İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

CHEMICAL CHARACTERIZATION OF AYVALIK MONOCULTIVAR OLIVE OILS

M.Sc. Thesis by
Food Engineer SİDAL ACUN

Department : Food Engineering

Programme: Food Engineering

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M.Sc. Thesis by
Food Engineer Sidal ACUN
(506051508)

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Supervisor (Chairman):	**Prof. Dr. Artemis KARAALİ**
Members of the Examining Committee	**Prof. Dr. Güldem Üstün**
	Yrd. Doç. Dr. Gürbüz Güneş

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AYVALIK ZEYTİNYAĞLARININ KİMYASAL KARAKTERİZASYONU

YÜKSEK LİSANS TEZİ

Sidal ACUN
(506051508)

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Tez Danışmanı: **Prof. Dr. Artemis KARAALİ**

Diğer Jüri Üyeleri: **Prof. Dr. Güldem Üstün**

Yrd. Doç. Dr. Gürbüz Güneş

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ÖZET

Akdeniz'e kıyısı olan ülkelerde yetişen zeytinyağı, Akdeniz diyetinde önemli olduğu kadar bu ülkelerin sosyal ve ekonomik yaşamlarında da önemli bir yere sahiptir. Türkiye üretim ve ihracatta dünyada beşinci sırada yer almaktadır. Ayvalık Türkiye'deki başlıca zeytin türüdür. Bu çalışmada, Çanakkale bölgesinden alınan Ayvalık tipi zeytinyağlarının kimyasal karakterizasyonu incelenmiştir. Naturel sızma (EVOO), organik (OGOO), naturel birinci (OO-1), naturel ikinci (OO-2) ve rafine zeytinyağı (RFOO) olmak üzere beş farklı sınıftan ticari Ayvalik tipi homojen zeytinyağı kullanılmıştır. Zeytinyağı örnekleri, Türkiye'nin Çanakkale bölgesi'nden 2005/06 ve 2006/07 yıllarında hasat edilmiş, Ayvalık tipi zeytinden elde edilmiştir. Zeytinyağların kimyasal karakterizasyonu için kalite parametrelerine (FFA, iyodin, K_{232} veK_{270} değerleri), yağ asidi içeriğine (Gaz Kromatografisi (GC)), fenolik madde profiline ve miktarına (Sıvı Kromatografisi-Kütle Spektroskopisi (LC-MS) ve Folin-Ciocalteu metodu) bakılmıştır. Analizlerin sonucunda, genel olarak alınan örneklerin kalite parametreleri (FFA, iyodin, K_{232} veK_{270} değerleri) ve yağ asidi içerikleri Avrupa Birliği Zeytinyağ Standardı ile uyumluluk göstermiştir. GC ile bakılan yağ asidi komposizyonu sonucu, literatürdeki birçok zeytinyağında olduğu gibi, başlıca yağ asidi oleik asit olarak saptanmıştır. Bununla birlikte, Türkiye Meteoroloji Müdürlüğü'nden Çanakkale bölgesinin 2005/06 ve 2006/07 hasat yıllarına ait iklimsel verileri alınmıştır. 2006 hasat yılına ait yağların 2005 yılındakilere oranla daha az toplam tekli doymamış yağ asidi (MUFA) içermesi, 2006 yılı yazının kurak geçmesi ile yorumlanmıştır. Ayrıca, iki yıla ait yağların toplam fenolik madde içeriklerinde istatiksel farkın bulunmaması, her iki yılın yaz ayları boyunca toplam yağış miktarının çok yakın olmasıyla açıklanmıştır. Folin-Ciocalteu metoduna göre ölçülen yağların toplam fenolik madde içerikleri, LC-MS analizi sonucunda bulunan fenolik maddelerin toplamından daha yüksek çıkmıştır. LC-MS analizi sonucunda, genel olarak 2005 yılına ait yağların 2006 yağlarına oranla daha yüksek oranda hidroksitirosol ve tirosol içermesi, 2005 yılına ait yağların depoda belli bir sure beklemiş olmasıyla açıklanmıştır. Ayvalık tipi zeytinyağlarının fenolik madde profilinde, tirosol, *para*-kumarik asit, vanilin, luteolin and apigenin, dekarboksimetil oleuropein aglikon, 1-asetoksipinoresinol, pinoresinol, olueropein aglikonun aldehit formu and ligstrosit aglikonun aldehit formunun yanı sıra başlıca fenolik madde olarak Pinoresinol tespit edilmiştir. Ayvalık zeytinyağlarında saptanan Pinoresinol'un diğer birçok ülkedeki zeytinyağ türlerine göre daha yüksek konsantrasyonda bulunması coğrafik bir belirti olarak sayılabileceğinden önemli bir bulgudur.

SUMMARY

Olive oil has been producing in the countries surrounding the Mediterranean Sea and has played an important role in the diet of the people in this area as well as their economy and culture. Turkey is one of the world's fifth largest producers and exporters of olive oil, and Ayvalik is the major olive-producing cultivar in Turkey.
The aim of this study is to investigate the chemical characterization of Ayvalik monocultivar olive oils. From this aspect, five different classes of commercial Ayvalik monocultivar olive oil samples, which are Extra Virgin Olive Oil (EVOO), Organic Virgin Olive Oil (OGOO), Virgin Olive Oil (OO-1), and Ordinary Virgin Olive Oil (OO-2) and Refined Olive Oil (RFOO) were examined. Olive fruits were harvested in two consecutive seasons (2005/06 and 2006/07) from the Canakkale olive-growing region of Turkey. For the chemical characterization of oils, the quality parameters, as ffa, iodine value, UV characteristics (K_{232} and K_{270}), fatty acid composition (Gas Chromatography (GC)), polyphenols (Liquid Chromatography–Mass Spectroscopy (LC-MS) and Folin-Ciocalteu method) were evaluated. As a result of quality parameters (FFA, iodine value and UV characteristics) and fatty acid composition analysis, all samples met the limits set on the European Standard for Olive Oils and Olive Pomace Oils with minor deviations, because of extended storage period of 2005 samples. While assaying fatty acid composition in GC, oleic acid was found the dominant fatty acid like most olive oils indicated in the literature. Moreover, climatic data of Canakkale Region were obtained from the Turkish State Meteorological Service belonging to 2005/06 and 2006/07 years. From this aspect, dry summer in 2006 crop season induced lower total monounsatured fatty acid (MUFA) percentage and iodine value of 2006 samples than samples belong to 2005. On the other hand, due to the similarity between accumulated rainfall during summer, significant differences in total phenolic content of samples belonging to two years were not observed. In general, increasing trend was observed in respective total phenolics content by Folin when compared to total LC-MS values. Furthermore, with regard of LC-MS analysis, hydroxytyrosol and tyrosol content of 2005 crop season olive oils were higher than 2006 season oils due to extended storage period of 2005 samples. On the other hand, besides, tyrosol, p-coumaric acid, vanilin, luteolin and apigenin, decarboxymethyl oleuropein aglycon, 1-acetoxypinoresinol, aldehydic form of olueropein aglycon and aldehydic form of ligstroside aglycon, the main phenolic compound detected in Ayvalik monocultivar oil was pinoresinol, which was found higher amount than many olive oils from other countries. This important finding can be used proposed to be as a geographical marker of Ayvalik monocultivar olive oil.

1. INTRODUCTION

Recent statistics show that the people settled around the Mediterranean Sea have a relatively high life expectancy, so Mediterranean diet is being investigated extensively. Olive oil is the fat source of this diet (Harwood and Yaqoob, 2002), and is claimed for its high for nutritional value and beneficial effect on health (Wahrburg et al., 2002).

In last decades, the world has become more aware of the importance of the olive oil, not only for health aspects, but also for its economical value (Wahrburg et al., 2002). There exists an extensive literature on the quality of the olive oil from many different countries.

The International Olive Oil Council (IOOC) is the world agency that set the standards for olive oil. However, the standardization of olive oil is not an easy task due to many variations: the varieties of olives, climatic conditions and technological aspects (Luchetti, 2002).

2. LITERATURE

2.1 The olive tree

Olive trees grow naturally especially in the Mediterranean area. The oldest known and slow-growing olive tree is called *Olea europea*, since it is mostly cultivated in southern Europe (Lucetti, 2002). The most advantageous characteristic of the olive tree is its resistance to unfavorable conditions such as drought and rocky soils. However, climate of the Mediterranean area induces higher yield of the olive tree because of frequency of rainfall (Berenguer, 2006).
Due to the periodicity characteristics of the olive tree, it yields better product in one crop year and less in the following one. (Kiritsakis, 1990).

2.2 Olive Oil Formation

Oil formation in an olive fruit starts in the middle of July in general, and reaches the maximum amount almost at the end of January depending on the variety (Kiritsakis, 1990). The olive fruit with 15-40 % oil is the most suitable cultivar for the production of olive oil. In addition, olives with ratio of pulp/kernel 4:1 to 8:1 are suitable for olive oil production, whereas those with ratio of 7:1 to 10:1 are suitable for table olives.

2.3 Olive Oil

Olive oil is quite unique since it is edible even without any chemical treatment and it is the main source of fat in the Mediterranean diet (Carrasco-Pancorbo et.al, 2005). It is a quite stable oil against oxidative deterioration because of its high percentage of monounsaturated fatty acid content and the antioxidative activity of its minor constituents such as phenolic compounds (Carrasco-Pancorbo et.al, 2005). Olive oil can be produced either as “heterocultivar” or as “monocultivar” oil. Characterisation of olive oil has become one of the most researched subjects in the food science. For

the characterization of monocultivar olive oil, there are many factors that have to be taken into account, such as environmental (soil, climate), agronomic (irrigation, fertilization), cultivation (harvesting, ripeness), technological factors (post-harvest storage, extraction system) (Aparicio and Luna, 2002).

2.4 Turkish Olive Fruit Cultivars

Olive trees have been grown along the Aegean coast of Turkey for over 8000 years.

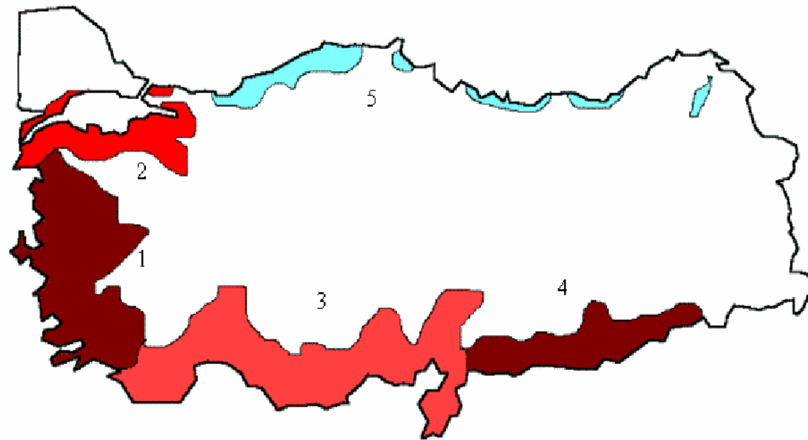
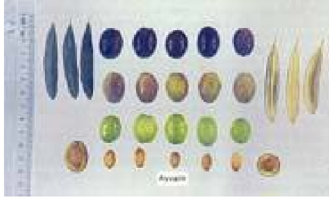


Figure 2.1: Map of Olive Production Sites in Turkey (Ozkaya, 1999).

Turkey has a wide range of olive orchards and olive fruit cultivars, as shown Table 2.1, Fig 2.1, Fig 2.2, Ayvalik (Edremit Yaglik) is one of the major olive producing areas in Aegean region of Turkey and it is most commonly used for olive oil production in Turkey. These olives are either cylindrical shaped or almost circular, and contain 24,72 % oil and 85, 26 % pulp on the average (Ozkaya, 1999).

Table 2.1: Regional Distribution of Major Turkish Olive Cultivars in Turkey (Ozkaya, 1999).

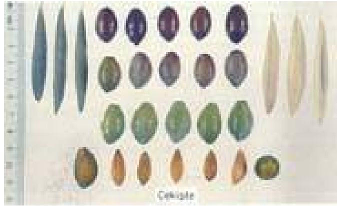
Aegean Region (1)	**Marmara Region (2)**	**Mediterranean Region (3)**	**Southeast Anatolia Region (4)**	**Black sea Region (5)**
Ayvalik (Edremit yaglik, Sakran, Midilli, Ada) **Cakır Cekiste** (Kirma, Memeli) **Cilli** (Tekir, Provens,Goloz) **Domat** **Erkence** (Izmir yaglik) **Gemlik** (Triliye, Kaplik, Kivircik, Kara) **Izmir Sofralik** **Memeli** (Emiralem, Akzeytin, Cekiste) **Uslu** **Memecik** (Tas arasi, Asiyel, Tekir, Gulumbe, Sehir, Yaglik) **Kiraz**	**Ayvalik** (Edremit yaglik, Sakran, Midilli, Ada) **Celebi** (Iznik celebi) **Edincik su** (Erdek su) **Gemlik** (Triliye, Kaplik, Kivircik, Kara) **Karamursel Su** (Kalamata) **Samanli** (Tatli zeytin)	**Gemlik** (Triliye, Kaplik, Kivircik, Kara) **Memecik** (Tas arasi, Asiyel, Tekir, Gulumbe, Sehir, Yaglik) **Büyük Topak** **Ulak** (Toprak Asi) **Egriburun** **Saurani** (Savrani) **Tavsan yüregi** (ters yaprak) **Uslu**	**Ayvalik** (Edremit yaglik, Sakran, Midilli, Ada) **Egriburun** **Halhali** **Kalembezi** **Kan Celebi** **Kilis Yaglik** **Nizip Yaglik** **Yag Celebi** **Hasebi** **Sari Ulak** **Gemlik** (Triliye, Kaplik, Kivircik, Kara) **Memecik** (Tas arasi, Asiyel, Tekir, Gulumbe, Sehir, Yaglik)	**Gemlik** (Triliye, Kaplik, Kivircik, Kara) **Memecik** (Tas arasi, Asiyel, Tekir, Gulumbe, Sehir, Yaglik)



Ayvalik

Buyuk Topakulak

Cakir



Cekiste

Celebi

Cilli

Domat

Edincik

Egriburun

Erkence

Gemlik

Halhali

Izmir Sofralik

Kalembezi

Kancelebi

Karamursel Su

Kilis Yaglik

Kiraz

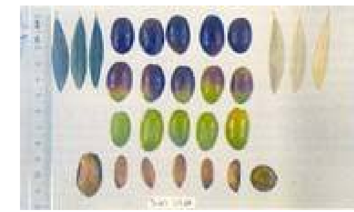
Memecik

Memeli

Nizip Yaglik

Samanli

Sari Hasebi



Sari Ulak

Savrani

Tavsan Yuregi

Uslu

Yag Celebi

Figure 2.2: Turkish Olive Fruit Cultivars (Anon, 2006)

2.5 Turkish Olive Oil

Turkey now produces one-third of the olives consumed in the European Union, and is World's fifth largest producers of olive oil (Table 2.2) (FAO, 2006).

