acid content in olive-pomace oil with time in the presence of varying amounts of molecular sieves. In the absence of molecular sieve, FFA content decreased from 32 to 18 % within 60 min but a gradual increase was observed later. The possible explanation is that the accumulation of water favors the reverse reaction that is the hydrolysis of the glycerides. Similar result has been reported by Hog *et al.* (1985). They found that on the synthesis of glycerides from glycerol and free fatty acids using *Chromobacterium* lipase, a steady increase in

glyceride synthesis was observed below an optimal water content of about 4 %. Chang *et al.* (1991) reported that at water concentrations higher than the value of 8 % (w/v), the amount of oleic acid was abruptly increased because the hydrolysis reaction became significant. Omar *et al.* (1987) investigated the effect of water content on the hydrolysis of olive oil and the esterification of oleic acid and glycerol. They found that increasing water content resulted in a decrease in the esterification degree, whereas the hydrolysis degree increased rapidly with increasing water content.

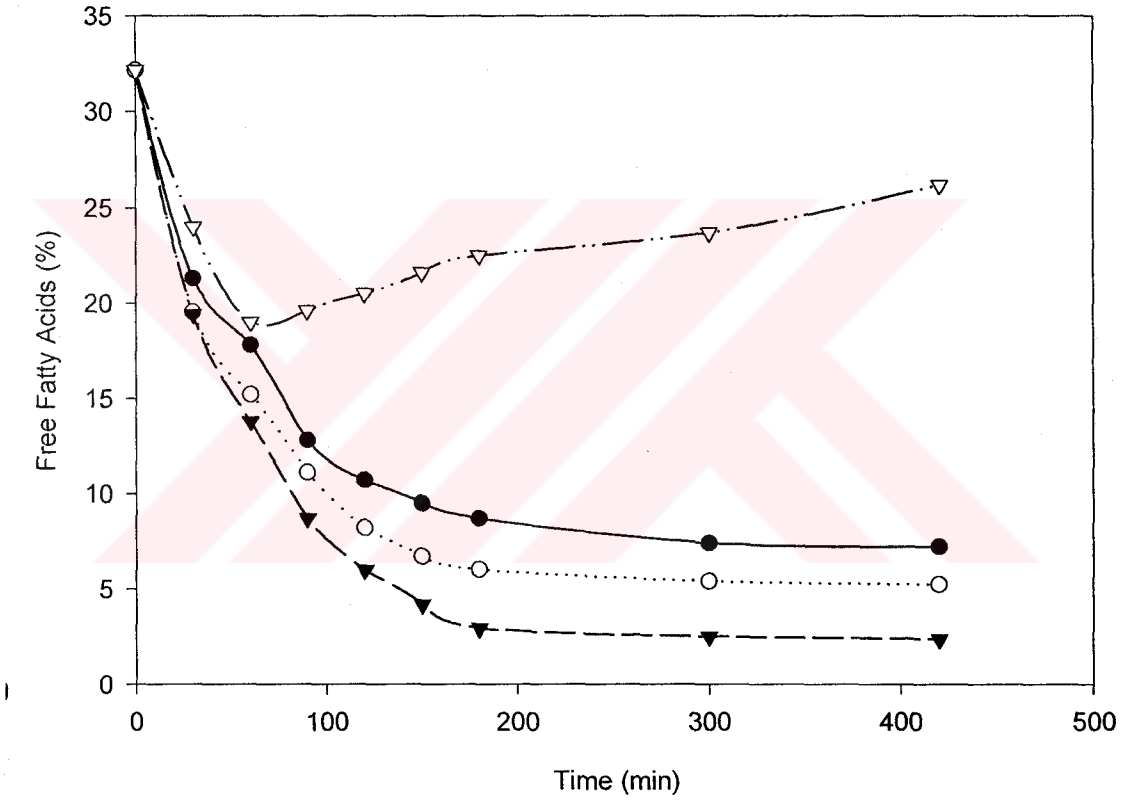

Figure 13. The effect of molecular sieve on the reduction of free fatty acids in olive-pomace oil as a function of time at 50°C. (∇) No sieve; (●) 350 mg sieve; (○) 50 mg sieve; (▼) 750 mg sieve.

When the amount of molecular sieve introduced into reaction media was increased, the FFA content in the medium reduced greatly. Initial FFA content (32 %) reduced to 9.58, 5.23 and 2.36 % with the molecular sieves of 350, 500 and 750 mg

respectively by the end of the reaction period of 420 min. A significant change ($p < 0.05$) was observed in the FFA content depending on the amount of molecular sieve introduced into the reaction. If it is calculated for the maximum amount of available free fatty acid content (32 %) in the reaction medium, the minimum necessary amount of molecular sieve is 225 mg according to its water absorption capacity. This calculation was based on the initial FFA content of 32 % corresponding to 0.0025 mol FFA in terms of oleic acid. Theoretically calculated amount of water produced was found to be 45 mg according to esterification reaction. The quantity of molecular sieves was more than that necessary to completely absorb the amount of water that would be produced from the complete esterification of free fatty acids present, considering the high capacity of these sieves for absorption of water (20 %, w/w). However, increasing amount of molecular sieve increased the rate of esterification. This can be explained by the increasing binding sites of higher amounts of molecular sieves catalyzing the esterification reaction in a shorter time compared to the amount of molecular sieve which is just enough to bind the available water in the system.

4.2. The Effect of Enzyme Concentration

The effect of enzyme concentration on the esterification of free fatty acids in olive-pomace oil is given in Figure 14. A significant decrease ($p<0.05$) was observed in the FFA content up to 18.1 mg/mL of enzyme concentration. The similar results was found by Mutua and Akoh (1993), and they explained that any further increase in the enzyme concentration did not result in an increase in the amount of desired product, because the subsrate concentration becomes a limiting factor in the face of an excess of active enzyme sites. Giacometti *et al.* (2001) also reported that on the enzymatic esterification of glycerol with oleic acid, the increase in the concentration of *Lipozym* increased the conversion of oleic acid. However the results of Omar *et al.* (1987) were contrary to these findings. They stated that increasing enzyme amount did not remarkebly affect glyceride formation rate from oleic acid and glycerol.

