EFFECT OF HARVEST TIME, MALAXATION TEMPERATURE AND OLIVE VARIETY ON THE CHEMICAL CHARACTERISTICS OF OLIVE OILS

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Olusola Samuel JOLAYEMI

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We approve the thesis of **Olusola Samuel JOLAYEMI**
Examining Committee Members:

Prof. Dr. Figen TOKATLI
Department of Food Engineering,
Izmir Institute of Technology

Prof. Dr. Durmuş ÖZDEMIR
Department of Chemistry,
Izmir Institute of Technology

Prof. Dr. Figen KOREL
Department of Food Engineering,
Izmir Institute of Technology

Prof. Dr. Aytaç GÜMÜŞKESEN
Department of Food Engineering,
Ege University

Doç. Dr. Nur DİRİM
Department of Food Engineering,
Ege University

5 December 2016

Prof. Dr. Figen TOKATLI
Supervisor,
Department of Food Engineering,
Izmir Institute of Technology

Prof. Dr. Banu ÖZEN
Co-advisor,
Department of Food Engineering,
Izmir Institute of Technology

Prof. Dr. Ahmet YEMENİCİOĞLU
Head of the Department of
Food Engineering

Prof. Dr. Bilge KARAÇALI
Dean of Graduate School of
Engineering and Sciences

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ABSTRACT

EFFECT OF HARVEST TIME, MALAXATION TEMPERATURE AND OLIVE VARIETY ON THE CHEMICAL CHARACTERISTICS OF OLIVE OILS

Changes in chemical and quality characteristics of olive oils were evaluated with respect to pre and post-harvest factors such as: olive type, harvest time, malaxation temperature, and storage at room temperature. Additionally, discriminative and predictive capacities of UV-vis, near infrared (NIR), mid infrared (MIR) spectra and electronic nose data on olive oils by using multivariate statistical tools were studied.

Varietal and harvest time differences were the most significant factors influencing the quality and chemical properties of Ayvalik and Memecik olive oils. Malaxation temperature was significant on a number of phenolic compounds such as tyrosol, hydroxytyrosol and pinoresinol, and peroxide value. Olive oils of early and mid-harvest were higher in phenolic alcohols, and pigments content, whereas peroxide values, linoleic and stearic acids characterized late harvest oils.

Storage for 15 months at room temperature in dark facilitated evolution of some important phenols, while increasing the content of ethyl and methyl esters at varying degrees among varieties. There were no significant changes in fatty acids and acidity contents of the oils. However, total phenol content and oxidative stability declined and a significant depletion of colors and pigments contents occurred. Multivariate regression analysis indicated that lipid-based variables are the most consistent contributors (positive or negative) to olive oil oxidative stability.

UV-vis, MIR, NIR spectroscopies and e-nose data were excellent varietal and harvest season discriminating tools. Pigments were well predicted by UV-vis, while MIR performs better in the prediction of fatty acids, alkyl esters, oxidative stability and free fatty acid.

ÖZET

HASAT ZAMANI, MALAKSÖR SICAKLIĞI VE ZEYTİN TİPİNİN SIZMA ZEYTİNYAĞLARININ KİMYASAL ÖZELLİKLERİ ÜZERİNE ETKİSİ

Bu çalışmada, zeytin yağlarının kimyasal kompozisyonları ve kalite parametrelerindeki değişiklikler, zeytin tipi, hasat zamanı, malaksör sıcaklığı ve depolama zamanına göre incelenmiştir. Ayrıca, ultraviyole-görünür (UV-vis), yakın-kızılötesi (NIR), orta-kızılötesi (MIR) ve elektronik burun (e-burun) tekniklerinin yağ örneklerini sınıflandırma ve bazı kimyasal parametreleri tahminleme kapasiteleri çok değişkenli istatistiksel tekniklerle çalışılmıştır.

Ayvalık ve Memecik zeytinyağlarının kimyasal ve kalite özelliklerinin farklılıklar gösterdiği ve bu değişkenlerin en fazla hasat zamanından etkilendiği tespit edilmiştir. Malaksiyon sıcaklığı bazı fenolik maddeler (tirosol, hidroksitirosol, pinoresinol) ve peroksit değeri üzerinde etkili bulunmuştur. Erken ve orta hasat dönemlerinde elde edilen yağlar fenolik alkoller ve klorofil, karoten pigmentleri açısından daha zenginken, geç hasat yağlarını karakterize eden parametreler yüksek peroksit değeri, linoleik ve stearik asit konsantrasyonları olarak bulunmuştur.