The olive oil industry in Turkey mostly produces refined olive oil (70-75 %), and also olive oil by mixing extra virgin olive oil (almost 80 %) and refined olive oil (almost 20 %) which is named as "Riviera" in Turkey. These last two types are the most preferred by consumers in Turkey. Besides the characteristic odor and taste of virgin olive oil, the cost contributes to this choice (Ozkaya, 1999).

Table 2.2: The Five Top Countries in Olive Production and Export (FAO, 2006).

	Quantity produced olives (1000 tonnes)		**Export quantity (1000 tonnes)**	
Country	**2003**	**2004**	**2003**	**2004**
Italy	4,424.13	5,202.67	1,546.91	3,561.76
Spain	2,698.14	1,371.70	2,756.56	5,611.18
Greece	741.85	1,103.70	432.22	514.01
Turkey	655.6	947.39	513.95	471.31
Tunisia	277.58	277	195.04	1,691.57

2.6 Olive Oil Classification

Codex Alimentarius, International Olive Oil Council Standards and the European Comission Regulation 2568/91 and its amendments describe the quality and authenticity characteristics of olive oil in detail. However, commercial olive oil types are classified as below only according to their free acidity contents.

a) Extra virgin olive oil: Virgin olive oil with a free acidity, expressed as oleic acid, of not more than 0.8 gram per 100 grams.

b) Virgin olive oil: Virgin olive oil with a free acidity, expressed as oleic acid, of not more than 2.0 grams per 100 grams.

c) Ordinary virgin olive oil: Virgin olive oil with a free acidity, expressed asoleic acid, of not more than 3.3 grams per 100 grams.

d) Refined olive oil: Olive oil obtained from virgin olive oils by refiningmethods which do not lead to alterations in the initial glyceridic structure. It has a free acidity, expressed as oleic acid, of not more than 0.3 grams per 100 grams.

e) Olive oil: Oil consisting of a blend of refined olive oil and virgin olive oilssuitable for human consumption,. It has a free acidity, expressed as oleic acid, of not more than 1 grams per 100 grams.

f) Refined olive-pomace oil: Oil obtained from crude olive-pomace oil by refining methods which do not lead to alterations in the initial glyceridic structure. It has a free acidity, expressed as oleic acid, of not more than 0.3 grams per 100 grams.

g) Olive-pomace oil: Oil consisting of a blend of refined olive-pomace oil and virgin olive oils. It has a free acidity, expressed as oleic acid, of not more than 1 gram per 100 grams.

2.7 Olive Oil Chemistry

2.7.1 The olive fruit

Oval shaped olive fruit has two main parts, the pericarp (skin and pulp) and the endocarp (seed). Skin and pulp parts of olive contain 96-98 % of oil, and seed contains 2-4 % (Kiritsakis, 1990). 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 %. Others constituents are pectins, organic acids, pigments and glycosides of phenols (Boskou, 1996).

Oleuropein, ester of elenolic acid with 3,4-dihydroxyphenylethanol (hydroxytyrosol), is the typical phenolic compound for olive that gives its bitter taste to the fruit. During ripening, oleuropein and ligstroside, another phenolic compound in olive fruits, hydrolize to hydroxytyrosol and tyrosol which are the two main polar polyphenols found in the olive oil (Fig. 2.3) (Vissers et al.,2001).

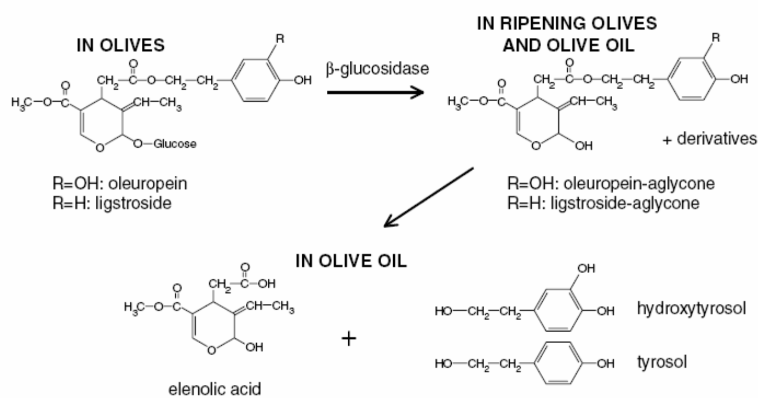


Figure 2.3: Hydrolysis Reactions of Phenolic Aglycones into Tyrosol and Hydroxytyrosol (Vissers et al., 2001).

One liter of olive oil is produced from almost five kilograms of olives (Luchetti et al., 2002).

2.7.2 Olive oil composition

Olive oil is mainly composed of triacylglycerols, free fatty acids and some 0,5-1 % of nonglyceridic constituents (Boskou, 1996). Some sources define chemical composition of olive oil according to its saponifiable fraction (TAG, FFA, phosphatides etc.) and its unsaponifiable fraction (fatty acids, hydrocarbons etc.) (Kiritsakis, 1990), whereas some others according to its minor and major components (Boskou, 1996; Servili and Montedoro, 2002).

Fatty acids and triacylglycerols are the major components of olive oil and represent about 98 % of total oil weight and remaining 2 % of olive oil is made up of volatile compounds, sterols etc., as the minor components (Servili and Montedoro, 2002).

Olive oil chemical content varies mostly due to agronomic (maturity, climate, crop season, production area etc.) and technological aspects (Servili and Montedoro, 2002; Aparicio and Luna, 2002; Salvador et al., 2003).

2.7.2.1 Fatty acids

Depending on olive variety, climatic conditions before and during harvest, production site and some other factors, the fatty acid composition of olive oil varies widely (Kiritsakis, 1990; Boskou, 1996; Luchetti, 2002). The main fatty acid in olive

oil is the monounsaturated oleic acid (18:1) which is the most concentrated fatty acid (70-80 % in weight) in olives. Stearic (18:0), linoleic (18:2), palmitic (16:0) and palmitoleic (16:1) are the other main fatty acids.

Due to higher percentage of monounsaturated fatty acid and lower percentage of saturated fatty acid in its composition, virgin olive oil has more stability to oxidation than many other vegetable oils (Kiritsakis, 1990). In Table 2.3, the limits for the fatty acid contents in olive oil as set by European Union Commission standard for olive oils and olive pomace oils are presented:

Table 2.3: Fatty Acid Composition as Determined By Gas Chromatography (% Total Fatty Acids) (EU, 2002).

Fatty acid	Virgin olive oils	Olive oil Refined olive oil	Olive-pomace oils
C14:0	0.0 – 0.05	0.0 – 0.05	0.0 – 0.05
C16:0	7.5 – 20.0	7.5 – 20.0	7.5 – 20.0
C16:1	0.3 – 3.5	0.3 – 3.5	0.3 – 3.5
C17:0	0.0 – 0.3	0.0 – 0.3	0.0 – 0.3
C17:1	0.0 – 0.3	0.0 – 0.3	0.0 – 0.3
C18:0	0.5 – 5.0	0.5 – 5.0	0.5 – 5.0
C18:1	55.0 – 83.0	55.0 – 83.0	55.0 – 83.0
C18:2	3.5 – 21.0	3.5 – 21.0	3.5 – 21.0
C18:3	0.0 – 0.9	0.0 – 0.9	0.0 – 0.9
C20:0	0.0 – 0.6	0.0 – 0.6	0.0 – 0.6
C20:1	0.0 – 0.4	0.0 – 0.4	0.0 – 0.4
C22:0	0.0 – 0.2	0.0 – 0.2	0.0 – 0.3
C24:0	0.0 – 0.2	0.0 – 0.2	0.0 – 0.2
Trans fatty acids			
C18:1 T	0.0. – 0.05	0.0 – 0.20	0.0 – 0.40
C18:2 T + C18:3 T	0.0 – 0.05	0.0 – 0.30	0.0 – 0.35

2.7.2.2 Triacylglycerols (TAG)

A triacylglycerol (also known as triacylglyceride) is a glyceride in which the glycerol is esterified with three fatty acids. The major TAG in olive oil are OOO (40-59 %), POO (12-20 %), OOL (12,5-20 %), POL (5,5-7 %) and SOO (3-7 %) (P=Palmitic, O=Oleic, S=Stearic, and L=Linoleic) (Kiritsakis, 1990; Boskou, 1996).

2.7.2.3 Tocopherols

Tocopherols are important antioxidative compounds not only contributing to the stability of olive oil, but also for its health aspects, such as prevention against cardiovascular diseases and cancer (Wahrburg et al., 2002).

The tocopherol content decreases gradually during olive maturation, processing and storage stages. Olive oil contains mainly α-tocopherol which varies from few ppm to 300 ppm. Due to the loss of α-tocopherol during refining, it is recomended to add this compound to the oil for future stability (Blekas et al., 1995).

2.7.2.4 Hydrocarbons

Squalene is the major hydrocarbon in the olive oil and is quite abundant as compared with other vegetable oils. Squalene is the indicator of sterol formation (Kiritsakis, 1990).

2.7.2.5 Sterols

Sterols are the quality indicators of olive oil, which is in the range between 180 and 265 mg of 100 g oil. There are mainly four types of sterols in olive oil: 4α-desmethylsterols, 4α-methylsterols, 4,4-dimethylsterols and triterpene dialcohols (Boskou, 1996). Storage time and processing affect the sterol composition (Kiritsakis, 1990) and refining process decreases the sterols in the olive oil (Boskou, 1996).

2.7.2.6 Pigments

Pigments are not only responsible for contributing its greenish yellow to gold color to olive oil, but also for their prooxidant and antioxidant effects (Kiritsakis, 1990; Boskou, 1996). Chlorophylls, pheophytins and carotenoids are the dominant pigment types of olive oil.

Olive maturity and the extraction system affect the pigment concentration of the oil (Boskou, 1996). Olive oil possesses higher concentration of chlorophyll and carotenoids when obtained by centrifugation system than by percolation and pressure extraction (Kiritsakis, 1990; Boskou, 1996).

2.7.2.7 Phenolic compounds

Phenolic compounds posses benzene ring bearing one or more hyroxy groups with different derivatives, and those compounds with repeating phenols are named as "polyphenols". They can be obtained from the polar fraction of olive oil by extraction (Boskou, 1996; Carrasco-Pancorbo et al., 2005).

Olive oil contains different classes of polyphenols (Table 2.4 and Figure 2.4), and secoiridoids are the main phenolic compounds in olive oil which are derivatives of the secoiridoid glucosides of olive fruits (Servili and Montedoro, 2002). The other polyphenol groups are phenolic acids consisting of two subgroups, hydroxybenzoic acids (gallic, p-hydroxybenzoic, protocatechuic, vanillic, syringic acids and etc. (C_6-C_1 structure)) and hydroxycinnamic acids (caffeic, ferulic, p-coumaric, sinapic acids and etc. (C_6-C_3)) (Balasundram et al., 2006). Also, olive oil contains flavone compounds (C_6-C_3-C_6 structure) (Servili and Montedoro, 2002). In spite of some similarities in polyphenol content of olive oil and olive fruit, olive oil also possesses some different phenolic compounds (Table 2.4).

European Union standard gives the limits for many quality criteria, such as acidity, peroxide value etc. for individual classes of olive oil. However it does not state the limits for the total phenolic content and percentage of individual phenolic compounds in the olive oil. According to various research reports, olive oil's total polyphenol concentration may range between 50 and 1000 mg/kg (Servili and Montedoro, 2002). Polyphenols in olive oil are affected by both cultivation and processing methods (Boskou, 1996).

Polyphenols not only contribute to flavor (bitterness etc.), aroma and the stability of the olive oil (Aparicio and Luna, 2002; Salvador et al., 2003), but also contribute positively to human health, because polyphenols are important bioactive compounds due to their antioxidant, anti-allergenic, anti-artherogenic, anti-microbial etc. properties (Balasundram et al., 2006).

Recent studies have proven that polyphenols of olive oil are potent antioxidants, (Valavanidis et al., 2001), which exert free radical scavenging, hydrogen atom or electron donating and metal chelation properties exhibiting antioxidant activity of their related structures. Higher degree of hydroxylation results in higher antioxidant activity. Also, hydroxybenzoic acids have lower antioxidant activity than hydroxycinnamic acids (Balasundram et al., 2006).

Table 2.4: The Main Phenolic Compounds in Virgin Olive Oil and Olive Fruit (Servili and Montedoro, 2002).