4.3. The Effect of Temperature

A significant decrease ($p<0.05$) was observed in FFA content with increasing reaction time at all temperatures (Figure 15). The decrease in FFA content is sharper at higher temperatures. FFA content reached into the equilibrium in almost 120 minutes at 60°C. However the time to reach into the equilibrium was around 300

minutes at 40 and 50°C. Two-way ANOVA (temperature x time) and multiple range test (LSD) were applied to see the net effect of temperature on free fatty acid content during 420 minute period at three different temperatures (40, 50 and 60°C). ANOVA showed that the effect of temperature on free fatty acid content was significant ($p < 0.05$) at 95 % confidence interval. Results of multiple range test showed that increasing temperature from 40 to 60°C significantly affected ($p < 0.05$) the free fatty acid reduction.

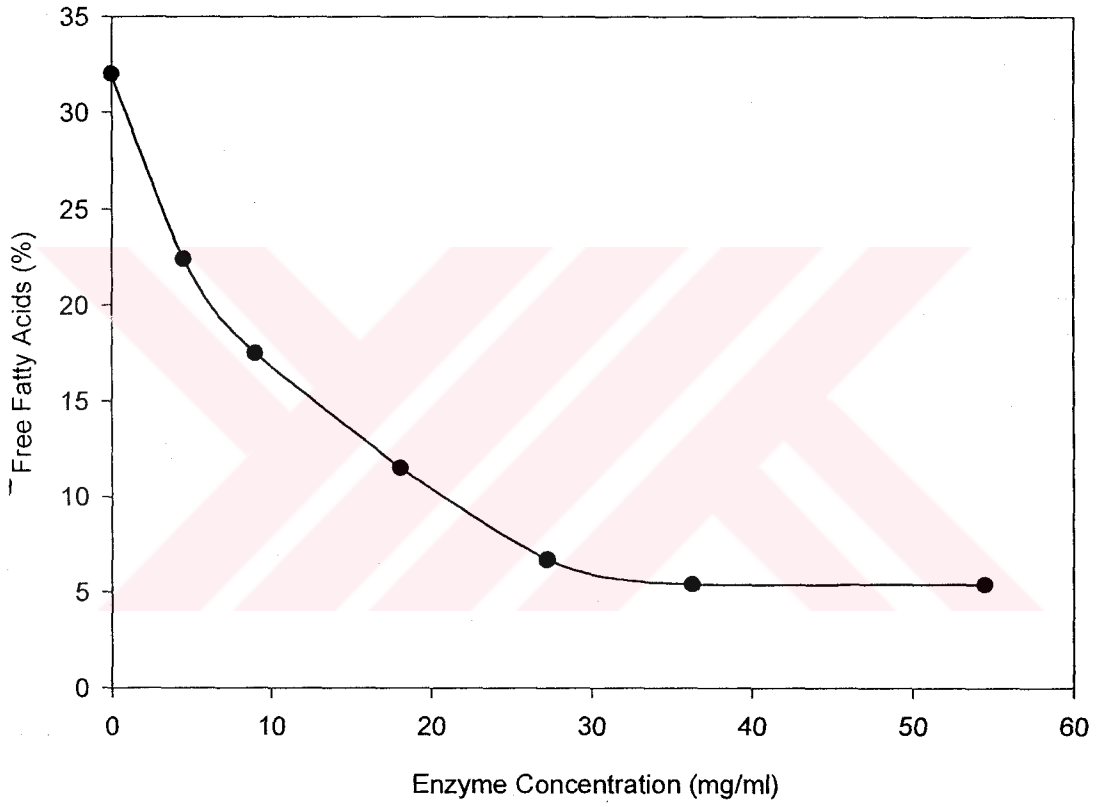

Figure 14. Free fatty acids content in olive-pomace oil as a function of enzyme concentration within 60 min at 50°C.

Various models have been used to describe the kinetics of the interesterification and glycerolysis reactions (Kyotani *et al.*, 1988b; Xu *et al.*, 1999). The models based on the first order kinetics have been found to fit best with the experimental data in most of these studies.

The following equation (Bozkurt *et al.*, 1999) was used to fit FFA reduction data by using non-linear regression analysis (two-parameter fitting of n and k).

$$n \neq 1 \qquad C^{(1-n)} - C_0{}^{(1-n)} = (1-n)\,kt$$

where C is the concentration at time t, C_0 is the concentration at time zero, n is the reaction order, k is the rate constant and t is the time. C_0 values were calculated to check the suitability of the model. The high values of R^2 show a close agreement between the experimental results and theoritical values predicted by the model (Table 6). Rate constants found in this study was in a range of 4.5×10^{-3} and 9.7×10^{-3} g FFA/100 g oil/min. These results were comparable with the findings of Lortie *et al.* (1993) for the synthesis of mono-, di- and triacylglycerols from the oleic acid.