Oda sıcaklığında ve karanlıkta 15 ay depolanan zeytinyağların toplam fenol, renk pigmenti konsantrasyonları ve oksidatif stabilitelerinde azalma, etil ve metil ester miktarlarında ise artış gözlenmiştir. Yağ asitleri kompozisyonlarında ve serbest asitlik değerlerinde istatistiki olarak önemli değişiklikler görülmemiştir. Fenolik maddelerden hidroksitirosol, tirosol ve pinoresinol konsantrasyonlarında artış tespit edilmiştir.

UV-vis, MIR, NIR ve e-burun verileri zeytinyağlarının varyete ve hasat zamanına göre sınıflandırılmasında etkili olmuştur. Pigment konsantrasyonları UV-vis ile daha doğru tahmin edilirken, MIR, yağ asitleri, alkil esterleri, oksidatif kararlılık ve serbest yağ asidi parametrelerinin tahmininde daha iyi sonuçlar vermiştir.

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LIST OF ABBREVIATIONS

% CC	Percent correct classification
$^1O_2^*$	Excited singlet oxygen
$^3O_2$	Ground state triplet oxygen
2der	Second derivative
3,4-DHPEA	3,4-dihydroxyphenyl-ethanol
3,4-DHPEA-EDA	3,4-dihydroxyphenyl-ethanol-elenolic acid diahdehyde
a*	Green-Red chromatic
AA	Antioxidant Activity
b*	Yellow-Blue chromatic
C	Chroma
COX	Cyclooxygenase
DAD	Diode Array Detector
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
EE	Ethyl esters
EEC	European Union Commission
EFSA	European Food Safety Authority
EPA	Eicosapentaenoic acid
EVOO	Extra Virgin Olive Oil
FAAE	Fatty acid alkyl esters
FAEE	Fatty acid ethyl esters
FAME	Fatty acid methyl esters
FFA	Free Fatty Acids
GA	Gallic acid
GC-FID	Gas Chromatography Flame Ionizing Detector
GC-MS	Gas Chromatography Mass Spectroscopy
HDL	High Density Lipoprotein
HPLC	High Performance Liquid Chromatography
L	Light-Dark chromatic
H	Hue color chromatic
IOC	International Olive oil Council

ISTD	Internal Standard
NIR	Near Infrared Spectroscopy
LOX	Lipoxygenase
LOO	Lampante olive oil
LDL	Low Density Lipoprotein
MUFA	Monounsaturated fatty acid
MIR	mid Infrared Spectroscopy
ME	Methyl esters
MOS	Metal oxide semi-conductor sensor
OPLS-DA	Orthogonal projections to Latent Structures Discriminant Analysis
OSI	Oxidative stability index
O-der	Oleuropein derivative
PC	Principal Component
PCA	Principal Component Analysis
PDO	Protected Designation of Origin
PEN	Portable Electronic Nose
PGI	Protected Geographical Indication
p-HPEA	*p*-hydroxyphenyl-ethanol
p-HPEA-EDA	*p*-hydroxyphenyl-ethanol- elenolic acid diahdehyde
PLS-DA	Partial Least Square Discriminant Analysis
POD	Peroxidase
PPO	Polyphenoloxidase
PUFA	Polyunsaturated fatty acid
PV	Peroxide Value
R^2	Degree of Fitness
R^2_{cal}	Quality of calibration model
R^2cv	Coefficient of cross-validation
R^2_{val}	Coefficient of validation
$RMSEC_V$	Root Mean Square error of cross-validation
RMSEC	Root Mean Square error of calibration
RMSEP	Root Mean Square error of prediction
SFA	Saturated fatty acid
SIMCA	Soft Independent Modelling Class Analogy
SNV	Standard Normal Variate

TPA	Total phenolic acid
TPC	Total phenol content
UV-vis	Ultraviolet-Visible radiation
VIP	Variable Influence on Projection
VOO	Virgin olive oil

CHAPTER 1

INTRODUCTION

Mediterranean constitutes the region of the world endowed with vast olive groves responsible for over 90% of the world production. The relatively higher life expectancy of this region may not be a surprise, considering the varieties of nutritious food substances that constitute their diets. As one of the main components of these diets, olive oil possesses unique characteristics that depend on both agronomical, processing and storage conditions of olive fruits. Parameters such as olive cultivar, variety, genetic, fertilizer and irrigation systems, degree of ripening or maturity and harvesting time, constitute the main primary quality determinants in virgin olive oil. Olive fruits obtained under suitable agricultural conditions do not automatically guarantee oil of acceptable organoleptic and nutritional status. Effective and appropriate operational choices in conjunction with high quality raw materials are required, in order to produce higher grade olive oils. This production process, which is solely by mechanical pressing, devoid of any external chemical inclusion or elevated temperature, must be carefully modulated. Essential operational variables such as type of olive mill (two or three phase), malaxation conditions (time-temperature, headspace, impeller type etc.) influence the "virginity" of olive oil, from sensory and nutritional perspectives (Reboredo-Rodríguez et al., 2014, Fregapane & Salvador, 2013).