Virgin Olive Oil	Olive Fruit
Phenolic acids and derivatives	**Antocyanins**
Vanillic acid	Cyanidin-3-glucoside
Syringic acid	Cyanidin-3-rutinoside
p-Coumaric acid	Cyanidin-3-caffeyglucoside
o-Coumaric acid	Cyanidin-3-caffeylrutinoside
Gallic acid	Delphinidin3-rhamosyglucosid-
7	
Caffeic acid	xyloside
Protocatechuic acid	
p-Hidroxybenzoic acid	**Flavonoids**
Ferulic acid	Quercetin-3-rutinoside
Cinnamic acid	
4-(acetoxyethil)-1,2-Dihydroxybenzene	**Flavones**
Benzoic acid	Luteolin-7-glucoside
	Luteolin-5-glucoside
Phenolic alcohols	Apigenin-7-glucoside
(3,4-Dihyroxyphenyl) ethanol (3,4-DHPEA)	
(*p*-Hydroxyphenyl) ethanol (p-HPEA)	**Phenolic acids**
(3,4-Dihdroxyphenyl) ethanol-glucoside	Clorogenic acid
	Caffeic acid
Secoiridoids	*p*-Hidroxybenzoic acid
Dialdehydic form of elenolic acid linked	Protocatechuic acid
to 3,4-DHPEA (3,4-DHPEA-EDA)	Vanillic acid
Dialdehydic form of elenolic acid linked	Syringic acid
to *p*-HPEA (p-HPEA-EDA)	*p*-Coumaric acid
Oleuropein aglycon (3,4-DHPEA-EA)	*o*-Coumaric acid
Ligstroside aglycon	Ferulic acid
Oleuropein	Sinapic acid
p-HPEA-derivative	Benzoic acid
	Cinnamic acid
Lignans	Gallic acid
(+)-1-Acetoxypinoresinol	
(+)-Pinoresinol	
(+)-1-Hydroxypinoresinol	
	Phenolic alcohols
	(3,4-Dihyroxyphenyl) ethanol (3,4DHPEA)
	(*p*-Hydroxyphenyl) ethanol (*p*- HPEA)
Flavones	
Apigenin	
Luteolin	**Secoiridoids**
	Oleuropein
	Demethyloleuropein
	Ligstroside
	Nüzhenide
	Hydroxycinnamic acid derivatives
	Verbascoside

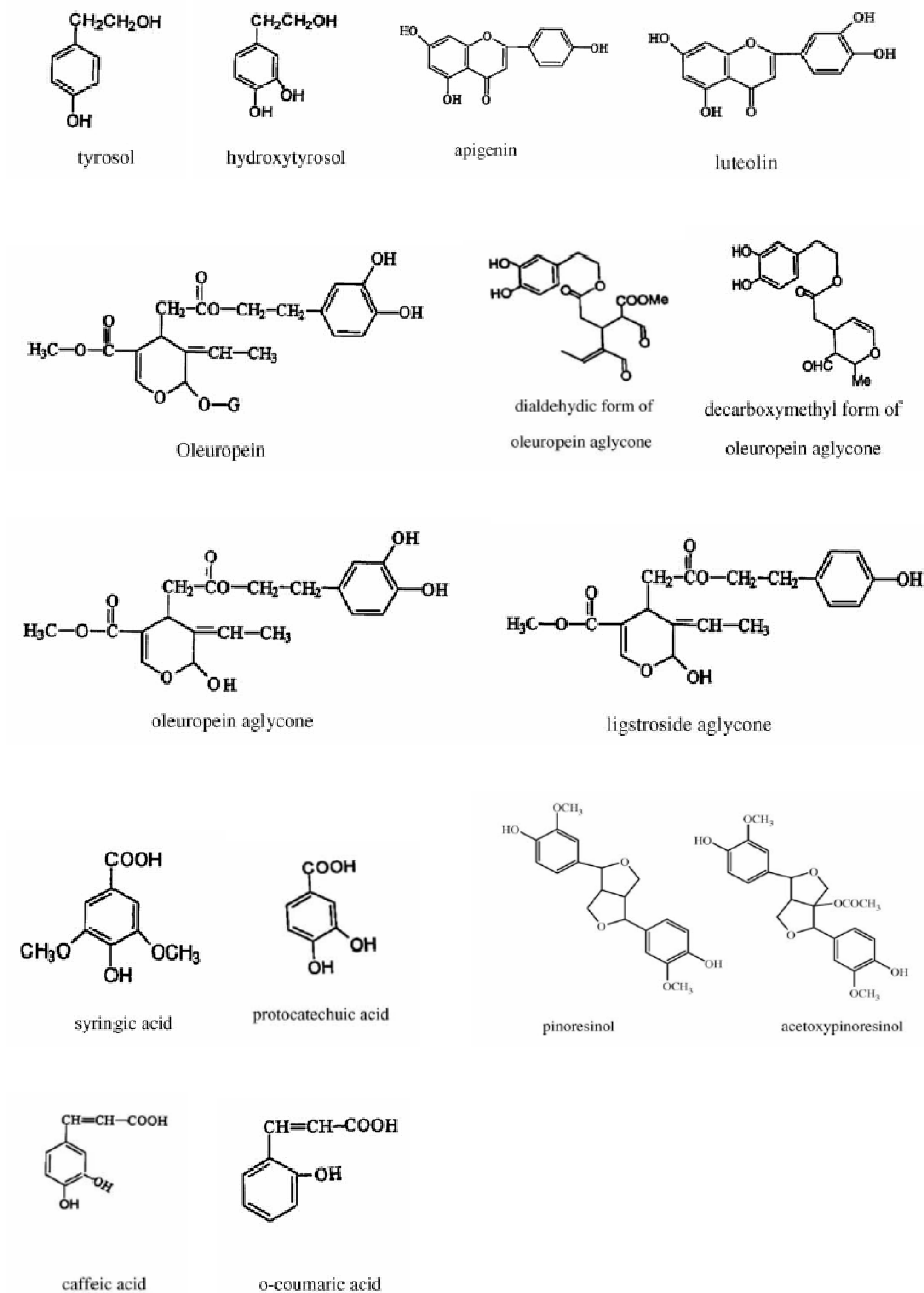


Figure 2.4: Major Olive Oil Phenolic Compounds (Boskou, 2006).

2.7.2.8 Determination of phenolic compounds in olive oil

An extensive literature exists concerning the detection and quantification methods of phenolic compounds in olive oil. Quantitative determination methods of phenolic

compounds in olive oil may be classified as spectrophotometric and chromatographic determination (Carrasco-Pancorbo et al., 2005).

As a spectrophotometric method, Folin-Ciocalteau is the most widely used non-specific colorimetric assay that determines total phenolic compounds in olive oil (Singleton et al., 1999). However determination of individual phenolic compounds in olive oil is assessed by chromatographic methods which contain three basic steps: extraction from the oil sample, analytical separation, and quantification (Carrasco-Pancorbo et al., 2005).

Liquid-liquid (LLE) and solid phase extraction (SPE) are two main techniques used for extraction of polar fraction from olive oil. Additionally, analytical separation can also be performed by thin-layer chromatography (TLC), high performance liquid chromatography (HPLC), gas chromatography (GC) and capillary zone electrophoresis (CZE), as well as by spectrophotometric methods (Carrasco-Pancorbo et al., 2005).

HPLC is the most reliable technique for quantitative detailed analysis of polyphenols and has been mainly used with UV detection, especially by photodiode array detector (Carrasco-Pancorbo et al., 2005). As well as HPLC-DAD, HPLC-MS/MS (HPLC-Mass Spectrometry) technique also shows good precision, recovery, linearity and sensitivity and most of phenolic compounds in olive oil can be detected and quantified this way (Torre-Carbot et al., 2005).

2.8 Olive Oil Processing

Processing is one of the most important factors that affect the quality of olive oil, besides cultivar, harvesting, and storage conditions. (Giovacchino et al., 2002). The olive oil processing entails harvesting, leaf removal and washing, crushing, malaxation, extraction and rarely filtration (Fig. 2.5). Although olive oil can be consumed without any chemical treatment, oils with very high free acidity should be refined in order to make edible. Refining process entails alkali refining, winterization, bleaching and deodorization steps (Fig. 2.5).

The aim of oil refining is the removal and the reduction of minor components, such as volatile components, pigments etc. that may affect the olive oil quality. Phenolic components are also eliminated by refining the oil (Garcia et al., 2006).

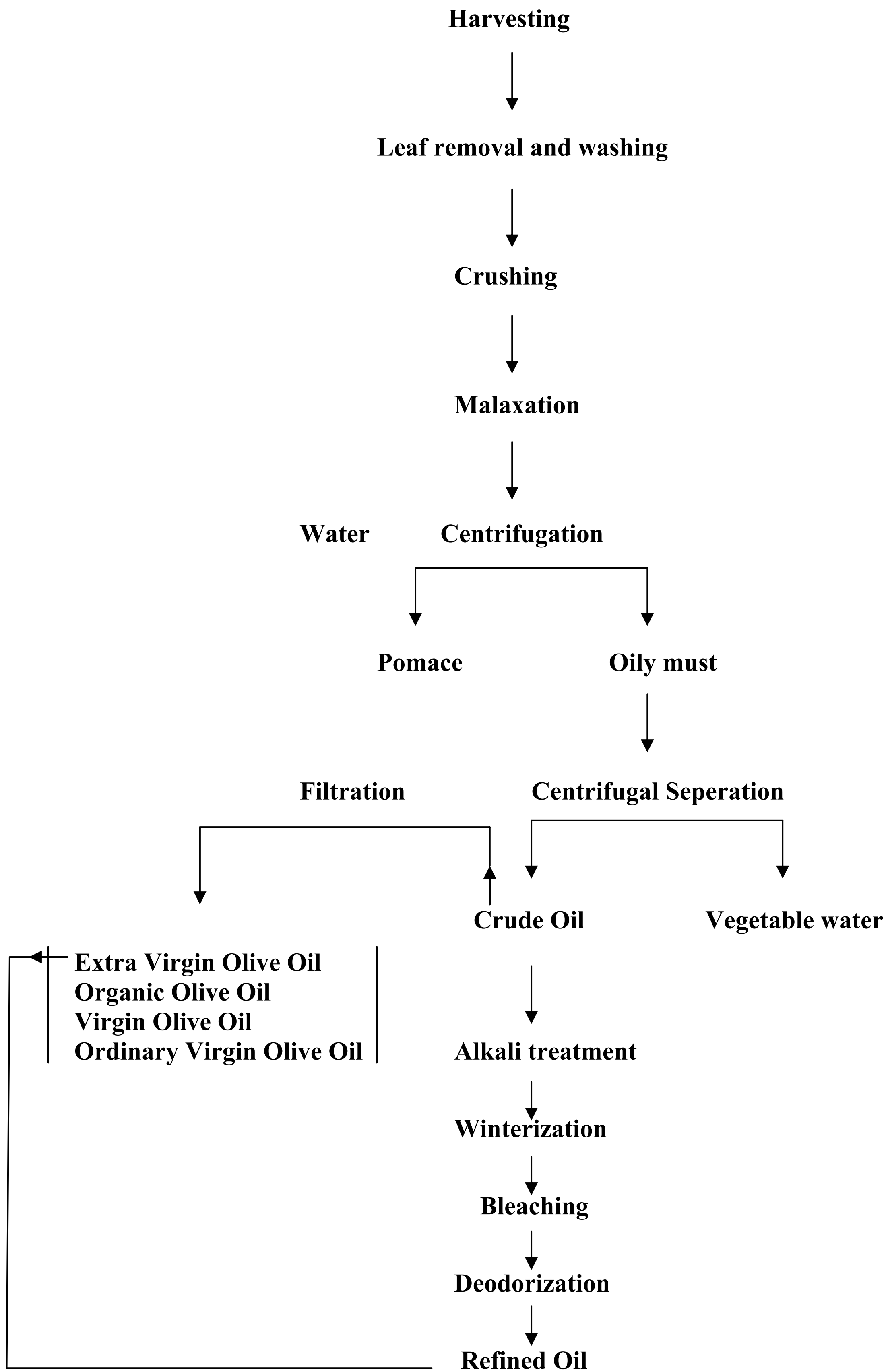


Figure 2.5: Flow Diagram of Olive Oil Processing (Boskou, 1996; Garcia et al., 2006).

2.8.1 Harvesting

Olive harvesting is the first stage for olive oil production. Depending on cultivar techniques, tree size and shape, and orchard terrain, different olive harvesting methods are used: harvesting from tree (by hand picking and machine shaking) and harvest on the ground (by using rollers, brushes and machines) (Boskou, 1996).

Maturity stage of the olive fruit also has important effects with regard to oil recovery and quality. During olive ripening, some chemical transformations occur in the drupe. The quality parameters, peroxide value, free fatty acid (FFA) and spectrophotometric absorption in the UV, do not change significantly during ripening, but phenolic substances and volatile compound of oil do change (Boskou, 1996). Artajo et al. (2006) stated that ripening of olives results in increase of some phenolic compounds such as hydroxytyrosol and some flavonoids. On the other hand, Gimeno et al. (2002) presented conflicting results that oils produced from green olives had higher contents of β-carotene and total phenolic compounds than found in ripe olives.

Following the maturation of olives, harvesting is realized during autumn and winter seasons (Kiritsakis, 1990).

2.8.2 Leaf removal and washing

After harvesting, the first process step which aims to remove all the impurities from olives (such as leaves, twigs, dust, soil, stones and the other foreign materials) for subsequent mechanical safety of crusher and decanter and prevention of organoleptic effects of leaves (Boskou, 1996; Giovacchino et al., 2002). Di Giovacchino et al. (2002) stated that when the olives are processed with their leaves, a fresh-cut grass aroma (trans-2-hexenal and cis-3-hexenal) and much greener color (chlorophyll) are perceived by tasters.

Deleafing and washing process can damage the over-riped olives, especially when the olives are picked by hand; removing foreign materials with simple separator is acceptable; but on the other hand, olive washing is necessary for more hygienic operation (Boskou, 1996; Giovacchino et al., 2002).

2.8.3 Crushing

The aim of crushing is the removal of oil from broken flesh cells and helping the formation of larger oil drops (Boskou, 1996).

Stone mills (gentle) or metallic crushers (violent) are used for crushing of olives (Boskou, 1996; Giovacchino et al., 2002). Although stone mills and metallic crushers do not influence olive oil's quality parameters (FFA, peroxide value etc.), olive oil obtained from violent metallic crushers possesses a higher total phenolic content, as well as a more bitter taste (Giovacchino et al., 2002). Besides these organoleptic and nutritional influences, metallic crushers have also higher capacity (Boskou, 1996). On the other hand, while olive paste temperature increases to 13-15 ºC with above the ambient temperature by metallic crusher, it is about 4-5°C higher by stone mill (Giovacchino et al., 2002). Higher temperature in the crusher during olive processing leads to a shorter quality preservation period of the oils.

During olives crushing, secoiridoid aglycons are produced due to hydrolysis of oleuropein, demethyloleuropein and ligstroside by the β-glucosidases (Servili and Montedoro, 2002).

2.8.4 Malaxation

After crushing, olive paste has to be malaxed to collect any free oil and increase the oil drop size (Giovacchino et al., 2002). Formation of large droplets and breaking up the oil-water emulsion are the main objectives of this mixing stage (Boskou, 1996). Semicylindrical vats with heated jacket fitted to a horizontal shaft, rotating arms and different sized and shaped stainless steel blades are parts of the malaxation equipments (Giovacchino et al., 2002).

Malaxation is an important step governed by two factors, time and temperature (Boskou, 1996). There is a direct correlation between oil extraction yield and those two factors (Boskou, 1996; Giovacchino et al., 2002). Di Giovacchino stated that for three phases centrifugal decanter, the increasing of malaxation time (15-90 min.) and temperature (about 30 °C) lead to increases in the oil extraction yield (Giovacchino et al., 2002). Furthermore, he pointed out that there is a slight decrease (10-20 %) on total phenols between 15 and 90 minute malaxation times (Giovacchino et al., 2002).

In addition, according to the study of Ranalli et al., the amount of the phenolics and secoiridoid derivatives increased, when malaxation temperature was increased to 30 ºC (Ranalli et al., 2005). Moreover, increasing time and temperature above these, limits (30 °C and 90 min.) of malaxation process resulted in decreasing the quantity of secoiridoid aglycons and phenolic alcohols (Servili and Montedoro, 2002; Servili et al., 2004).

2.8.5 Extraction

After malaxation, separation of oil from the olive paste is achieved by extraction step which is the last stage for virgin olive oil.