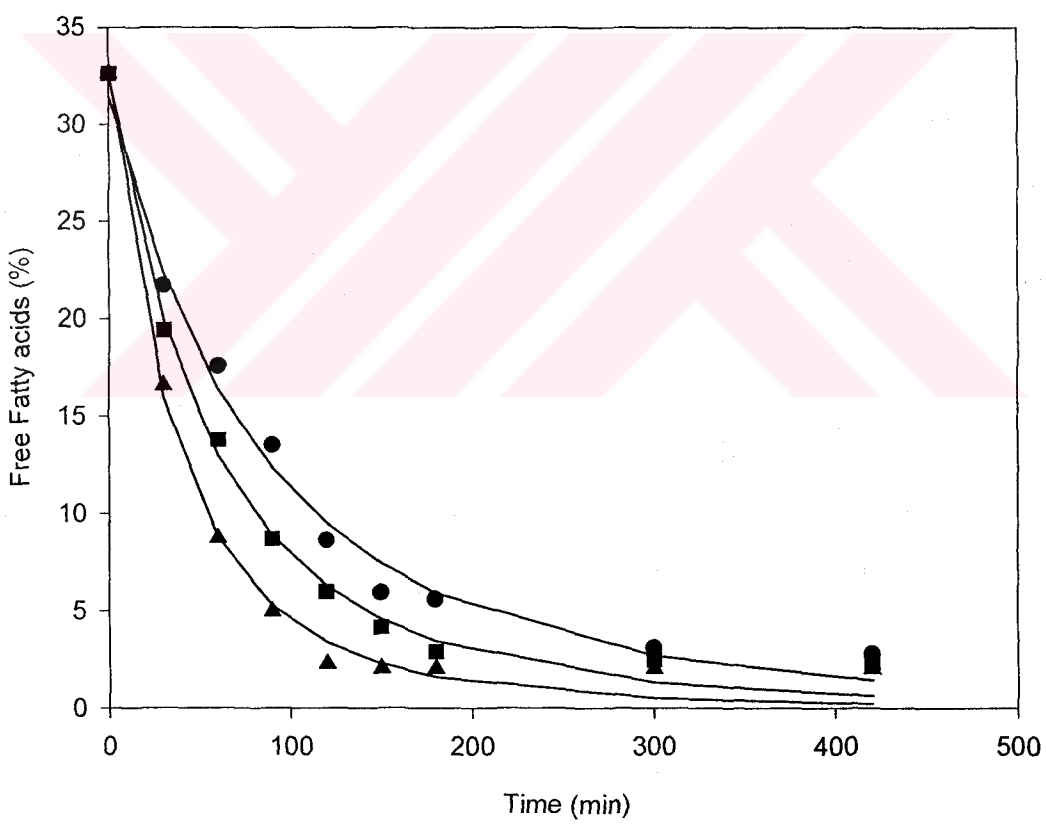

Figure 15. Free fatty acids reduction in olive-pomace oil as a function of time at various temperatures (continuous lines refer to the predicted model). (●) 40°C; (■) 50 °C; (▲) 60°C.

The reaction orders, rate constants and their correlation coefficients (R^2) for FFA reduction in esterification reaction are given in Table 6. As seen, reaction orders for FFA reduction were found to be 1.3. It was reported in the literature that the reaction order of triolein synthesis was found to be pseudo-first order (Lortie *et al.*, 1993), and interesterification of oils and fats followed a first order kinetics (Kyotani *et al.*, 1988a).

Table 6. The reaction orders, rate constants and R^2 values for free fatty acids (FFA) decrease in olive-pomace oil.

Temperature (°C)	n	k (g FFA/100 g oil.min)	C_0 (g FFA/100 g oil)	R^2
40	1.2915	0.004536±0.0002	32.45±0.895	0.9916
50	1.2915	0.006368±0.0004	32.63±1.387	0.9912
60	1.2915	0.009702±0.0005	32.79±1.124	0.9893

The Arrhenius relationship was applied to find the activation energy (Ea). The activation energy for FFA reduction in esterification reaction of free fatty acids of olive oil was 32.89 kJ mol^{-1} with an R^2 of 0.9987.

4.4. The Effect of Glycerol Content

Figure 16 shows the compositions of reaction mixtures in the absence of glycerol with respect of FFA, MAG, DAG and TAG as a function of time during the enzymatic esterification. As it is seen, FFA concentration sharply decreases from 32 to 23 % in the first 60 min of the reaction. Following this sharp decrease in FFA concentration, there is a slow rate of decrease up to a FFA concentration of 21 % at 8 hours. In earlier studies (Yamane *et al.*, 1994; Ergan and Andre, 1989), the hydrolysis and synthesis reactions in a similar system were followed for 72 hours at 34 °C. It has been observed that the change in the compositions reached almost equilibrium in the first 10 hours. However, it has been reported again in similar systems by using commercial immobilized lipases such as Novozym 435 that the equilibrium level was reached in a shorter time than 2 hours (Elfman-Börjesson and Harröd, 1999). The time course to reach into equilibrium is an agreement with those studies. MAG was followed a similar pattern of decrease during the time course of

the reaction. MAG concentration decreased from 20 to 17 % within the first 60 min and reached to 14.5 % by the end of the reaction. While the substrate FFA and intermediate MAG concentrations decreased, DAG and TAG concentrations increased for the overall reaction. DAG concentration increased sharply from 15 to 26 % in the first 60 min and it was almost constant in the following 6 hours of the reaction. A linear slow increase was observed in TAG concentration from 33 to 40 % during the reaction. This increase in TAG was found to be significant statically ($p < 0.05$). The rate of increase for TAG is around 0.875 g TAG/g oil/h. The decrease in FFA and MAG concentrations and the increase in DAG concentration within first 60 min of the reaction indicates that free fatty acids bound to available monoglycerides and as a result, formed diglycerides. The constant DAG concentration after the first hour of the reaction shows that while DAG formed, FFA also bound to DAG and formed TAG.