Nutritionally, there are several chemical compounds making olive oil indispensable as an essential, health-significant part of human diet. One of such is monounsaturated oleic acid composition of its triacyglycerols. Oleic acid does not only contribute to the storage stability of olive oil, but also promotes substantial health benefits in consumers (Rondanini, Castro, Searles & Rousseaux, 2014; Tamborrino et al., 2010). Other appealing chemical constituents of olive and olive oil are polyphenolic compounds, which belong to different classes based on their molecular weights and structures. Phenolic acids, phenyl ethyl alcohols, hydroxylisochromans, flavonoids, lignans and secoiridoids are the most characterized among others (Bendini et al., 2007). Phenols contribute to the sensory properties of olive oils; they are good antioxidants, enhance stability during storage and are health related components. The consumption of 5 mg per 20 g of hydroxytyrosol and or its derivatives daily was recommended as

capable of preventing oxidative damages in human system (EFSA, 2011, EFSA, 2012). Quality authentication and certification is one of the most controversial aspects of olive oil regulation. A recently adopted system of quality assessment of olive oil by International Olive Council and European Union is the ethyl and methyl ester compositions in conjunction with wax contents (EEC, 2013; IOC, 2010).

Olive oils of poor and defective quality can pass the rigorous panels of expert's assessment undetected. Evaluations of olive oil authenticity have further shifted toward rapid fingerprinting techniques such as spectroscopy and electronic sensing. This is to overcome the hurdles of chemical analysis which include: expensive chemical reagents, extensive extraction procedure, expertise and technical requirements, time and a lot of waste generation (Forina et al., 2015). Different regions of electromagnetic spectrum in conjunction with chemometric techniques have been employed to predict the differences in olive oils origin, harvest time, and chemical variables. There are quite a number of reports devoted to the application of UV-vis, mid (MIR) and near (NIR) infrared spectroscopy to characterize various aspects of olive oils including Protected Designation of Origin (PDO) and Protected Geographical Indication (PGI) confirmation (Sinelli et al., 2008, Sinelli et al., 2010; Terouzi, et al., 2013). In the same vein, electronic senses such as: e-nose, e-tongue and e-eyes applied separately or together, are considered to be close to becoming effective replacements to human olfactory, gustatory and vision systems, in quality assessment of olive oils (Pierpauli, et al., 2008; Lerma-García et al., 2010; Haddi et al., 2013).

In view of all these aforementioned, the objectives of this thesis study are to determine the effects of malaxation temperature, olive variety, harvest time on chemical properties of olive oil; to evaluate changes in olive oil chemical parameters after 15-month of dark storage at room temperature; and to assess the predictive, pattern recognition and discriminative capacities of UV-vis, near infrared (NIR), mid infrared (MIR) spectra and electronic nose data on olive oils using multivariate analytical techniques such as principal component analysis (PCA) and orthogonal partial least square discriminant analysis (OPLS-DA).

CHAPTER 2

OLIVE

2.1. World Production

There are approximate 23 million acres of olives (9.4 million ha) with an output of about 1.5 million tons of table olives and 16 million tons of olives out of which 2.56 million tons of oil is extracted. As at 2016, the olive oil production was reported as 2.9 million tons. Spain has about one-quarter of the world acreage, with 5.98 million acres (2.42 million ha) of olive trees under cultivation and 36 % of the oil production (approx. 800, 000 t/year) making it number one top producer in the world (Fig. 2.1). Italy is ranked second, with about 25% of the world's oil production (520, 000 t/year). The third on the rank is Greece with about 17% (approx. 400,000 t/year) of the world's oil production. Turkey occupies the 5th position with about 4.2% of world production. These figures have not significantly changed since 2003 (IOC, 2015).