There are three extraction systems that are used in commercial plants: pressure, centrifugation and percolation system (Boskou, 1996; Giovacchino et al., 2002). While pressure and percolation are older extraction systems, centrifugation is the newest and most widespread continuous system. It is based on the principle of application of centrifugal force on the olive paste and its dilution with or without warm water (Giovacchino et al., 2002). Centrifugation is achieved by high speed rotating machines and applying centrifugal force in the horizontal centrifuges or decanters. Three-phases decanter (50-100 l water/100 kg olive pastes), three-phases decanter (10-30 l water/100 kg olive pastes) and dual-phases decanter are the centrifugation system types (Servili et al., 2004). There is no water used in dual phases centrifugation as a difference from three-phases.

The amount of added water and temperature of mixing are to other important points that affect oil extraction yield and also its nutritional quality. Critical limits for centrifugation are 20-25 °C temperature and 1:0,7 to 1:1,2 for paste/water ratio (Boskou, 1996).

The oil quality parameters (FFA, peroxide value etc.) do not change significantly with different extraction systems (Boskou, 1996; Giovacchino et al., 2002). This is also in agreement with results reported by Gimeno et al. (2002). On the other hand, some authors have reported that extraction method affects the oils chemical characteristics (Torres and Maestri, 2006). The antioxidant compounds, induction time and chlorophyll pigments significantly change, especially by using 3-phase centrifugation system. Addition of water leads to removal and dissolving of

polyphenols from oil and so induction time becomes shorter and this means lower resistance to autooxidation. Also more chrophyll is released by centrifugation (Boskou, 1996). In addition, the two-phase centrifugal decanter preserves more phenolic content than the three-phase method because of the high water-solubility characteristics of phenolic compounds (Gimeno et al., 2002; Salvador et al., 2003). Furthermore, three phase centrifugation system produce black olive mill wastewater which is a highly polluting organic compounds, that induces an environmental problem in all olive-producing countries, such as Spain, Italy, Greece, Tunisia and Turkey (Lesage-Meessen et al., 2001).

2.8.6 Filtration

In general, olive oil in industry uses filtration after extraction and before bottling for better consumer acceptance, but this process induces removal of some of the water-soluble polyphenols from the oil, and thus resulting in decreased stability of olive oil.

2.8.7 Alkali refining

In this step, after heating the oil pomace to 80-90 °C and adding 0,05-0,1% of phosphoric acid, it is mixed for 5-10 minutes and subsequently 20-30 % of sodium hydroxide solution is applied for neutralization of high acidity oil. The soapy pastes are seperated from the neutralized oil in vertical centrifuge (Boskou, 1996).

As it is stated by Garcia et al. (2006), o-diphenols (hydroxytyrosol, catechol and hydroxytyrosol acetate) and flavonoids (luteolin and apigenin) are mostly eliminated during the alkaline treatments.

2.8.8 Bleaching

After winterization, the oil is heated to 90 ºC and residual water in the oil is eliminated in vacuum-sealed reactors. Then the oil is transferred to another reactor and 2-6 % bleaching earth is added, depending on the intensity of the initial color. After heating of the oil/bleaching earth mixture to 80-90 ºC, it is agitated for 20-30 minutes (Boskou, 1996).

Garcia et al. stated that some flavonoids (luteolin and apigenin) are completely removed after bleaching step (Garcia et al., 2006).

2.8.9 Winterization

Winterization may be carried out either after alkali refining or after bleaching. After alkali refining, the oil is cooled to 5-10 ºC, agitated 4 hours for the formation of wax crystals, then 5 % water is added at room temperature and heated to 12-15 ºC for seperation of oily and aqueous phases that contain soaps and wax particles. After the aqueous phase is heated to 20-25 ºC, the aqueous phase separation with wax from the neutralized and dewaxed oil is done in the vertical centrifuge and latter following the addition of hot water, soaps are eliminated in a washing centrifuge (Boskou, 1996).

Winterization can also be done after bleaching and before deodorization by using hexane (1:2-1:4 oil/hexane) and the mixture is cooled to 15-20 °C for the formation of wax crystals (Boskou, 1996).

2.8.10 Deodorization

Deodorization eliminates undesirable volatile compounds which give an unpleasant odor to oil. For this aim, steam (about 1% of oil) is injected to oil about 2-3 hours in the reactor that is conditioned at 200-230 ºC at a pressure of 1-3 mbar. After deodorization, the oil is cooled to 25 °C under nitrogen (Boskou, 1996).

As it is stated by Garcia et al., although deodorization step removes most polyphenols from the oil, very low amounts of lignans may still be found in the oil (Garcia et al., 2006). Also, according to the literature, during refining of olive oil, almost 50% losses of tocopherol occur, especially in deodorization step, so the addition of α-tocopherol in refined oil is beneficial for the stability of oil (Blekas et al., 1995).

3. MATERIAL AND METHODS

Five commercial olive oil samples were obtained from specialized retailers, from two harvesting seasons: 2005/06 and 2006/07. Ayvalik (Edremit yaglik) cultivar olives were harvested from olive growing region of Canakkale, Turkey. The Extra Virgin Olive Oil (EVOO), Organic Virgin Olive Oil (OGOO), Virgin Olive Oil (OO-1), and Ordinary Virgin Olive Oil (OO-2) samples were obtained from one company, while Refined Olive Oil (RFOO) sample was supplied by another company. However, all olive oil samples originated from the same region and the same cultivar. Although EVOO and OGOO are expressed with different names, the only difference between these two is the original olive fruits for OGOO which was produced by organic farming.

All olives were harvested in October, November and December by hand using a rake and transferred in 20 kg perforated boxes to the plant and immediately processed to olive oil.

2005/06 season olive oils were kept in an inert Nitrogen atmosphere till 20th of May 2006 in stainless steel tanks. Then the olive oil samples were filled in transparent glass bottles and exhibited for sale at room temperature till October 2006. However the 2006/07 season olive oil samples were bottled immediately and kept at +4°C.

2005/06 season oils were carried out in the period of October-December 2006, while 2006/07 season was analyzed in January-March 2007. During analyses, all samples were kept at + 4 °C and were protected from light.

Climatic data of Canakkale region were obtained from the Turkish State Meteorological Service. The figures of annual patterns of air temperature and daily rainfall belonging 2005 and 2006 harvest years were shown in the appendix (Figure A.10, A.11 and A.12).

The aim of this study is to investigate the chemical characterization of Ayvalik monocultivar olive oils belong to two crop seasons, 2005/06 – 2006/07.

3.1 Olive Oil Quality Parameters

3.1.1 Free fatty acidity (FFA)

3.1.1.1 Reagents

Hexane/isopropanol (50/50, v/v)
Phenolphtalein indicator, 1 % solution in isopropanol
NaOH solution 0,1 N accurately standardized with acid potassium phthalate, $KHC_8H_4O_4$ (AOCS Specification H12-52)

3.1.1.2 Method

Determination of free fatty acidity was carried out following official method Ca 5a-40 and modified by Verhe et al. (2000).

Briefly, hexane/ isopropanol mixture (50/50, v/v) was prepared, heated and stirred for degasing. Following the adding of the two droplets of phenophtalein indicator into the mixture, almost three droplets of sodium hydroxide was added to mixture untill light pink color appeared. Afterwards, 1,5 g of olive oil was added to the mixture and immediately titrated with sodium hydroxide until color remains pale pink for 15 to 30 seconds.

The FFA was calculated by the equation:

% FFA as oleic acid = (ml NaOH x N x 28,2) / W
W: weight of the sample (g)
N: normality of the NaOH solution

Free fatty acidity was expressed as the percent of oleic acid.

3.1.2 Ultra-Violet light (UV) absorption characteristic

3.1.2.1 Reagents

n-hexane (HPLC grade)

3.1.2.2 Method

Ultra-violet light (UV) absorption K_{232} and K_{270} were determined according to the Official Method of the European Community No L 248/33-35, 1991.

To determine spectrophotometric indices, K232 and K270, the amount of 0,10 g of olive oil was dissolved till 10 ml with n-hexane. Samples were analysed in triplicate in 10 mm quartz cuvettes, using a Varian Cary 50 Probe UV-Visible Spectrophotometer. The specific extinctions (K_λ) was calculated according to the following formula:

$K_\lambda = E_\lambda / (c \times s)$

K_λ : spesific extinction at λ wavelength
E_λ : extinction measured at λ wavelength
c : solution concentration in g / 100 ml
s : thickness of the cuvette in cm (1 cm)

All measured extinction coefficient were between 0,1 - 0,8.

3.1.3 Iodine value

The iodine value of fat is the number of grams of halogen absorbed by 100 grams of the fat, and expressed as the weight of iodine. It is a measurement of the degree of unsaturation. Iodine values were calculated from fatty acid percentages by using the formula (Torres et al. 2006).

IV = (% palmitoleic x 1,001) + (% oleic x 0,899) + (% linoleic x 1,814) + (% linolenic x 2,737)

3.2 Fatty Acids Composition

3.2.1 Reagents

Saturated NACl solution (ACROS ORGANICS)
BF_3 / methanol reagent (20 % BF_3 solution in methanol) (MERCK)
Iso-octane (RIEDEL DE HAEN)
$MgSO_4$ (dry)

NaOH (0,5 N solution in methanol)

Internal standard: nonadecanoic acid in iso-octane 5 mg/ml (FLUKA, Switzerland)

3.2.2 Method

Fatty acids methyl esters (FAME) method was analyzed by gas chromatography (GC) to identify and quantify the individual fatty acid in the oils. The procedure was adapted from AOCS Official method Ce 1f-96.

Briefly, approximately 0,05 mg (3 drops) oil was weighed into a 10 ml tube, subjected to alkaline saponification (2 ml, 0,5 N NaOH) and blowed in nitrogen during 1 minute. After heating the test tube for 7 minutes, it was cooled for 5 minutes. Then BF_3 / methanol reagent was added to the tube and closed tube was shaken by vortex for 30 seconds. The tube was heated for 5 minutes. Internal standard (6 ml) and NaCl solution (5 ml) were added to the tube, flushed with N (nitrogen) for 1 minute, and again shaken for 30 seconds. And after, adding small amount of dry $MgSO_4$ and shaking, the sample was subsequently diluted with iso-octane (in original method hexane is used) into a vial until the end concentration of 1,5 to 1,8 mg/ml and injected into the GC. The content of fatty acids was expressed as percentages of fatty acid methyl esters.

The identification of FAME was carried out by Agilent Technologies 6890N gas chromatography using a 50mm long capillary column CP-Sil 88 (Varian-Chrompack) with a Flame Ionization Detector (FID). The column temperature was isothermal at 190 ºC and the injector and detector temperatures were 220 ºC. Carrier gas hydrogen was at 0,8 bar pressure and the quantity of injection was 1 µl. All measurements were carried out in triplicate.

3.3 Polyphenol Analysis

3.3.1 Reagents

Internal standards: ortho-coumaric acid (0,01 mg/ml in methanol) and para-hydroxyphenlyacetic acid (0,05 mg/ml in methanol) (ACROS ORGANICS)

External standards: hydroxytyrosol (CAYMAN CHEMICAL, USA), tyrosol (ACROS ORGANICS, Belgium), para-coumaric acid (SIGMA, Germany), vanilin (FLUKA, Switzerland), vanilic acid (FLUKA, USA), ferulic acid (SIGMA, Germany), luteolin (SIGMA, Germany) and apigenin (FLUKA, USA)

Methanol (LC-MS grade BIOSOLVE)
Hexane (LC-MS grade BIOSOLVE)
Ethylacetate (HPLC grade ROMIL CHEMICALS)
Water (LC-MS grade BIOSOLVE)
Methanol (HPLC grade BIOSOLVE)
Folin-Ciocalteau reagent (MERCK, Germany) (1:10 dilution in distilled water)
Sodium-carbonate, Na_2CO_3, 20 %
Gallic acid (SIGMA CHEMICAL, Switzerland)

3.3.2 Extraction of phenolic compounds

The phenolic fractions of olive oil were isolated by using solid-phase extraction method according to the procedure of Mateos et al. (2001).

In brief, 2,5 ± 0,001 g of olive oil were weighed four times into pear shaped flasks, and 500 µl of a solution of the internal standards was added to three of them. The solvent was evaporated in a rotary evaporator at 35 ºC under vacuum, and then each sample was dissolved in 6 ml of hexane.

Four diol-bonded solid phase SPE C_{18} cartridges (Supelco, Bellefonte, PA) were placed in a vacuum elution apparatus (Alltech) and conditioned by consecutive passing of 6 ml methanol and 6 ml hexane. Then, the vacuum was released to prevent the column from drying, and sample solutions were applied to the columns, which were subsequently washed with 2x3 ml hexane and 4 ml hexane/ethylacetate (90:10, v/v), respectively. Finally, the retained fraction was eluted with 10 ml of methanol

and evaporated in a rotary evaporator under vacuum. The dry residue was dissolved in 500 µl of methanol-water (1:1, v/v) for LC-MS analyze and was dissolved in 1 ml of methanol for total phenolic content analyze.

Before LC-MS analysis, the samples were filtered through a syringe filters (Acrodisc Syringe filters, Cr 13 mm; 0,45 PTFE Membrane) to a vial.

3.3.3 Determination of individual phenolic compounds by LC-MS analysis

3.3.3.1 LC-MS analysis condition

Phenolics were analyzed by both reversed-phase high performance liquid chromatography (HPLC) coupled with diode array UV detection and Mass Spectroscopy (MS). The Agilent 1100 LC-MSD chromatographic system equipped with a quaternary pump, C18 column (Phenomenex-Luna 4,6x250 mm, Ø 5 µm, 100A pore size), security guard column (Phenomenex C18, ODS, Octadecyl), vacuum degasser, an autosampler, 1100 6-port autoinjector valve, quaternary pump were used. Detection was performed at 280 and 320 nm. The elution solvents used were A (0,2% acetic acid in water), B (methanol), D (acetonitril). B and D solvents were mixed in a 50:50 (v/v).

Flow rate was 1 ml/min and run time 72 min. The run was performed at 35°C. The sample injection volume was 20 µl. Identification of compounds was achieved by comparing their retention time values absorbance spectra, and m/z ratios to those of the standards. Data was collected and processed using Agilent software v A.09.03.