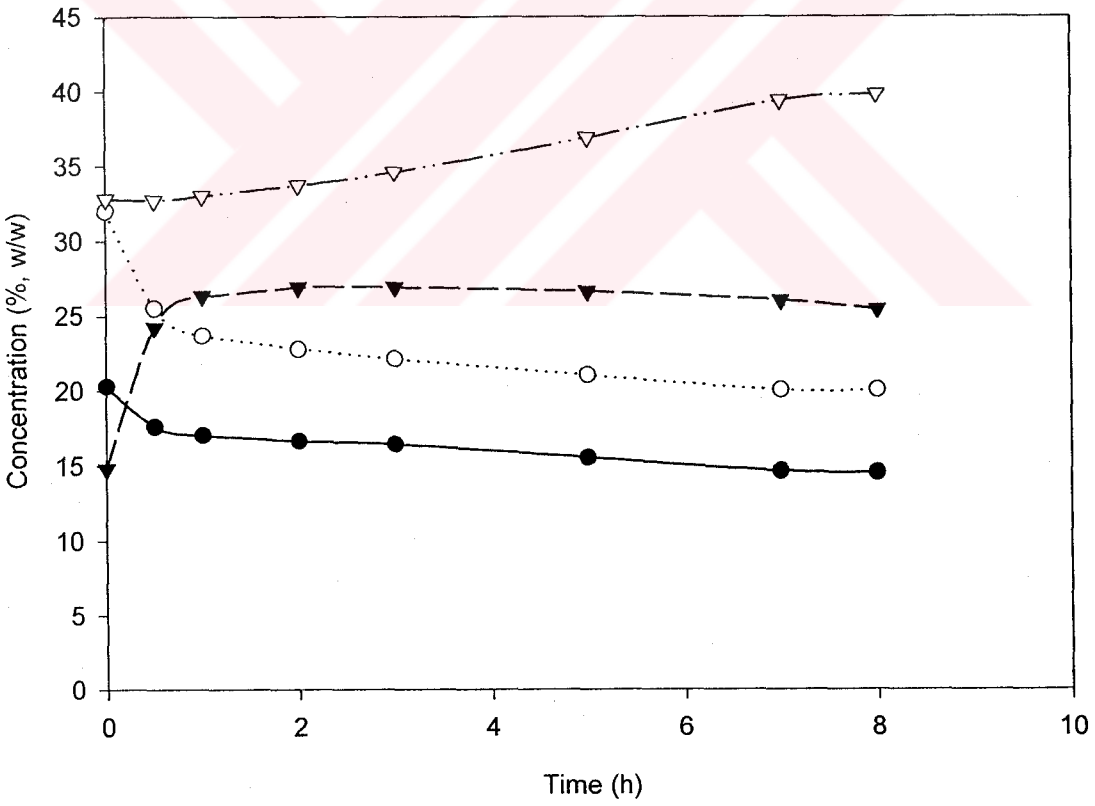

Figure 16. The change in glyceride composition of olive-pomace oil with time in the absence of glycerol. (○) FFA; (●) MAG; (▼) DAG; (▽) TAG.

All these results show that in the absence of glycerol, monoglycerides behave as a substrate more than an intermediate in the first stage (60 minutes) of the reaction. In the following stage, both of the intermediates, MAG and DAG, are constant because their rates of production and conversion into TAG is the same. So, it is possible to say that there is a net conversion of FFA into TAG in this second stage of the reaction. But, the results show that the ester, especially TAG, yield of this reaction is quite low. There is only 20 % increase in TAG on its own basis. As it is described earlier, the synthesis reaction catalyzed by immobilized enzymes needs a water free medium. It has been explained that glycerol behaves both as a substrate for glycerolysis and a powerful water binder that reduces the water activity of the reaction medium (Ferreira-Dias and Fonseca, 1995b). Although, the enough molecular sieve was used to bind the produced water during the reaction, absence of glycerol is a possible reason for the direct contact of water with the reaction components when it is produced. Other than that, FFA might have a higher affinity to glycerol to bind than MAG and DAG.

Detailed changes in FFA and glycerides (MAG, DAG, TAG) composition of the reaction mixture in the presence of glycerol with a molar ratio of 1/6 during the course of esterification reaction are given in Figure 17. FFA concentration showed an exponential decrease from 32 to 12 %. The concentration of MAG remained almost constant throughout the reaction with a final yield of 18 %. Only a slight increase and then a quick decrease was observed in the first 3 hours. DAG concentration followed a similar pattern to that of no-glycerol case. Equilibrium was almost reached in 2 h with a DAG concentration of 28 %. Again a similar increase trend in TAG concentration was observed. It increased from 33 to 43 %. This increase was significant statically ($p<0.05$). Slightly different patterns followed by the glycerides might be explained by the presence of glycerol in a molar ratio of Glycerol/FFA of 1/6.

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The higher degree of conversion into triglycerides was obtained with a glycerol to FFA molar ratio of 1/3 (Figure 18). Triglyceride concentration raised from 33 to 78 % while FFA concentration reduced from 32 to 2.5 % at the end of 8 hours of reaction time. In contrast to results shown in Figures 16 and 17 there was no significant change ($p< 0.05$) in diglyceride concentration. DAG concentration showed a slight increase from 15 to 19 % within the first 30 min and remains steadily

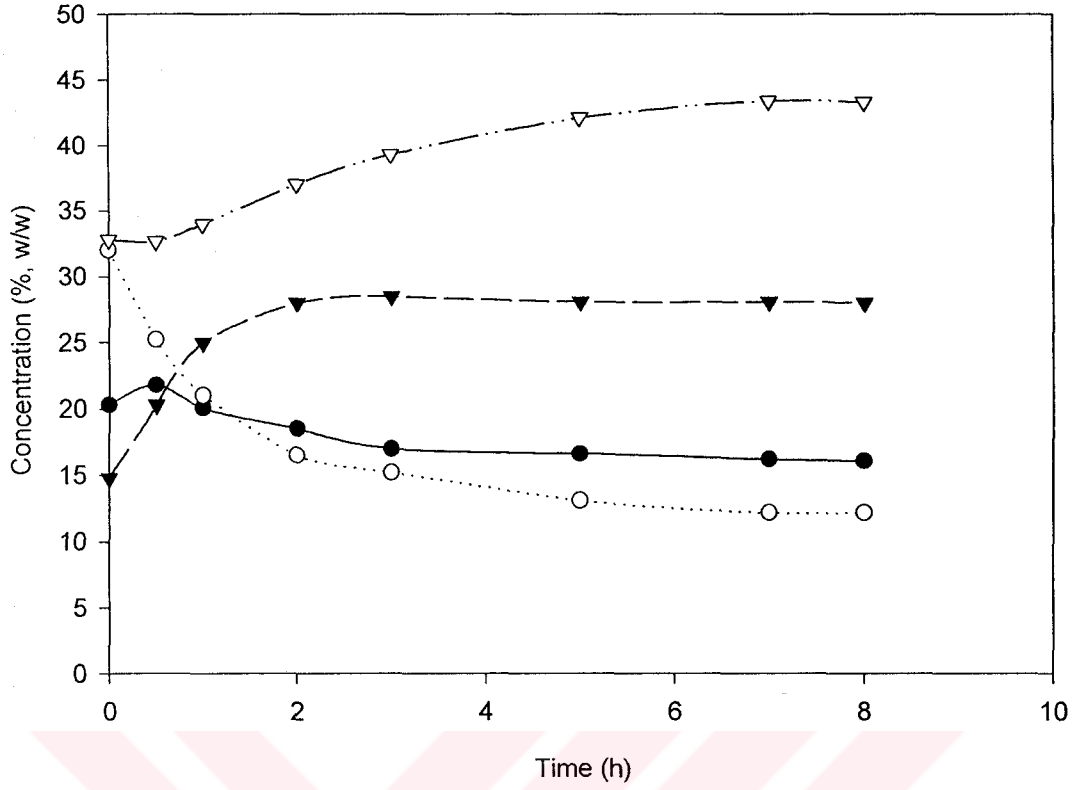

Figure 17. The change in glyceride composition of olive-pomace oil with time in the presence of glycerol (glycerol to FFA molar ratio of 1/6). (○) FFA; (●) MAG; (▼) DAG; (▽) TAG.