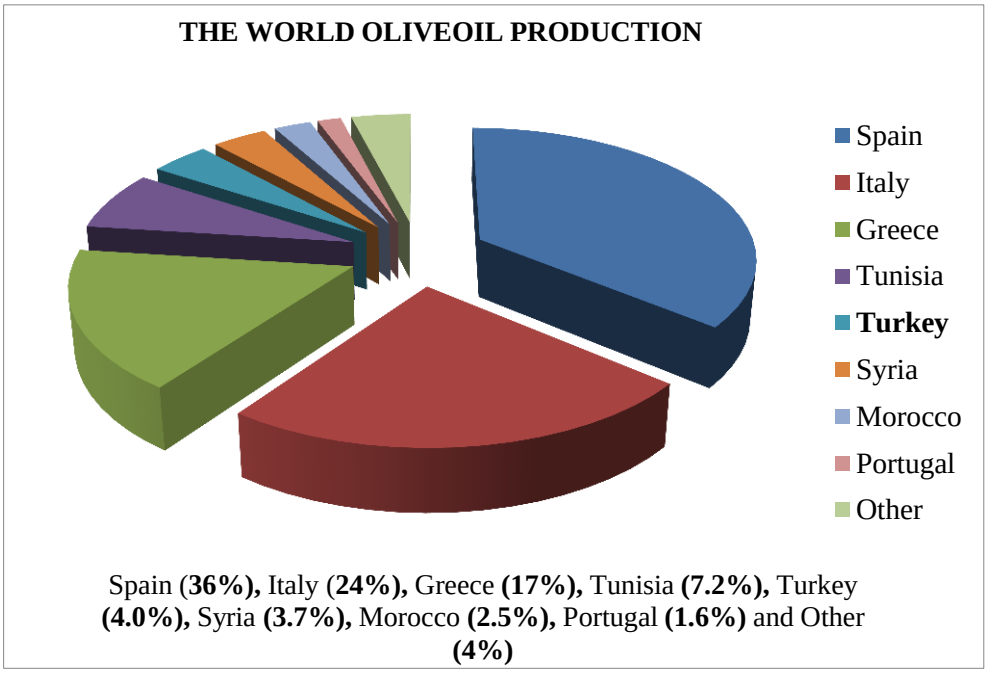

Figure 2.1. World olive oil production (Source: IOC, 2015)

2.2. Turkish Olives

As at the year 2010, olive agricultural area in Turkey amounts to 79,000 ha of land, about 72% of the total cultivation is primarily for oil production. This figure varies from season to season and over the last five harvest years, olive acreage has experienced a great deal of expansion. (IOC, 2012).

Turkey is characterized with the longest coastline among the countries in Mediterranean Sea, thereby making it one of the main producers of olives. There are five regions in Turkey where olives are grown: Aegean, Marmara, Mediterranean, South eastern Anatolia and Black Sea, each with its distinctive pedioclimatic properties (IOC, 2012)

In the Aegean Region, 80% of the olives produced are processed into olive oil and 20% go for table production (Ilyasoglu et al., 2011). The humid and steamy air blowing in from the North Aegean Sea; when blended with oxygen helps create ideal climatic and environmental conditions suitable for the proliferation of Ayvalik olive variety. The Marmara region, on the other hand, is responsible for over 40% of all the table olives produced in Turkey. The cultivation of olive in the Mediterranean region lies between Taurus Mountains and the coastline. 68% of olives produced in this region go to oil production and 32% for table olive processing. South-eastern Anatolia, region has 86% of olive production for oil and 14% for table olives. Lastly, the Black Sea region is usually known for subsistence production of table olives for self-consumption (IOC, 2012).

2.2.1. Varieties

Olive cultivars differ in seasonal crop load, fruit size, and flesh-to-stone ratio, rate of ripening and pattern, degree of adaptability, productivity, stress response, disease resistance and many more. These characteristics variation among olive and its product depend primarily on its genetic make-up. Climatic adaptations influence the phenotypic stability of olive fruits (Leon, Martin, & Rallo, 2004), thereby giving rise to different varieties with individual peculiarity. According to World Catalogue of Olive Varieties (IOC, 2012) eleven main olive varieties were reported in Turkey: Ayvalik, Çekiste, Çelebi, Domat, Erkence, Gemlik, Izmir Sofralik, Memecik, Memeli, and Uslu. Two of

the most economic important varieties (Ayvalik, Memecik) and a local variety (Erkence) were studied with respects to their agronomical behavior and performances.

***Ayvalik*:** This is a highly vigorous cultivar adaptable to relatively arid areas. It is cultivated along the northern Aegean coast, accounting for about 25% of olive acreage in Turkey. It has high productivity and intermediate fruit ripening. The fruit has over 24% oil content, which is reasonably high. Owing to the exceptional aromatic, quality and other chemical properties of its oils, Ayvalik is considered as one of the most promising olive cultivar in Turkey. It is also very amenable to mechanical harvesting procedure because of its upright erect growth habit. It is very suitable for split green olives and black olives production; having 5.6 flesh to stone ratio. It is resistant to olive fly.

***Memecik*:** This variety is highly adaptable to various soil and pedioclimatic conditions. It is closely similar to Ayvalik in its rooting ability and intermediate fruits bearing and flowering times. It has a very high productivity. Harvesting time is intermediate, when fruit is for green pickling and fruity aromatic oil. It has a high oil yield and a high flesh-to-stone ratio. This variety is becoming popular for black pickling as well. It is cold-tolerant and can survive extreme drought conditions, but moderately vulnerable to olive fly.