3.3.3.2 Quantification of individual phenolic compounds

Phenolic compounds were quantified by using reference compounds. Quantification of all phenolic components was carried out at 280 nm, using *o*-coumaric acid as internal standard. Following compounds were quantified hydroxytyrosol, tyrosol, p-coumaric acid, vanilin, vanilic acid, ferulic acid, luteolin and apigenin. For these compounds calibration curves were obtained where the response ratio (formula 3.3.3.2.a) of the area of the reference component over the area of internal standard (Figure 3.1 and Table 3.1) was calculated. The solution of standards were prepared at different concentrations: 0,1; 0,08; 0,0512; 0,0328; 0,0210; 0,0107 mg/ml. On the other hand p-hydroxyphenlyacetic acid was used as the standard for calculation of dialdehydic form of decarboxymethyl oleuropein aglycon, pinoresinol, 1-

acetoxypinoresinol, aldehydic form of olueropein aglycon and aldehydic form of ligstroside aglycon using the response factor as reported by Mateos et al. (2001).

$$\text{Response ratio} = \frac{\text{Absorbance of component x Concentration of standard}}{\text{Absorbance of standard x Concentration of component}} \quad (3.3.3.2a)$$

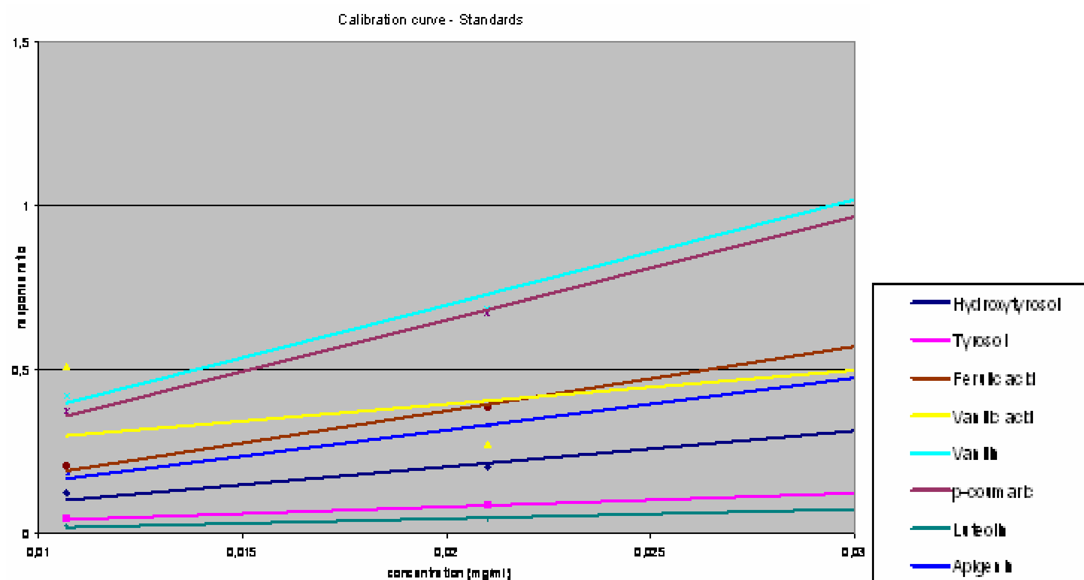


Figure 3.1: Calibration Curves of Standards: hydroxytyrosol, tyrosol, vanilic acid, vanilin, p-coumaric acid, ferulic acid, luteolin and apigenin

Phenolic compounds in olive oil were determined by comparing with standard compounds, as mentioned above, on the basis of their retention times, molecular masses and their spectrometric properties. The content of each phenol present in the polar fraction of the oil samples is expressed in mg/kg of olive oil.

Table 3.1: Intercept, Slope and R^2 Values of Standards

Component	Intercept	Slope	R^2
Hydroxytyrosol	-0,014	10,962	0,999
Tyrosol	-0,001	4,188	0,999
Vanilic acid	0,189	10,347	0,896
Vanilin	0,056	32,075	0,998
p-coumaric acid	0,024	31,434	1,000
ferulic acid	-0,018	19,667	1,000
Luteolin	-0,011	2,873	0,999
Apigenin	-0,002	15,929	0,999

3.3.4 Determination of total phenolic content by Folin-Ciocalteu method

The total phenol content of the oils was determined colorimetrically using the Folin-Ciocalteau reagent according to the method of Gutfinger (1981). However this method does not detect the individual phenolic compounds of olive oil. Gallic acid was used as a standard for the calibration curve to calculate the total amount of all phenolic compounds in samples. Results were expressed as gallic acid equivalents (mg GAE/kg of oil).

3.3.4.1 Standard curve

The calibration curve was constructed using standard solutions of gallic acid in MeOH within range of 0 - 50 mg/ml, as shown in Figure 3.2.

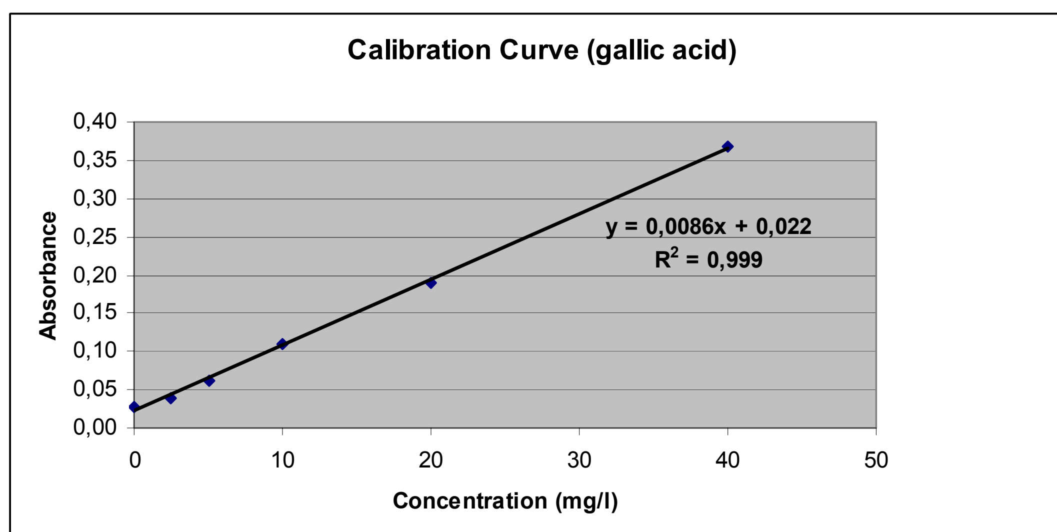


Figure 3.2: Calibration Curve for Folin-Ciocalteau Assay

3.3.4.2 Method

Briefly, the olive oil was extracted by using solid-phase extraction method (Mateos et al., 2001) without addition of the internal standard. The obtained dry phenolic extract was diluted in 1 ml MeOH. Further it was added into 100 ml volumetric flask previously filled with 60 ml deionized water. After addition of 5 ml (1:10) dilution of Folin-Ciocalteau solution, the mixture was left for 5 min. Then 15 ml %20 sodium carbonate solution and distilled water were successively added to volume. The solution was kept in a dark place. The absorbance of the solution was measured after

2h against a blank (water) using the calibration curve by UV-Visible Spectrophotometer (Carry 50 BIO, Australia) at 760 nm. The spectrophotometer was controlled with the software Carry Win UV version 3.00 from Varian. All measurements were carried out in triplicate and also, each sample was read three times in the spectrophotometer.

3.4 Statistical Analyses

Statistical differences were estimated by applying ANOVA and using the Tukey test at the 5 % level (P= 0.05) of significance for the pairwise comparisons between means. The Minitab computer program (release15-; Minitab Inc., Addison-Wesley Publishing Co., Reading, MA) was used.

4. RESULTS AND DISCUSSION

The samples were characterized for their quality indices, as shown in the Table 4.1. For these aspects, all samples conformed to the respective European Standard for Olive Oils and Olive Pomace Oils (EU, 2002) designated for each class though with some minor deviations, due to differences arising from extended storage period of 2005 samples.

Table 4.1: Quality Characteristic Ranges of All Analyzed Samples Belong to Two Crop Seasons.

Quality characteristics	2005 Crop Season	2006 Crop Season
FFA (% oleic acid)	0,45 – 3,44	0,19 – 2,60
Iodine value	85,91 – 87,28	84,23 – 85,44
K 232	2,25 – 3,41	0,08 – 2,64
K270	0,31 – 1,34	0,05 – 0,34

4.1 Fatty Acid Composition

Fatty acid composition of samples is presented in Table 4.2. The major fatty acids of Ayvalik cultivar olive oils varied in the following ranges: oleic acid 68,28-73,25 %, palmitic acid 12,29-13,57 %, linoleic acid 9,63-11,93 %, stearic acid 2,26-2,90 %, palmitoleic acid 0,53-0,72 %, and linolenic acid 9,84-11,93 %. The distribution of fatty acid composition of samples is in agreement with the European Standard for Olive Oils and Olive Pomace Oils (EU, 2002) and Codex Alimentarius (Codex Alimentarius, 2001). The limits for fatty acids' levels found in literature were also listed in Table 2.3.

Moreover, the total saturated fatty acid (SFA) percentages, the total monounsaturated fatty acid (MUFA) percentages, the total polyunsaturated fatty acid (PUFA) percentages, the MUFA/SFA ratio and MUFA/PUFA ratio of samples were presented in the Table 4.2. The low oleic and high linoleic acid content leads to a low MUFA/PUFA ratio, and this data indicate low oxidative stability as was also stated

in Pardo and co-workers' study (Pardo et al., 2007). Furthermore, higher PUFA leads to increasing the oxidative susceptibility (Salvador et al., 2001).

Determining fatty acid composition is an important tool to distinguish among monocultivar olive oils belonging to particular cultivars, so some differences between monocultivar olive oils can be observed (Torres and Maestri, 2006; Pardo et al., 2007). On the other hand, the climate of the production area is an other important factor affecting fatty acid composition of olive oil. According to the climatic data of Canakkale region (Figure A.10, A.11 and A.12), the weather in 2006/07 was characterized by severe frosts in October-December and dry summer when compared with 2005/06. During summer period (June-August), there was no big difference on accumulated rainfall in between 2005/06 (392 mm) and 2006/07 (382 mm) crop seasons. Oils from dry and warm areas possess less unsaturated fatty acids than cool areas (Kiritsakis, 1990 and Romero et al., 2003). From temperature aspect, this information is in agreement with the data, which MUFA content of 2006 samples are lower than 2005 samples due to dry summer, found in this study as shown in the Table 4.2. Also, iodine values of 2006 samples are lower than samples belonging to 2005 due to high temperature in 2006.

Regarding statistical test Tukey, no significat differences were observed in oils belonging to 2005 crop season, while 2006 samples revealed some significant differences (Table 4.2). However, in general, RFOO had higher mean value of oleic acid percentage than the others. This is also in aggrement with results reported by Satue et al. (1995), where the RFOO had higher content of oleic acid than EVOO, both of are from California. Furthermore, the oleic acid content of Ayvalik olive oil, categories EVOO and OGOO were in the lower range than olive oils from different countries, such as Spain (*Picual* and *Cornicabra,*) (Pardo et al., 2007), Italy (*Liguria* ve *Coratina*) (Boskou, 1996) and higher range than California (*Californian*) olive oil (Satue et al., 1995).

Table 4.2: Olive Oil Fatty Acid Composition (expressed in % FAME), SFAs, MUFAs, PUFAs, (MUFAs/SFAs) and (MUFAs/PUFAs) in All Analysed Samples with Standard Deviations.

FATTY ACID	EVOO		OGOO		OO-1		OO-2		RFOO	
	2005/06	2006/07	2005/06	2006/07	2005/06	2006/07	2005/06	2006/07	2005/06	2006/07
Palmitic acid, C16:0	12,41 ± 0,09	13,16± 0,05	12,82 ± 0,14	13,57 ± 0,28	12,68 ± 0,23	13,24 ± 0,30	13,11 ± 0,19	13,40 ± 0,25	12,29 ± 0,11	12,32 ± 0,11
Palmitoleic acid, C16:1	0,59 ± 0,01	0,67 ± 0,00	0,53 ± 0,02	0,72 ± 0,01	0,54 ± 0,02	0,65 ± 0,03	0,61 ± 0,02	0,69 ± 0,02	0,65 ± 0,01	0,68 ± 0,02
Heptadecanoic acid, C17:0	0,27± 0,01	0,29± 0,00	0,27± 0,01	0,25± 0,02	0,25± 0,01	0,30± 0,01	0,26± 0,02	0,29± 0,00	0,25± 0,02	0,25± 0,00
Heptedecenoic acid, C17:1	0,20± 0,01	0,22± 0,02	0,18± 0,01	0,20± 0,02	0,18 ± 0,00	0,21± 0,02	0,19± 0,01	0,21± 0,01	0,14 ± 0,00	0,13 ± 0,00
Stearic acid, C18:0	2,56 ± 0,05	2,5 ± 0,03	2,49 ± 0,07	2,26 ± 0,12	2,53 ± 0,06	2,59 ± 0,02	2,74 ± 0,02	2,61 ± 0,05	2,90 ± 0,01	2,76 ± 0,01
Oleic acid, C18:1	72,32 ± 0,63	69,50 ± 0,33	72,03 ± 1,21	69,08 ± 1,89	71,63 ± 1,65	68,91 ± 1,45	70,00 ± 0,47	68,28 ± 1,55	73,25 ± 0,37	72,01 ± 0,32
Linoleic acid, C18:2	10,31 ± 0,03	10,76 ± 0,03	10,43 ± 0,18	10,85 ± 0,08	10,92 ± 0,30	11,24 ± 0,43	11,79 ± 0,29	11,93 ± 0,39	9,63 ± 0,07	9,84± 0,15
Linolenic acid, C18:3	0,77 ± 0,01	0,67 ± 0,00	0,75 ± 0,03	0,63 ± 0,01	0,82 ± 0,06	0,70 ± 0,03	0,86 ± 0,02	0,63 ± 0,03	0,71 ± 0,01	0,72± 0,02
SFA	15,23	16,57	15,57	16,67	15,47	16,79	16,12	16,96	15,57	16,00
MUFA	73,27	70,68	72,88	70,26	72,45	70,03	70,94	69,47	72,88	73,11
PUFA	11,50	12,75	11,55	13,07	12,08	13,18	12,94	13,57	11,55	10,89
MUFAs/SFAs	4,81	4,27	4,68	4,22	4,68	4,17	4,40	4,10	4,68	4,57
MUFAs/PUFAs	6,37	5,54	6,32	5,37	6,00	5,31	5,48	5,12	6,32	6,72
Tukey Test	a	ae	ac	be	ae	ae	a	ae	a	ad

4.2 Phenolic Compounds (Folin-Ciocalteau and LC-MS Methods)

The total phenolic content of Ayvalik monocultivar olive oil ranged from 89,20 to 128,70 and from 44,26 to 129,16 mg GAE/kg in two successive crop seasons (2005/06 and 2006/07), respectively, as shown in Table 4.3 and Table 4.4.

The sum of quantified phenols in 2005 crop season oils varied from 14,57 to 93,28 mg/kg; while 2006 crop season oils were in the range 7,34 -121,96 mg/kg (Table 4.4 and 4.5).