constant during the rest of the reaction and reached to a final concentration of 16.4 %. Monoglyceride concentration remains constant up to 2 hours of reaction time but then a significant decrease ($p<0.05$) from 20 to 4.6 % was observed. As seen in the plot, FFA concentration reduced significantly up to 2 hours but then the rate of reduction slowed down. From this reaction progress it can be concluded that esterification of FFA with glycerol produced MAG within the two hours of the reaction and MAG produced converted to DAG at the same rate. While DAG formed, conversion of DAG to TAG started quickly. After this point MAG concentration started to decrease and reduction in FFA concentration slowed down and reached to lowest value at the end of 3 hours. The possible explanation is that the conversions of MAG into DAG and DAG into TAG are directly related with the consumption of FFA in olive-pomace oil. The constant DAG concentration after the first hour of the reaction explains that the production and conversion rates of DAG

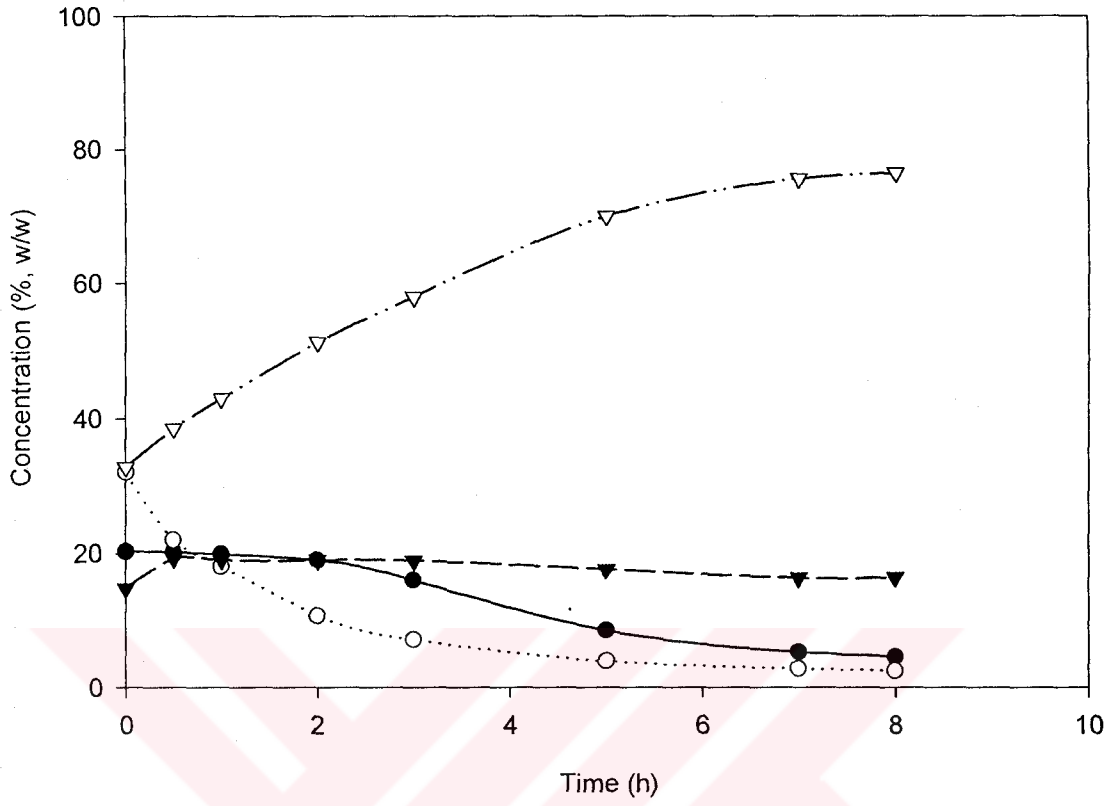

Figure 18. The change in glyceride composition of olive-pomace oil with time in the presence of glycerol (glycerol to FFA molar ratio of 1/3). (○) FFA; (●) MAG; (▼) DAG; (▽) TAG.

into TAG are equal. In the presence of limiting amount of glycerol, it seems to be possible to obtain higher conversion into TAG. Binding most of the FFA to available sites on glycerol and/or MAG and DAG would give higher concentration of TAG. When the excess amount of glycerol was added to the reaction medium, glycerol was converted into TAG in a lower degree with a high concentration of MAG and DAG. This might be explained by the possible random binding of FFA to available glycerol. It has also been reported by previous workers (McNeill *et al.*, 1990; Chang *et al.*, 1991) that in the enzymatic esterification of glycerol with oleic acid, an excess of glycerol was able to increase MAG production. The ratio of % triacyglycerol to % FFA for the reactions proceed at three different cases (no glycerol, Glycerol to FFA molar ratio of 1/3 and 1/6) is given in Figure 19. A significant increase ($p<0.05$) in % TAG/% FFA was observed for the limiting glycerol case. The ratio increased nearly

from 1 to 32 in the course of 8 hours reaction. This explains that the rate of FFA conversion into triacylglycerols is significantly higher ($p<0.05$) when glycerol is used in limiting amount.