***Erkence*:** This variety accounts for over 3 million trees in Turkey. The oil yield of the fruit is about 25% close to that of Ayvalik, Memecik and Memeli. However, it is basically used for the production of good but inferior quality olive oil compared to Ayvalik and Memecik cultivars. It is also suitable for green or black pickling. The fruit is easy to harvest because of low removal force and usually drops prior to harvesting. The fruit is highly vulnerable to disease (*Phoma oleae)* during ripening, especially in area prone to wet winds. This reduces its bitterness and causes brown coloration. When the fruit turns this particular color, it is known as "Hurma" olive (naturally de-bittered olives), which is ready for direct consumption.

2.3. Olive Oil

The liquid obtained solely from the fruit of the olive tree *(Olea europeae sativa),* is called olive oil. This is to the exclusion of oils obtained using solvents or re-esterification processes or mixture with oil of other kinds. Its market value is based on

some parameters, which are based on consumer protection against fraudulent, adulteration and misrepresentation that are so common in olive oil industries. The markets have been so buoyant over the years, extending into non-producing countries. In accordance to the International Olive Council designation, olive oils are placed in classes and they are priced accordingly (IOC, 2016).

Virgin olive oil: Oils obtained solely from healthy and wholesome olive fruits by mechanical and physical means absence of any chemical inclusion or extreme temperature is technically referred to virgin olive oil. The thermal conditions of extraction are expected to be within the minimum level. Virgin olive oil must not have undergone any secondary treatment such as washing, decanting or filtering, in order to fall within this category. It is fit for consumption as it without further processing (natural). These parameters are the primary basis on which classification of olive oil to extra virgin, virgin olive oil, ordinary virgin, and not fit for consumption is lampante olive oil.

***Extra virgin olive oil*;** has free acidity less than 0.8 g per 100 g. It is naturally considered as the finest and fruitiest of the olive oils and is therefore the most expensive. Extra virgin olive oil is mostly recognized and classified as based on its overall physicochemical and organoleptic properties.

Virgin olive oil; has free acidity less than 2 g per 100 g. It is suitable for consumption.

***Ordinary virgin olive oil*;** has free acidity below 3.3 g per 100 g. Ordinary virgin olive oil has undergone serious sensory and organoleptic defects that are easily detectable by trained experts. Apart from the defect in sensory attribute, there are corresponding defects in the essential chemical properties such as: decrease in phenolic compounds, increase in alkyl esters and wax contents.

***Lampante virgin olive oil*;** refers to olive oil that is not fit for consumption as it is. The free acidity is above 3.3 g per100 g and it recommended for refinement or technical use.

***Refined olive oil*;** is obtained from any category of olive oil, by refining methods. The refining process removes color, odor, and flavor from the olive oil, and leaves behind a very pure form of olive oil that is tasteless, colorless, and odorless and extremely low in free fatty acids (> 0.3g per100g).

***Olive oil as the blend of refined and virgin olive oils*;** which has free acidity greater than 1g per 100 g and may not necessarily be a blend of two different categories.

Crude olive-pomace oil; is intended for human consumption after refining; but usually for technical purpose.

***Olive-pomace oil*;** is obtained from chemical (solvents) or physical treatment of olive pomace. This does not include oils obtained from a process of re esterification. A blend of refined olive pomace oil and virgin olive oils can also fit for consumption.

2.4. Chemical Composition

The chemical composition of olive oil can be considered in two broad categories: saponifiables and unsaponifiables. The former comprises of triacylglycerol (TAG), and other lipid-based constituents, which together represent 98% of the oil chemical composition. The latter is mainly minor components such as phenolic, tocopherols, phytosterols, and color pigments.

2.4.1. Olive Oil Lipids

Fatty acids present in olive oil include palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic (C18:1), linoleic (C18:2), and linolenic (C18:3) mainly, and myristic (C14:0) margaric (C17:0), gadoleic (C20:1), 11-cis-vaccenic acid and eicosenoic acids in trace amounts. Oleic acid (C18:1) is the principal component constituting 55-75% total fatty acid. The range may change depending on some parameters such as growth location, latitude, climate, variety, and stage of maturity of the olive fruit (Beltrán, Del Rio, Sanchez, & Martinez, 2004). Polyunsaturated fatty acids (PUFAs) of 18 carbon atoms such as such as linoleic (18:2 ω-6), and α-linolenic (18:3 ω-3) are regarded as essential fatty acids because of their relevance in maintaining healthy human physiological system (Radcliffe, et al., 2016). Human depends on sufficient intake of EFAs as they are not synthesized in the body. The intake of PUFA should account for only 6–8% of calories from fat, while the consumption of saturated fatty acids should be kept moderate (approximately the same amount as polyunsaturated, with a ratio of 1:1). In the diet, the presence of ω-6 and ω-3 fatty acids in correct ratio is very important. Excess amount of linoleic acid can interrupt the endogenous synthesis of long-chain, hormone-like (prostaglandins and leukotrienes) important α-linolenic acid derivatives (eicosapentaenoic EPA acid and docosahexaenoic

acid DHA), with consequent damage to body (Viola & Viola, 2009). According to WHO recommendations, ratio of 5 to 10 between omega 3 and omega 6 fatty acids is very important for growth and development of brain. Other important functions attributed to appropriate ratio of omega 6 to omega 3 fatty acids are: anti-cancer, antiplatelet aggregation, anti-inflammatory, and protection against dryness of the skin as emphasized by Caporaso et al., (2015). Olive oil obtained under a well-controlled conditions of harvesting, processing and storage, has been found to meet this ratio, whereas the same cannot be said of other vegetable oils.