Table 4.3: Phenolic Compounds Identified in Ayvalik Monocultivar Olive Oil by LC-MS

LC-MS peak	Compound	RT (min)	m/z	Other ions	Response factor
1	Hydroxytyrosol	9,3	153,2	307,3	....
2	Tyrosol	11,8	137,2	273,2 604,0	....
3 (IS)	p-hydroxyphenylacetic acid	12,7	151,2	325,2 107,2	....
4	Vanilin	15,5	151,2	....	....
5	*p*-coumaric	16,1	163,2	119,5 349,2	....
6 (IS)	o-coumaric	22,3	163,2	119,5 241,3	....
7	luteolin	31,2	285,2	361,2 415,3	....
8	apigenin	36,3	269,2	377,2	....
...	Dialdehydic form of decarboxymethyl oleuropein aglycon	24,5	335,2	319,2 693,2	1,303
...	Pinoresinol	30,1	357,2	....	1,843
...	1-acetoxypinoresinol	31,5	415,3	393,2 361,2	0,197
...	aldehydic form of olueropein aglycon	36,2	377,2	333,3 361,2	1,587
...	aldehydic form of ligstroside aglycon	37,2	375,2	275,3	2,121

RT: Retention Time (minute)

m/z : mass charge value

The identified individual phenolic compounds were listed in the Table 4.3. Although eight standards were used in this study (Table 3.1) only hydroxytyrosol, tyrosol, vanilin, *p*-coumaric, luteolin and apigenin were found in analyzed samples. The other phenolic substances (ferulic acid and vanilic acid) normally found in olive oil could not be identified in the investigated samples. The chromatograms of olive oil samples together with the standards (p-hydroxyphenylacetic acid and *o*-coumaric acid) used for identification are shown in Figure A.1, A.2, A.3, A.4, A.5, A.6, A.7 A.8, and A.9 which are enclosed in appendix.

Table 4.4: Mean Values of the Quantified Phenolic Compounds Of Olive Oil Samples Belong To 2005 Crop Season with the Standard Deviations.

Olive Oil	Hydroxytyrosol (mg/kg)	Tyrosol (mg/kg)	Vanilin (mg/kg)	p-coumaric acid (mg/kg)	Luteolin (mg/kg)	Apigenin (mg/kg)	dialdehydic oleu. agly. (mg/kg)	pinoresinol (mg/kg)	1-acetoxy-pinoresinol (mg/kg)	aldehydic oleu. agly. (mg/kg)	aldehydic ligst. agly. (mg/kg)	LC/MS (mg/kg)	Total phenolic compounds (FOLIN) (mgGAE/kg)
EVOO	4,92 ± 0,29	9,94 ± 0,46	0,21 ± 0,11	0,58 ± 0,05	18,91 ± 1,14	1,32 ± 0,07	0,83 ± 0,17	50,84 ± 9,39	-	3,08 ± 0,60	2,67 ± 0,45	93,28	105,88 ± 15,32[a]
OGOO	3,28 ± 0,40	4,26 ± 0,68	0,78 ± 0,28	0,46 ± 0,10	28,81 ± 2,79	1,99 ± 0,15	0,41 ± 0,01	38,59 ± 1,00	-	2,04 ± 0,08	1,48 ± 0,03	82,10	89,20 ± 3,24[a]
OO-1	1,15 ± 0,15	8,84 ± 0,22	0,55 ± 0,07	0,63 ± 0,01	16,07 ± 1,34	1,69 ± 0,04	-	49,30 ± 1,02	-	2,62 ± 0,04	0,93 ± 0,06	81,79	89,57 ± 16,01[a]
OO-2	1,58 ± 0,09	4,32 ± 0,10	0,17 ± 0,06	1,13 ± 0,04	17,62 ± 1,36	1,07 ± 0,09	-	31,99 ± 4,86	-	1,21 ± 0,05	0,86 ± 0,05	59,94	128,70 ± 10,16[a]
RFOO	0,76 ± 0,10	3,72 ± 0,09	-	-	-	-	-	7,82 ± 0,26	1,98 ± 0,14	0,29 ± 0,01	-	14,57	104,57 ± 8,33[a]

dialdehydic oleu. agly. : dialdehydic form of decarboxymethyl oleuropein aglycon
aldehydic oleu. agly. : aldehydic form of olueropein aglycon
aldehydic ligst. agly.: aldehydic form of ligstroside aglycon

Table 4.5: Mean Values of the Quantified Phenolic Compounds Of Olive Oil Samples Belong To 2006 Crop Season with the Standard Deviations.

Olive Oil	Hydroxytyrosol (mg/kg)	Tyrosol (mg/kg)	Vanilin (mg/kg)	p-coumaric acid (mg/kg)	Luteolin (mg/kg)	Apigenin (mg/kg)	dialdehydic oleu. agly. (mg/kg)	pinoresinol (mg/kg)	1-acetoxy-pinoresinol (mg/kg)	aldehydic oleu. agly. (mg/kg)	aldehydic ligst. agly. (mg/kg)	LC/MS (mg/kg)	Total phenolic compounds (FOLIN) (mgGAE/kg))
EVOO	2,71 ± 0,42	4,18 ± 0,47	0,43 ± 0,13	0,41 ± 0,06	27,59 ± 6,68	2,12 ± 0,43	0,78 ± 0,11	59,68 ± 9,82	-	2,55 ± 0,42	2,14 ± 0,33	102,59	113,86 ± 21,59^{a}
OGOO	0,46 ± 0,05	2,07 ± 0,04	0,09 ± 0,11	0,71 ± 0,02	35,71 ± 2,32	2,93 ± 0,15	0,55 ± 0,09	77,07 ± 2,77	-	1,70 ± 0,07	0,66 ± 0,03	121,96	129,16 ± 16,73^{a}
OO-1	2,29 ± 0,21	4,64 ± 0,51	0,37 ± 0,29	0,66 ± 0,09	25,51 ± 2,22	1,70 ± 0,12	-	48,64 ± 4,44	-	2,24 ± 0,30	1,53 ± 0,61	87,59	114,05 ± 23,74^{a}
OO-2	1,27 ± 0,08	3,91 ± 0,27	0,28 ± 0,06	0,99 ± 0,07	22,04 ± 1,97	1,57 ± 0,14	-	43,54 ± 3,17	-	1,82 ± 0,16	1,23 ± 0,09	76,63	94,48 ± 5,18^{a}
RFOO	-	-	-	-	-	-	-	4,91 ± 0,16	2,43 ± 0,11	-	-	7,34	44,26 ± 0,14^{b}

dialdehydic oleu. agly. : dialdehydic form of decarboxymethyl oleuropein aglycon
aldehydic oleu. agly. : aldehydic form of olueropein aglycon
aldehydic ligst. agly.: aldehydic form of ligstroside aglycon

The main phenolic compound detected in Ayvalik monovarietal oil was pinoresinol followed by luteolin. It is important to underline that although there was a decrease in concentration of total phenolics was observed by ripening, according to the study of Garcia et al. (2002), pinoresinol was the least affected compound by about 25 % . On the other hand, Salvador et al. (2001) investigated each ripening stage with respects to total phenolics and stated that concentration of phenolic compounds increases until it reaches purple color and then start to decrease. Lignans (1-acetoxypinoresinol and pinoresinol) were found the most stable phenolic compound during storage according to the Morello et al.(2004) study.

It has previously been reported that vanilin, p-coumaric acids, and the flavonoids luteolin and apigenin decreased with time for Picual olive oil, and the storage effect was statistically significant for most of them. Also, in the same study, lignan peak was still detected after storage of olive oil under light and air conditions for 460 days (Brenes et al., 2001). In another study, after two years storage of olive oil under nitrogen in amber glass bottles, Picual olive oils still contained most of their antioxidants (Garcia et al., 2002).

Pinoresinol content of Cornicabra, Arbequina, Picual and Hojiblanca virgin olive oils were much more lower (2,70; 2,23; 2,32; 1,44 mg/kg olive oil; respectively) (Gomez-Alonso et al., 2002) than Ayvalik olive oil samples (Table 4.4 and 4.5). The quantification of Pinoresinol compound was according to the Mateos et al. (2001) similar to this study.

As seen in Table 4.4 and 4.5, 1-acetoxypinerosinol is present only in RFOO phenolics. Although this sample was same variety with the other oils, it was from a different production area. Salvador et al. (2003) studied Cornicabra virgin olive oils and also found that phenolic contents vary depending on the production area. Moreover, Garcia et al., 2006 stated that, even most of the polyphenols removed during refining, very low amounts of lignans may be found in the oil.

With regard to the climate of harvest season, the water deficiency in olive fruit induces production of phenolics, so lower rainfall during summer period in the harvest year can be related with the increase in the total polyphenol content (Romero et al., 2003). As mentioned before, accumulated rainfall during summer was similar in 2005 and 2006 crop seasons, so from this aspect, significant differences in total

phenolic content of samples belonging to two years should not be expected, as observed in this study (Table 4.4 and 4.5). Similarly, Salvador et al. (2003) did not find any influence of crop season on total phenolics of the olive oil when there were no differences in rainfall. On the other hand, hydroxytyrosol and tyrosol content of 2005 crop season olive oils were higher than 2006 season oils which can be explained by the acid hydrolysis of the secoiridoid aglycons due to the storage, extended period for 2005 samples (Garcia et al., 2002).

There are two main conceptions on the effects of the refining process of olive oil. While some researches show that refining process removes all the polyphenols from the oil, some others emphasize that some polyphenols, such as *o*-diphenols, are not removed by the refining process (Garcia et al., 2006). In this study, there was still some phenolic compounds in refined olive oil.

In general, there was an incresing trend in respective total phenolics content by Folin (Table 4.4 and 4.5) when compared to total LC-MS values. It is well known that, while total phenolics are determined colorimetrically, LC-MS analysis do not quantify all the phenolic compounds (Garcia et al., 2002). This was especially apparent in refined olive oil due to tocopherol antioxidant, which is known to be added to refined olive oil and it cannot be quantify in LC-MS.

5. CONCLUSION

This study was performed for the chemical characterization of Ayvalik monocultivar olive oil from the Canakkale region of Turkey. With this purpose, five classes of olive oils were obtained from the region belonging two consecutive crop seasons, 2005/06 and 2006/07. Also, climatic data of production area in 2005 and 2006 years was obtained from Turkish State Meteorological Service considering annual patterns of air temperature and daily rainfall. The study covered the analysis of quality parameters, as FFA, iodine value, UV characteristics (K_{232} and K_{270}), fatty acid composition, and phenolic compounds.

According to the results, it can be stated that quality characteristics and fatty acid composition of the samples generally met the limits as indicated in European Standard for Olive Oils and Olive Pomace Oils with some minor deviations. In Ayvalik cultivar olive oil, oleic acid was the dominant fatty acid, palmitic acid was the second prevalent fatty acid, followed by linoleic acid and linolenic acid was the lowest, like most monocultivar olive oils indicated in the literature.

For the identification of individual phenolic profile of Ayvalik cultivar olive oils, LC-MS was used and pinoresinol was detected as a main phenolic component followed by luteolin. Furthermore, pinoresinol content of the samples were found to be markedly higher than many olive oils from other countries. This important finding can be used proposed to be as a geographical marker of Ayvalik cultivar olive oil for determining the geographical origin. European legislation allows the labelling of virgin olive oils with the name of region where they are produced (Protected Denomination of Origin). Obtaining this certification will surely improve the commercial value of Ayvalik monocultivar olive oil, due to the growing demand in EU countries for agricultural produce with an identifiable geographical origin.

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Vissers, M.N., Zock, P.L., Roodenburg, A.J.C., Leenen, R., Katan, M.B., 2001. Olive oil phenols are absorbed in humans, *American Society for Nutritional Sciences*, 409-417.