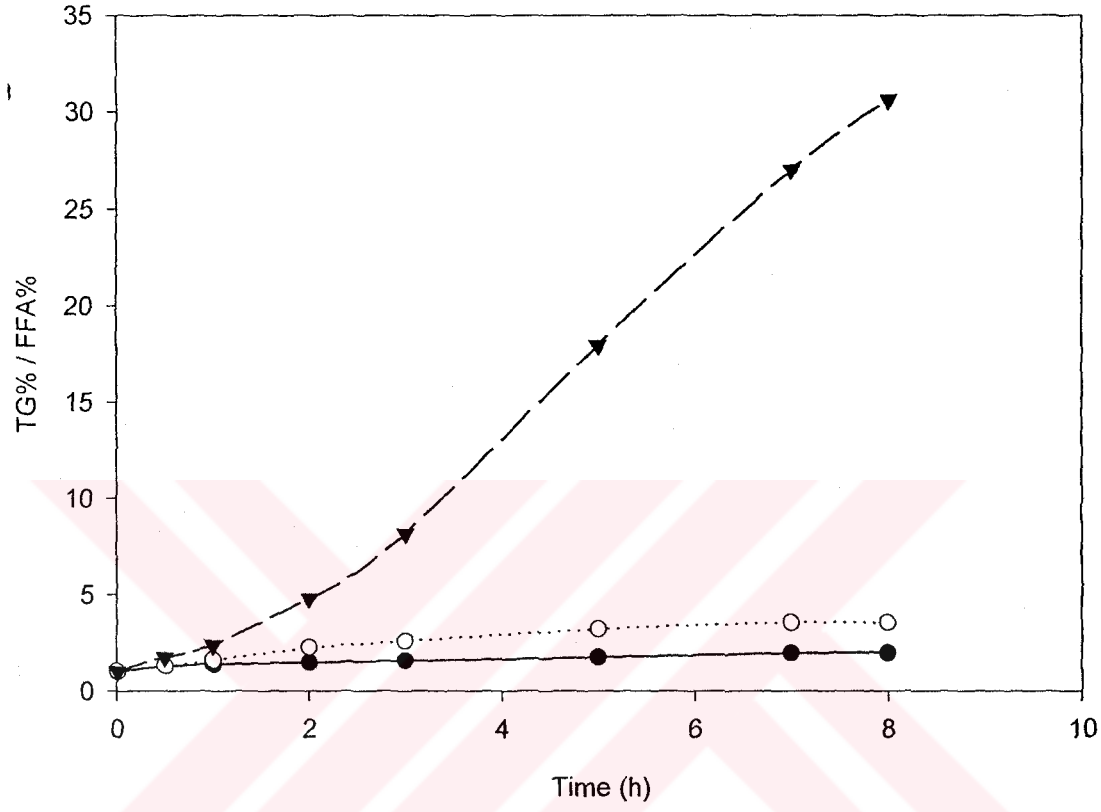

Figure 19. The change in the ratio of TAG % to FFA % with time in the absence and presence of glycerol. (●) in the absence of glycerol; (○) glycerol to FFA molar ratio of 1/6; (▼) glycerol to FFA molar ratio of 1/3.

The following aspects have to be considered in this reaction system:

- The use of solvent for the olive oil is advantageous in order to favor (a) the contact between immobilized lipase and glycerides, (b) the emulsification of glycerol in the organic medium, (c) product recovery (Ferreiara-Dias and Fonseca, 1995a). We used hexane as solvent in our reactions. It is well known that the kind of organic solvent strongly affects enzyme activity and selectivity in organic media (Parida and Dordick, 1991). A solvent is used to decrease the viscosity (Ison *et al.*, 1988) and increase the mass transfer of the substrates. A useful solvent for lipase-catalyzed esterifications should (i) be

nontoxic to permit use in a wide range of products, (ii) not affect the stability and activity of biocatalysts, (iii) form a suitable azeotropic mixture with water, and (iv) have a reasonable price. Only very few solvents fulfil most of these criteria. Hexane is one of these solvents which are permitted by (German) authorities (Yan *et al.*, 2002). Ferreira-Dias and Fonseca (1995a) used n-hexane for the production of monoacylglycerols by glycerolysis of olive oil with immobilized lipase and reported that n-hexane is used widely for the extraction and processing of fats and oils. Abraham *et al.* (1988) used a hexane to substrate ratio of 3, same with our ratio, and observed that the interesterification yield increased from 6.2 % to 18.7 %.

- The hydrophobic character of glycerides vs. the hydrophilic character of glycerol, which prevents the solubilization of glycerol in the organic medium (Ferreiara-Dias and Fonseca, 1995a). Since glycerol is not soluble in n-hexane, this alcohol had to be emulsified in the organic medium by vigorous stirring. The hydrophilic matrix captures glycerol molecules from the glycerol droplets. Thus, the access of glycerol to the enzyme depends on the mixing regime (Ferreiara-Dias and Fonseca, 1995b).
- The hydrophilic phase (glycerol) has a high viscosity and density, leading the glycerol accumulation in the bottom of the reaction vessel (Ferreiara-Dias and Fonseca, 1995a).
- We used RI detector for FFA analysis by HPLC, but UV detector for MAG, DAG and TAG analysis. As reported by Ergan & Andre (1989), it has been found that the refractive index (RI) detector is preferable for determination of FFA by HPLC. For all other compounds (MAG, DAG and TAG), the sensitivity of ultra violet (UV) detector (at 206 nm) was found to be better than that of the RI detector.
- The use of olive oil, a monounsaturated vegetable oil containing oleic acid as a major component and several other longer chain fatty acids (C_{16}-C_{22}), instead of a model system with only one type of glycerides increases the complexity of the system and thus of the analytical methods (Ferreiara-Dias and Fonseca, 1993).

CHAPTER V

CONCLUSIONS

In this work, enzymatic esterification was applied to highly acidic (32 % FFA) olive-pomace oil in order to reduce the acidity. Effects of molecular sieve, enzyme concentration, temperature and glycerol amount were investigated.