Another ratio of importance in olive oil is oleic/linoleic acids, which shows the stability of olive oil to oxidation, meaning less primary and secondary oxidative products in olive oil. High value is an indication of low susceptibility of the oil to oxidative degradation. This is based on the fact that oleic acid is about 10 to 40 times less vulnerable than linoleic acid. The decrease in bond dissociation energy associated with the addition of methylene-interrupted carbon in a triglyceride makes unsaturated fatty acids more prone to oxidation (McClements & Decker, 2008). Lowering of serum cholesterol is widely accepted as one of health significant benefits of oils having high level of MUFAs (Monounsaturated fatty acids) and lower in saturated fatty acids (SFAs). This claim places olive oil above every other vegetable oil nutritionally. Apart from other minor components exerting significant health impacts on olive oil high amount of MUFAs, low SFA, moderate PUFAs (essential fatty acids) makes virgin olive oil an indispensable commodity.

2.4.2. Olive Oil Phenols

Phenolic substances are highly complex components of olive oil and their concentrations depends on several factors including maturation stage, part of the fruit, variety, season, packaging, storage, agronomical variables and extraction techniques applied during production (Bendini et al., 2007; Gómez-Rico, Fregapane, & Salvador, 2008). The main classes of phenols in virgin olive oil are secoiridoids, phenolic acids, phenolic alcohols, hydroxyl-isocromans, flavonoids, and lignans (Fig. 2.2). The amounts range between 40 and 900mg/kg when estimated colorimetrically (Servili, Selvaggini, Esposto, Taticchi, Montedoro, & Morozzi, 2004). These biophenol compounds in conjunction with its antioxidant potentials play important role in

nutritional and organoleptic properties of virgin olive. Several phenolic acids, such as protocatechuic, *p*-hydroxybenzoic, vanillic acid, caffeic acid, syringic, *p*- and *o*-coumaric acid, ferulic acid, and cinnamic acid have been quantified chromatographically and the content is often less than 1mg per kg of olive oil (Bendini, et al., 2007). These compounds seem less potent in their antioxidant capacity, but capable of serving as fingerprint in the identification of olive variety.

Oleurropein, demethyloleuropein and ligstroside are the secoiridoids found in olive oil. The most abundant ones are the dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol (3,4-dihydroxyphenyl-ethanol) or *p*-hydroxyphenyl-ethanol (3,4-DHPEA or *p*-HPEA) (3,4-DHPEA-EDA or *p*-HPEA-EDA). Their concentrations are usually low in fresh oil but increase during storage due to hydrolysis to produce their derivatives (Hydroxytyrosol 3, 4-DHPEA and tyrosol *p*-HPEA respectively), which constitute the main olive oil phenolic alcohols. Flavonoids include flavones, flavonols, flavanones, flavanols, anthocyans and their glucosides derivatives (such as luteolin-7-glucoside and rutin. Luteolin and apigenin are the only form of flavonoids found in olive oil (Artajo, Romero, & Motilva, 2006). Lignans are another group of phenols recently quantified by Brenes et al., (2000) and Owen et al (2000). Lignans are characterized in the form of (+)-1-acetoxypinoresinol and (+)-1-pinoresinol as the most abundant phenolic compounds in olive oil. Lignans are potent antioxidants that are absent in seed oil and are only present in virgin olive oil. It may be capable of modulating cancer chemopreventive activities like every other natural antioxidant (Cecchi et al., 2017). Oleocanthal is an ester of tyrosol that is structurally related to olEECropein. It is a phenylethanoid form of natural phenolic compound. It is mainly found in extra virgin olive, and it is responsible for the burning sensation that accompanies consumption of fresh extra virgin olive oil (Cicerale et al., 2013). It has been described as having similar properties to that of anti-inflammatory molecule ibuprofen in inhibiting cyclooxygenase (COX)-1 and COX-2 (Beauchamp et al., 2005). Hydroxy-isochromans is also a relatively new class of phenolic compounds of extra-virgin olive oil and the presence of 1-phenyl-6, 7-dihydroxy-isochroman and 1-(3-methoxy-4-hydroxy) phenyl-6, 7-dihydroxy isochroman formed mainly during oil extraction, by reaction between hydroxytyrosol and vanillin or benzaldehyde (El Riachy, Priego-Capote, León, Rallo, & Luque de Castro, 2011)