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APPENDIX

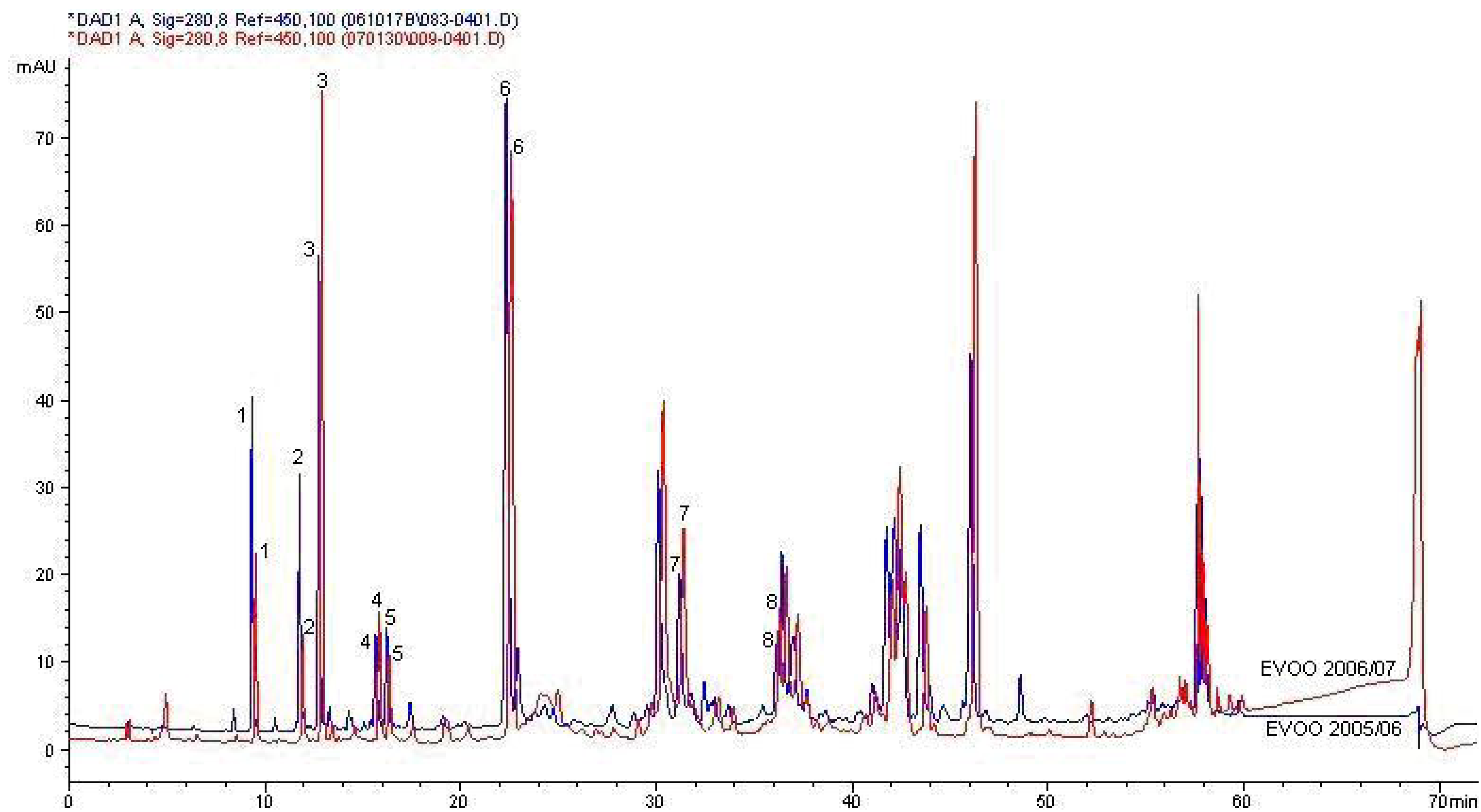

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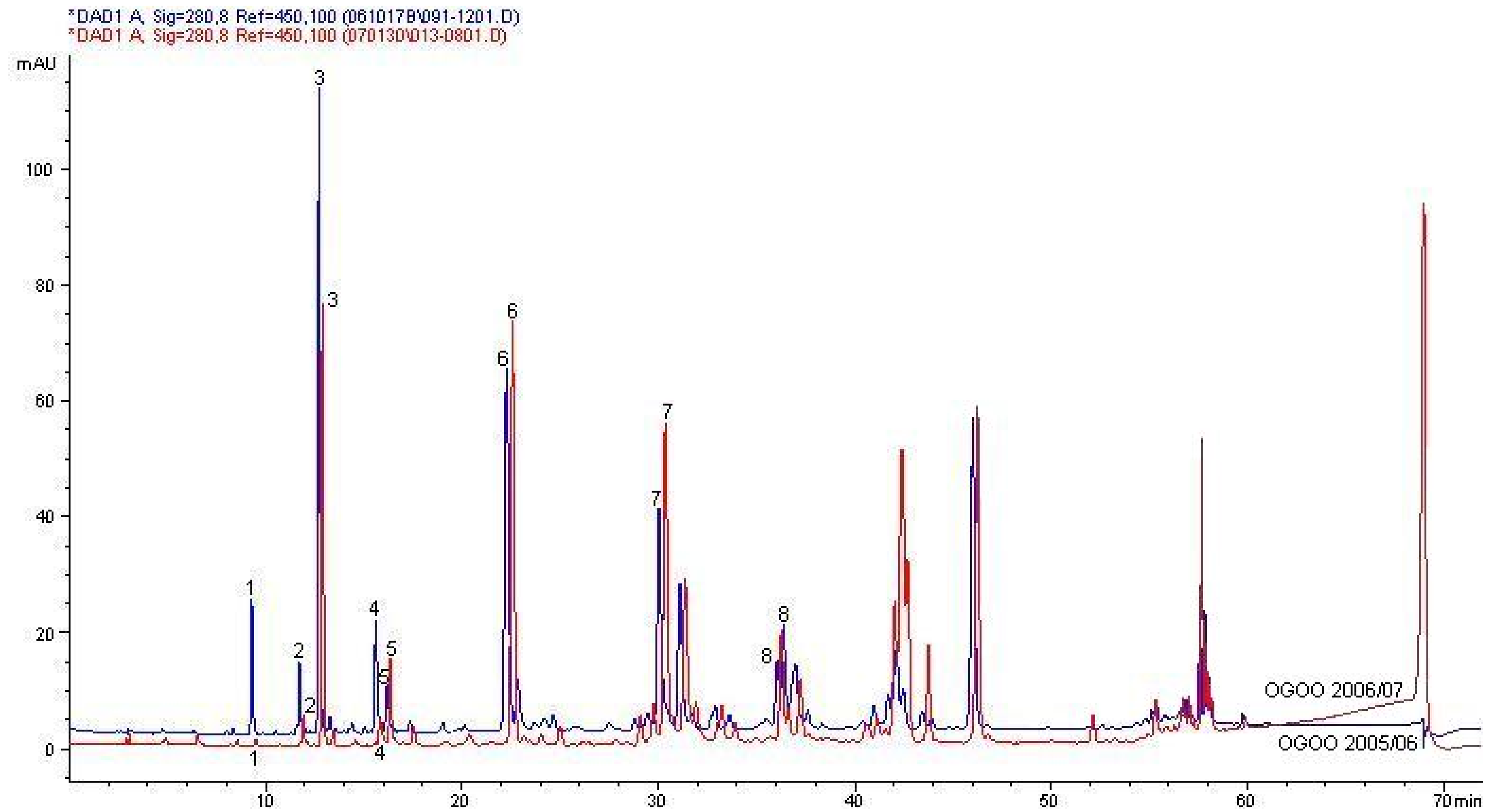
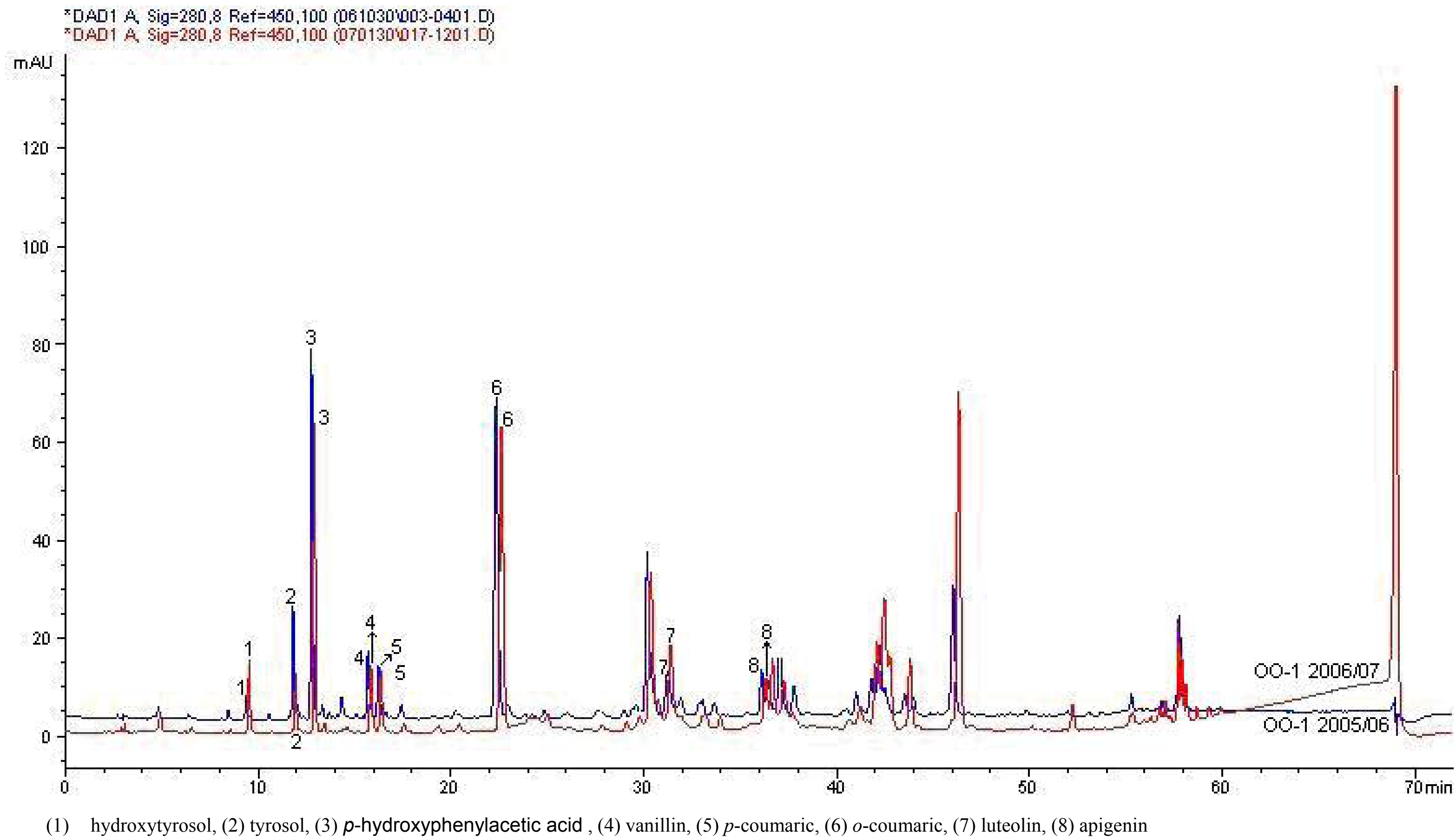
Figure A.1: The Chromatogram of EVOO in Two Consecutive Crop Seasons.



(1) hydroxytyrosol, (2) tyrosol, (3) *p*-hydroxyphenylacetic acid , (4) vanillin, (5) *p*-coumaric, (6) *o*-coumaric, (7) luteolin, (8) apigenin

Figure A.2: The Chromatogram of OGOO in Two Consecutive Crop Seasons.



(1) hydroxytyrosol, (2) tyrosol, (3) *p*-hydroxyphenylacetic acid , (4) vanillin, (5) *p*-coumaric, (6) *o*-coumaric, (7) luteolin, (8) apigenin

Figure A.3: The Chromatogram of OO-1 in Two Consecutive Crop Seasons.

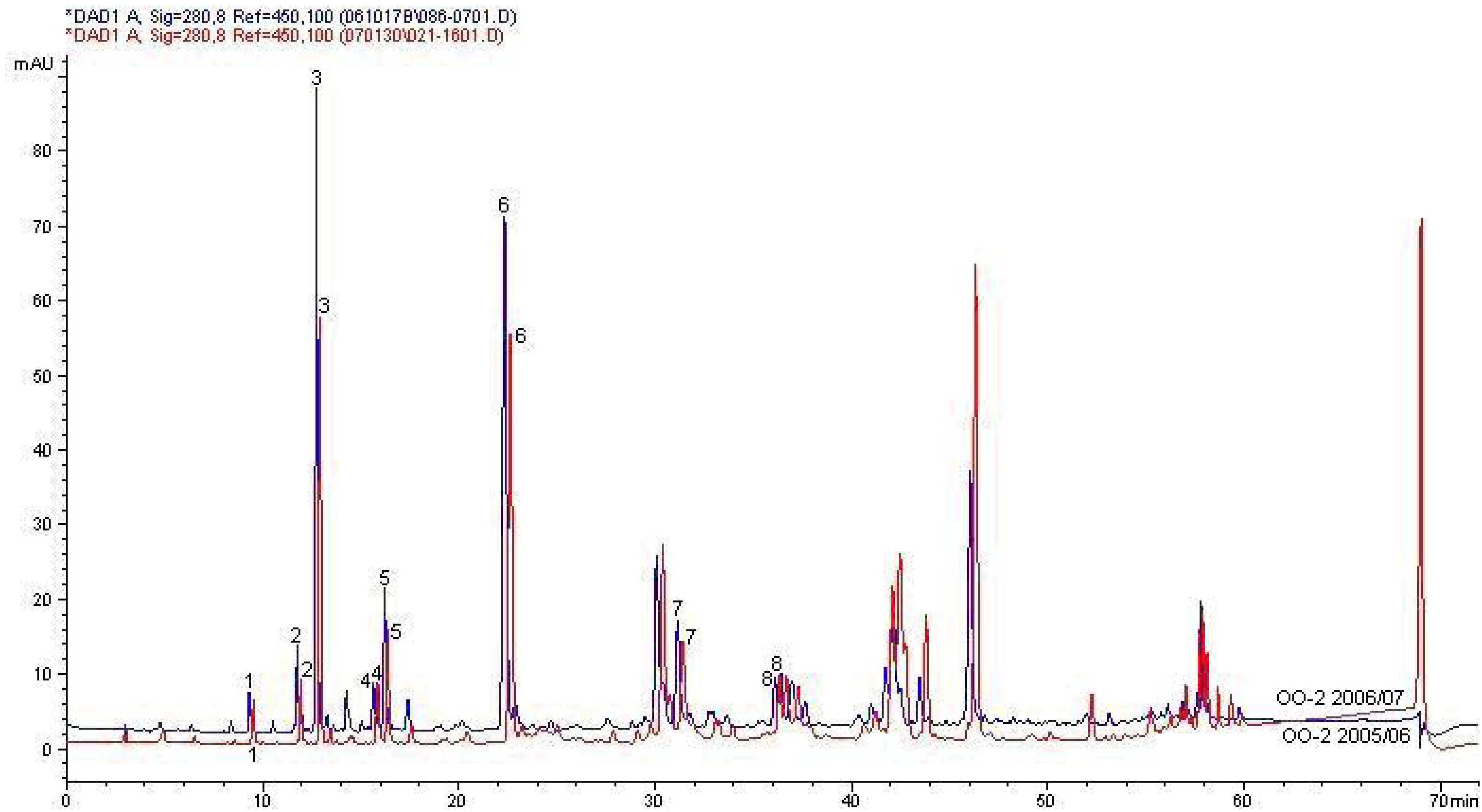


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Figure A.4: The Chromatogram of OO-2 in Two Consecutive Crop Seasons.