The following results were obtained from the studies,

- In the absence of molecular sieve, FFA content decreased from 32 to 18 % within 60 min but a gradual increase was observed later. When the amount of molecular sieve introduced into reaction medium was increased, the FFA content in the medium reduced greatly ($p<0.05$). Initial FFA content (32 %) reduced to 9.58, 5.23 and 2.36 % with the molecular sieve of 350, 500 and 750 mg, respectively by the end of the reaction period of 420 min.
- A significant decrease ($p<0.05$) was observed in the FFA content up to 18.1 mg/mL of enzyme concentration.
- A significant decrease ($p<0.05$) was observed in FFA content with increasing reaction time at all temperatures. The decrease in FFA content is sharper at higher temperatures. FFA content reached into equilibrium in almost 120 min at 60°C. However the time to reach into the equilibrium was around 300 min at 40 and 50°C.
- In the absence of glycerol, FFA concentration decreased from 32 to 21 % in 8 hours. MAG concentration decreased from 20 to 14.5 % by the end of the reaction. DAG concentration reached to 26 % and TAG concentration increased from 33 to 40 % at the end of the reaction. The increase in TAG was found to be significant statically ($p<0.05$).
- In the presence of glycerol with glycerol to FFA molar ratio of 1/6, FFA concentration decreased from 32 to 12 %. MAG concentration remained almost constant throughout the reaction with a final yield of 18 %. DAG concentration followed a similar pattern to that of no-glycerol case. TAG

concentration increased from 33 to 43 %. This increase was significant statically ($p<0.05$).

- When the excess amount of glycerol was added to the reaction medium, glycerol was converted into TAG in alower degree with a high concentration of MAG and DAG.
- The higher degree of conversion into triglycerides was obtained with a glycerol to FFA molar ratio of 1/3 (limiting glycerol case). TAG concentration raised from 33 to 78 % while FFA concentration reduced from 32 to 2.5 % at the end of 8 hours of reaction time. % TAG/ % FFA increased nearly from 1 to 32 in the course of 8 hours reaction.

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APPENDICES

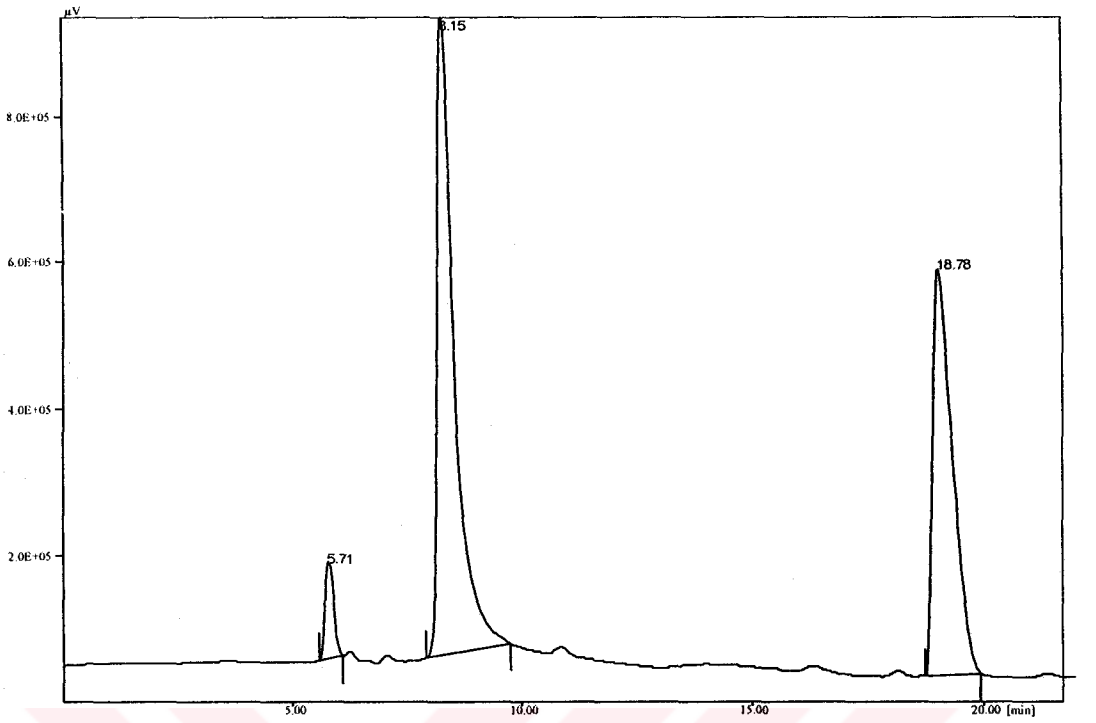

Figure A1. HPLC chromatogram of oleic acid and triolein mixture (oleic acid; RT: 8.15 min., triolein; RT: 18.78 min).

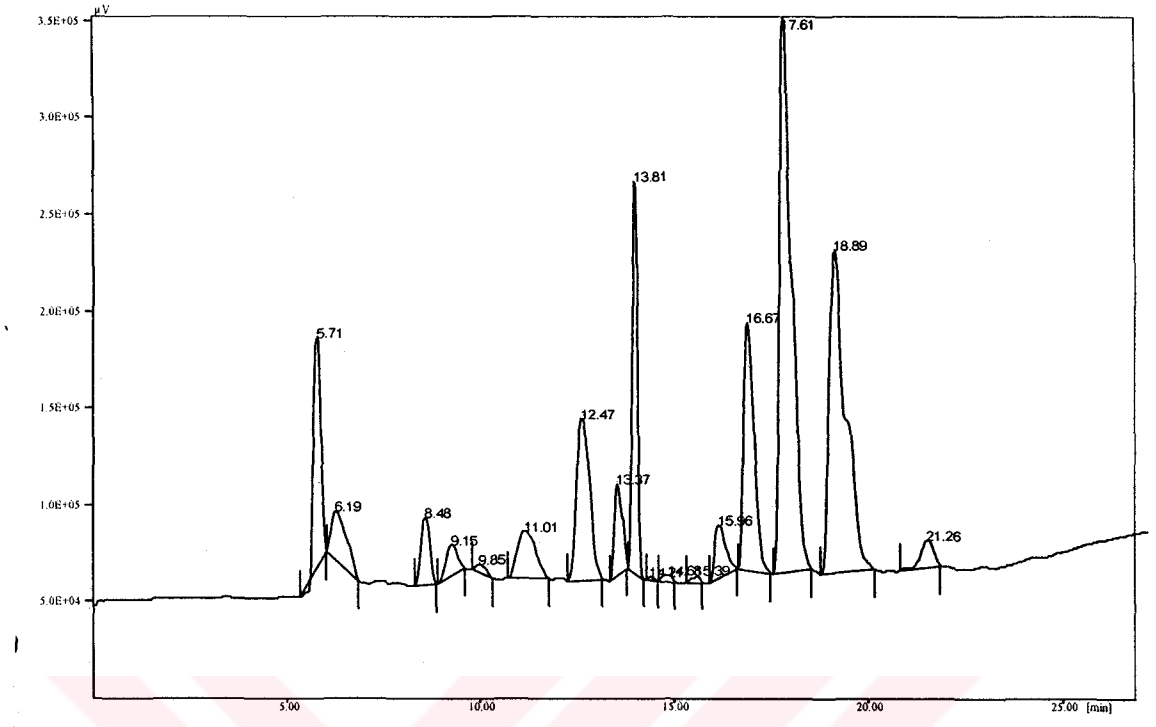

Figure A2. HPLC chromatogram of refined olive oil.