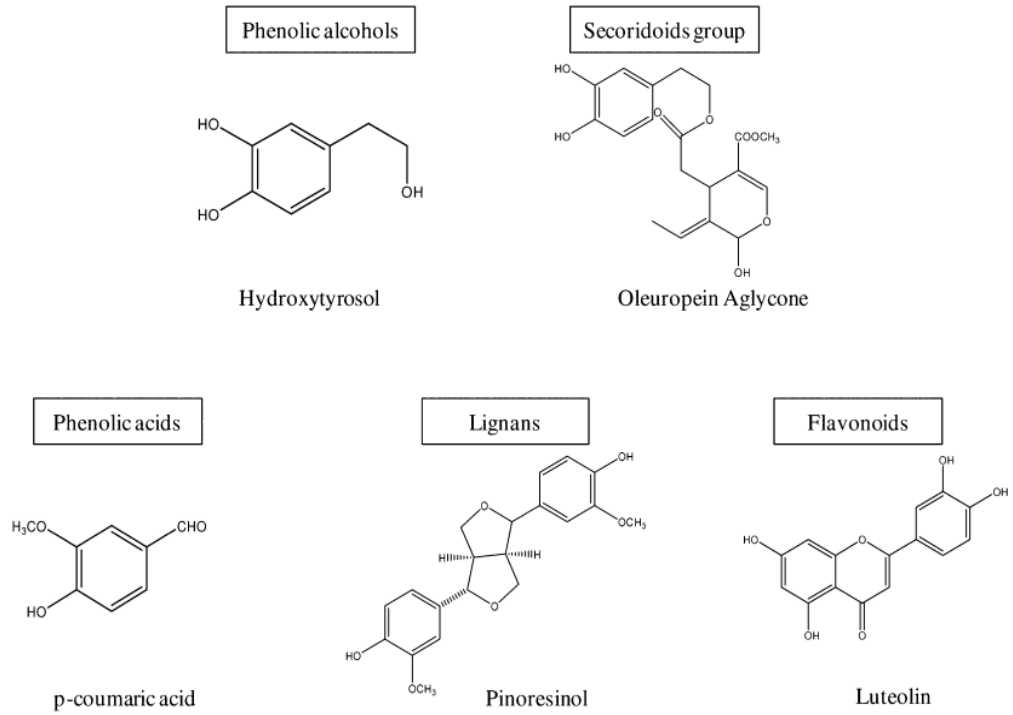

Figure 2.2. Structures of the main phenolic compounds from each family identified in virgin olive oil

2.4.3. Olive Oil Alkyl Esters

There are over 10 analytical parameters that can provide the means of accessing the authenticity of olive oil. The fulfillments of these standard requirements, is what qualifies olive oil to carry the label “extra virgin”. The most recent one among these parameters is the ethyl and methyl ester contents, sum of which is called alkyl esters. The proponent of this method as a quality assurance parameter of olive is borne out of the incessant adulteration, mislabeling, and misrepresentation fraudulent activities of olive oil that has become a global phenomenon (Perez-Camino et al., 2008). One of the most common and subtle way of olive oil adulteration that have achieved great success in eluding discovery, is addition of deodorized low quality olive oil with extra virgin olive oil. Fatty acid ethyl esters (FAEEs) and methyl esters (FAMEs) are formed by esterification of free fatty acids with low molecular weight alcohol functional groups such as ethanol and methanol. With the availability of alcohol groups, triglycerides molecules are converted into their corresponded methyl or ethyl esters; liberating glycerol as a byproduct. Ethyl and methyl ester contents of olive oils are known to be dependent on the initial health conditions of the fruits, hydrolytic and fermentative

process in olive fruit before extraction. As part of the fermentative metabolites, ethanol is accumulated at a rate proportional to many factors other than just fermentation. Olive cultivars, ripening rate and harvest time are some of the factors influencing ethanol accumulation in olive fruits (Beltran et al., 2015).

The major fatty acid alkyl esters found in olive oils are those corresponding to palmitic, oleic, stearic and linoleic acids. In high quality extra virgin olive oils, the content of methyl and ethyl esters are relatively close as reported in literature (Perez-Camino et al., 2008). A concentration of 35 mg/kg of alkyl esters have been the standard requirement (IOC, 2015). The ratio of ethyl and methyl ester is another parameter. A FAEEs/FAMEs ratio of less than 1.5 is expected in the case of extra virgin olive oils. Alkyl esters as quality parameters are currently indisputable especially when combined with other quality assessment procedures.