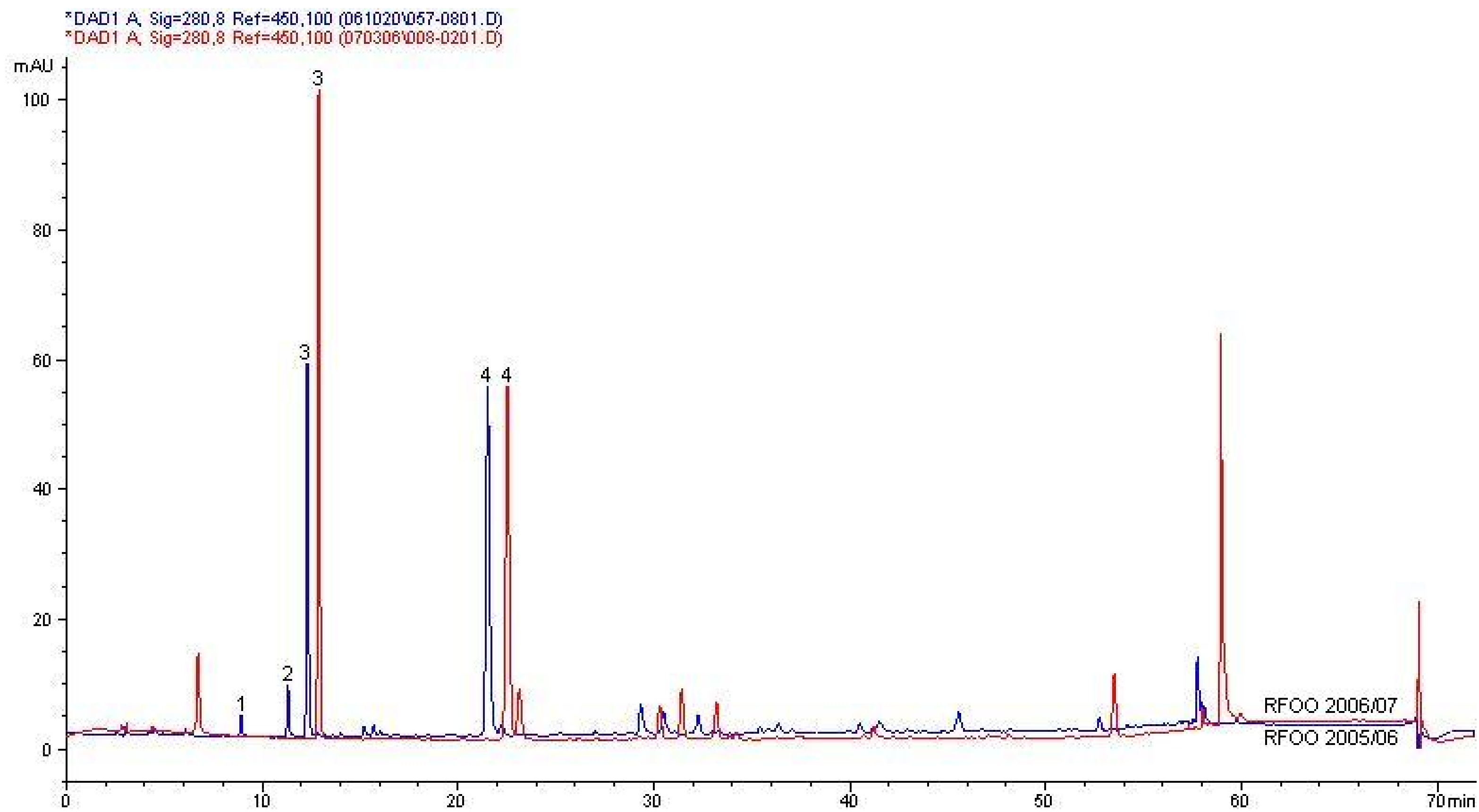

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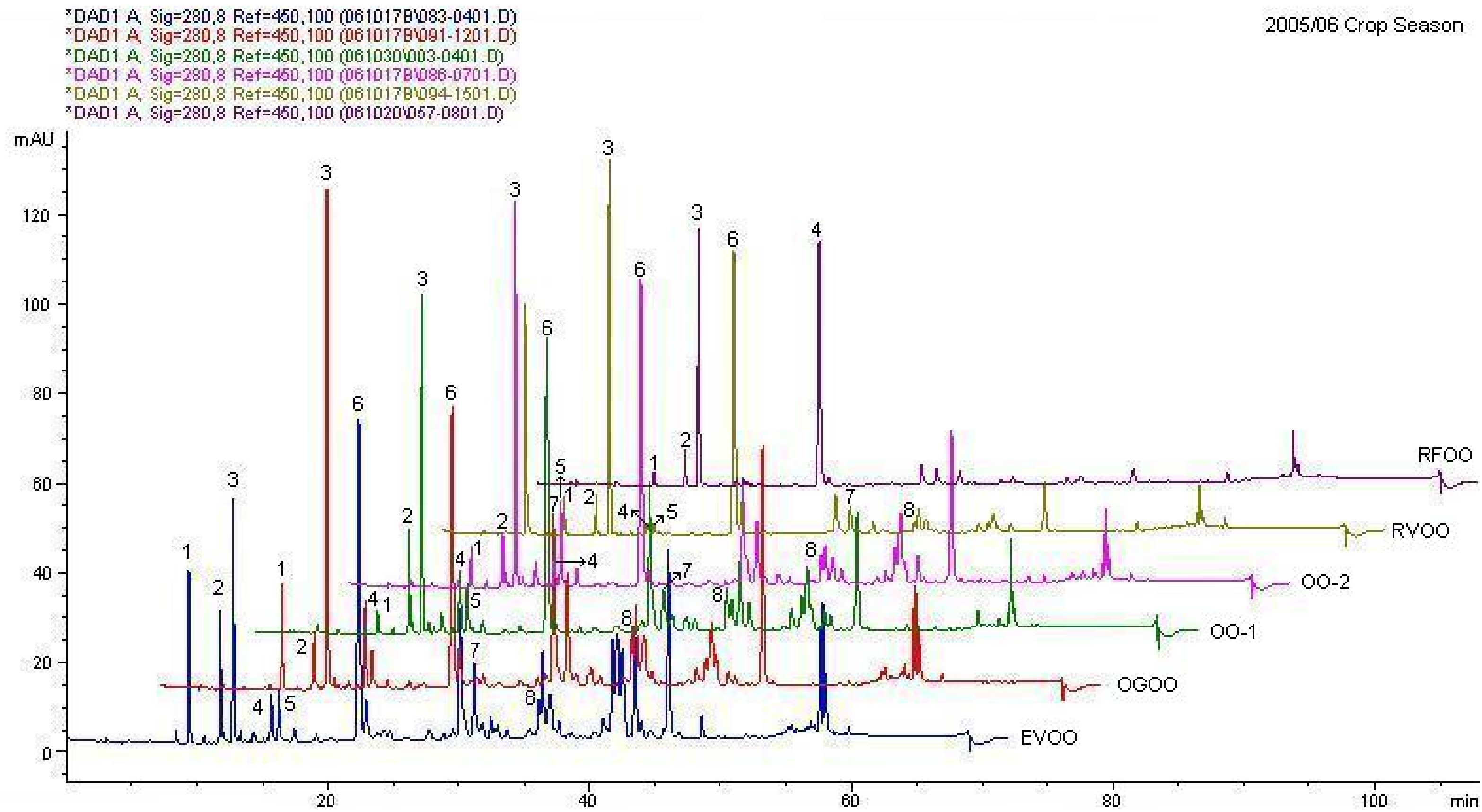
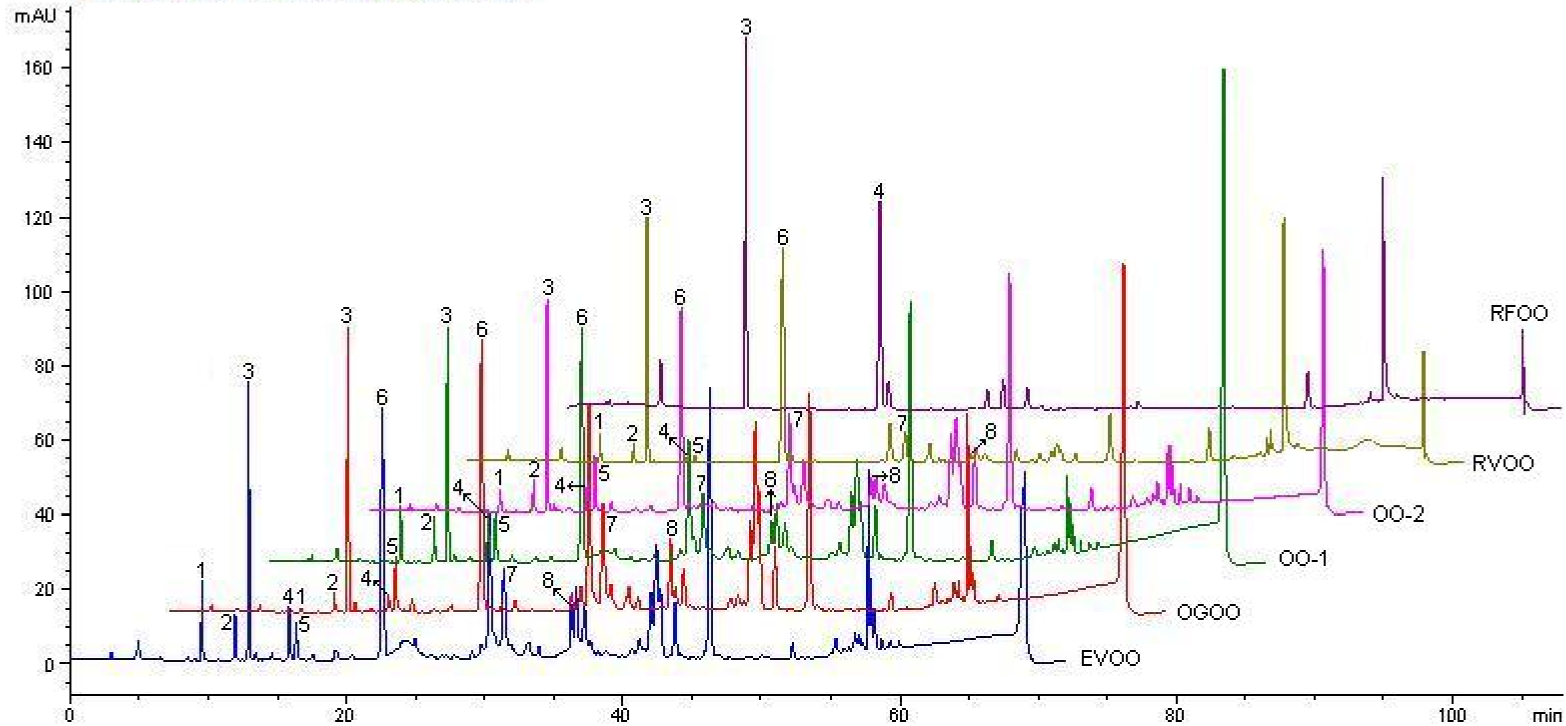
Figure A.5: The Chromatogram of RFOO in Two Consecutive Crop Seasons.



(1)hydroxytyrosol, (2) tyrosol, (3) *p*-hydroxyphenylacetic acid , (4) vanillin, (5) *p*-coumaric, (6) *o*-coumaric, (7) luteolin, (8) apigenin

Figure A.6: The Overlay Chromatograms of Samples Belong to 2005/06 Crop Season

Figure A.7: The Overlay Mass Spectra (MS) of Samples Belong to 2005/06 Crop Season



(1)hydroxytyrosol, (2) tyrosol, (3) *p*-hydroxyphenylacetic acid , (4) vanillin, (5) *p*-coumaric, (6) *o*-coumaric, (7) luteolin, (8) apigenin

Figure A.8: The Overlay Chromatograms of Samples Belong to 2006/07 Crop Season

Figure A.9: The Overlay Mass Spectra (MS) of Samples Belong to 2006/07 Crop Season

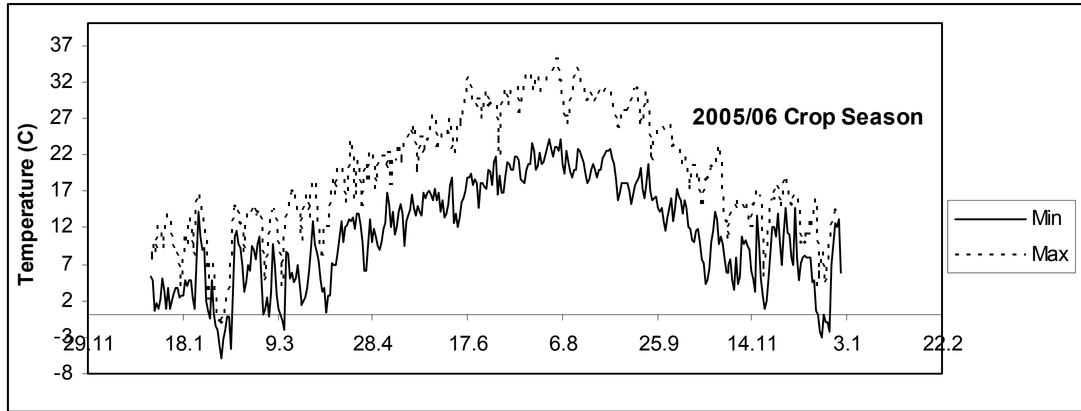


Figure A.10: Annual Pattern of Air Temperature in Canakkale Region for 2005/06

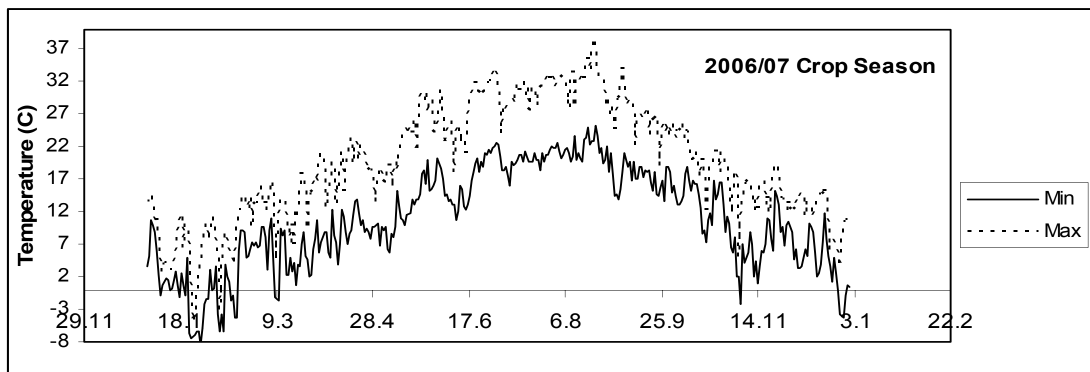


Figure A.11: Annual Pattern of Air Temperature in Canakkale Region for 2006/07

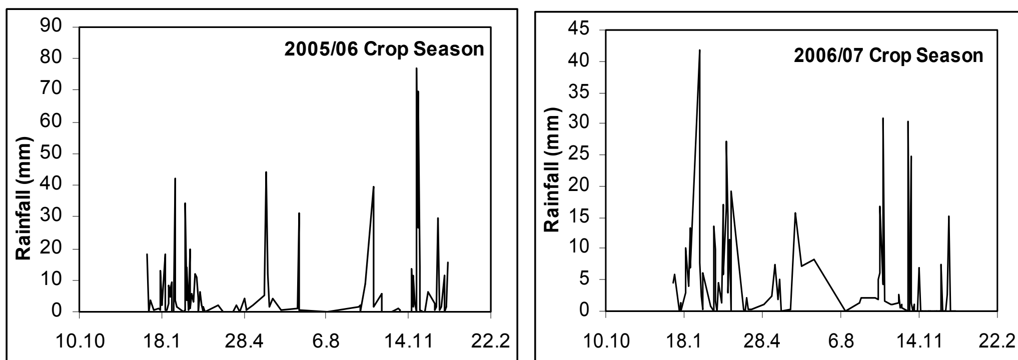


Figure A.12: Annual Patterns of Daily Rainfall (mm) for 2005/06 and 2006/07

CIRRICULUM VITAE

Sidal ACUN was born in Balikesir in 15/06/1981. She graduated from Caglayan High School in 1999 and graduated from Food Engineering Programme of Istanbul Technical University in 2005. At the same year, she graduated from Food Trade Association Programme of Anatolian University. In October 2006, she participated to Socrates/Erasmus Programme and went to Ghent University in Belgium to do experimental analysis.