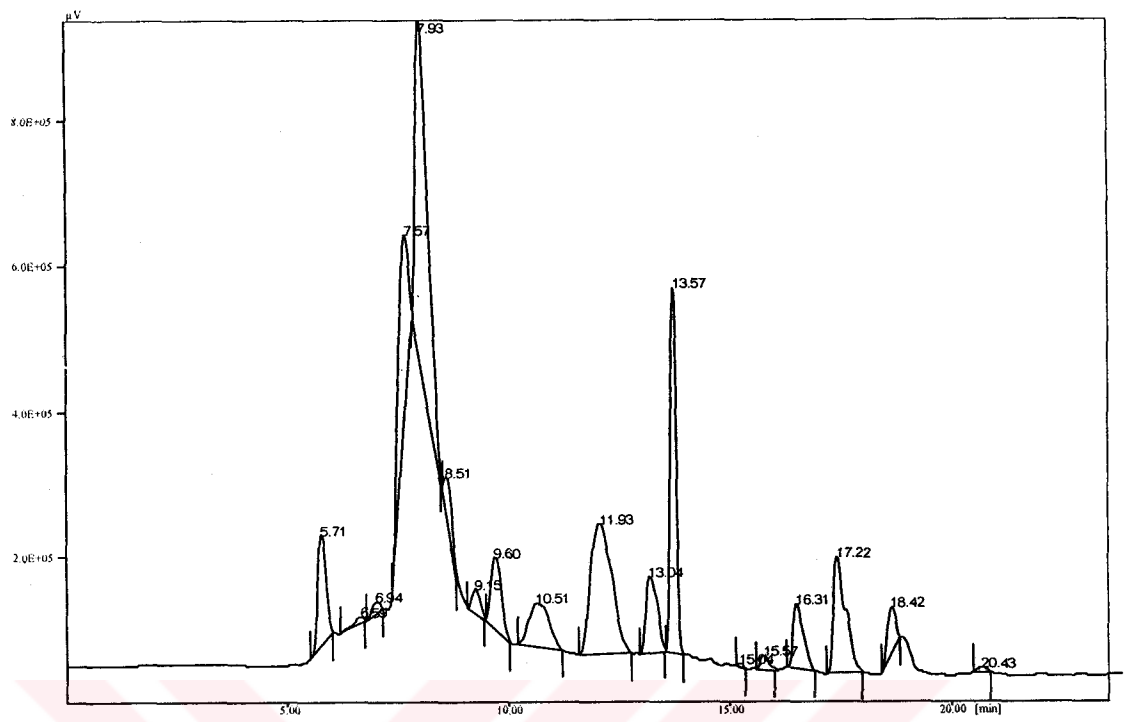

Figure A3. HPLC chromatogram of olive-pomace oil.

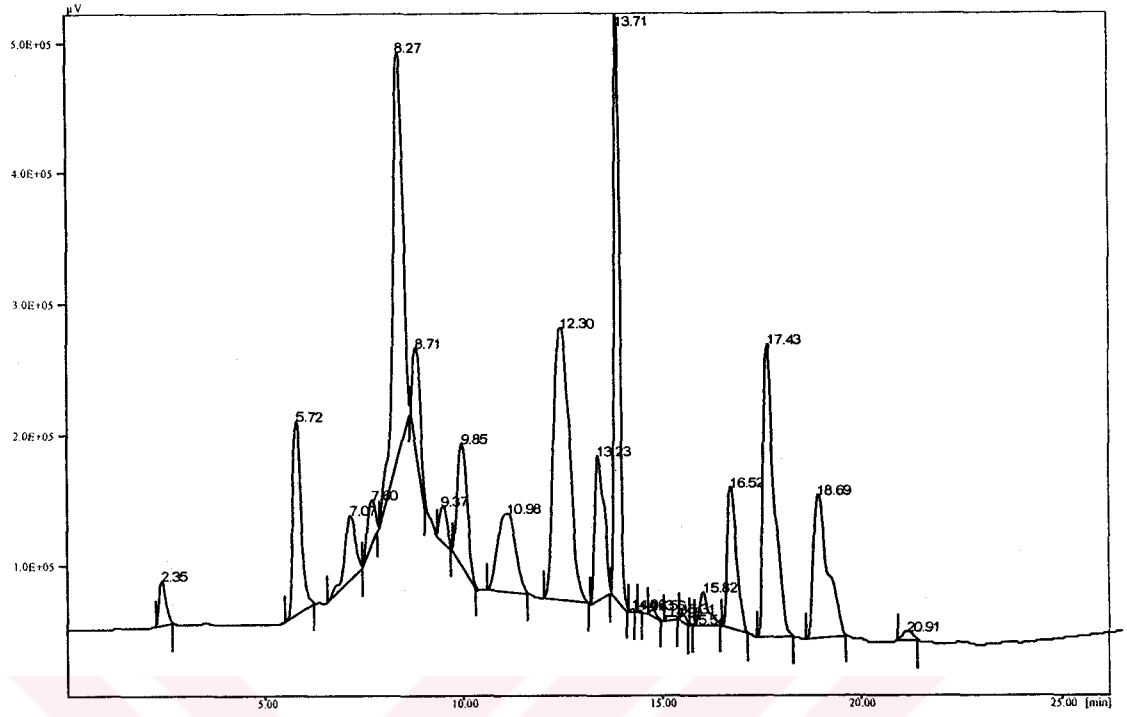

Figure A4. HPLC chromatogram of olive-pomace oil enzymatically esterified for 8 h (Gly/FFA molar ratio of 1/3).