2.4.4. Olive Oil Volatile and Aromatic Compounds

Olive oil obtained from healthy fruits, harvested at appropriate maturation stage and processed under a well-controlled extraction condition contains mainly volatiles derived from linoleic and linolenic acid decomposition through the lipoxygenase (LOX) pathway (Clodoveo et al., 2014). Hexanal, I-2-hexenal, (z)-3-hexenal, (z)-3-hexen-1-ol, hexan-1-ol, hexyl acetate and (z)-3-hexenyl acetate are the most abundant volatile compounds present in olive oil. These volatiles are responsible for the fruity and green perception peculiar to virgin olive oil. Olive oils produced from microbial infected, overripe, advanced stage fermented and over-stored fruits produce greater number of volatile compounds responsible for their sensory defects. Acids, esters, alcohols, aldehydes and ketones are the primary contributors to the fusty and wine-like defects associated with low quality olive oil (Morales, Luna, & Aparicio, 2005)

The 13-hydroperoxides are enzymatic product of LOX pathway from linoleic and linolenic acids (Padilla, Hernández, Sanz, & Martínez-Rivas, 2014). It is a secondary oxidative product and subsequently turned into C6 aldehydes and C12 oxoacids, from where C5 compounds are generated. The formation of these two aromatic compounds (C6 and C5) is affected by the degree of olive maturation or ripening, time-lag between olive harvest and processing, and extraction parameters (Angerosa et al., 1999). In addition to these volatiles, autoxidation decomposition

products of linolenic, linoleic and oleic acid produce aldehydes as the main hydrocarbon framework for olive oil volatiles. . Also, aldehydes, alcohols and ester derived from biochemical transformation of amino acids can contribute to the aroma of olive oil to a small degree.

2.4.5. Coloring Pigments

The pigment profile of virgin olive oil is ultimately determined by the fractions of chlorophyll and carotenoid that are initially present in the olive fruit prior to extraction and their derivatives during milling. The chlorophyll and carotenoids content of olive oil basically depend on the degree of maturation or ripening, variety, geographical origin and quality differences (Minguez-Mosquera et al., 1991). However, chlorophyll and carotenoids are good monitoring tools to evaluate the impact of storage on olive oil color degradation with respect to various storage conditions. Morelló et al., (2004) observed a noticeable and proportionate reduction in chlorophyll and carotenoid of olive oil over a long storage period. Chlorophylls in form of pheophytin, contents of olive ranges from 3.3 to 40 mg/kg, while other derivatives of pheophytins (chlorophyll a and b) are present in trace amounts (Gandul-Rojas & Gallardo-Guerrero, 2014. In olive fruit the rate of decrease in chlorophyll is greater than carotenoid during ripening, which implies that, the ratio of the two pigments decreases with maturation (Gallardo-Guerrero et al., 2002). Bleaching of chlorophyll occurs when exposed to light and under intense temperature. Apart from contributing to the color of the oil, chlorophyll exhibits antioxidative properties preventing skin aging and cancer. However, at the same time, chlorophyll is a photosensitizer capable of initiating oil oxidation when exposed to light by the conversion of ground state triplet oxygen ($^3O_2$) to reactive excited singlet oxygen ($^1O_2{}^*$). The main carotenoids found in olive oil are lutein and β-carotene, which is a precursor of vitamin A (Psomiadou & Tsimidou, 2001). Carotenoids are considered more potent antioxidant than chlorophyll because of its positive contribution to oxidative stability of olive oil (Jolayemi, Tokatli & Ozen, 2016).

2.4.6. Sterols and Tocopherols

Sterols are major part of unsaponifiable matters that are biosynthetically derived from squalene to form group of triterpenes. Phytosterols are similar to human cholesterols with respect to chemical structural and biological function (Fernández-Cuesta, León, Velasco, & De la Rosa, 2013). They are very applicable in the authentication test of olive oils. There are basically four classes of sterols in olive oil: Total phytosterols content of olive oil varies between 1000 and 2300 ppm (IOC, 2015). Cholesterol being the most important desmethylsterol compounds in animal fat, *β*-sitosterol constitutes almost 90% total plant sterols (Canabate-Diaz et al., 2007). The content of this compound and it derivatives in olive oil depends on various factors that affect other components of olive oil. Olive cultivar, maturation and ripening are the primary factors influencing the content of sterols in olive oil. It is noteworthy to state that storage of olive oil could hardly be found a legit parameter to monitor differences in sterol content of olive oil (Psomiadou & Tsimidou, 2002).

Tocopherols are the most prominent lipophilic phenols in VOO. Four types of tocopherols α-, β-, γ- and δ-tocopherol have been identified and reported in olive oil, with α-tocopherols constituting about 90% total amount of the compound (Beltrán et al., 2010). Several claims have been made on the influence of tocopherol in preventing the dreadful effects of reactive oxygen species (ROS) in physiological system. Examples is the cell aging management, improve immune system and prevention of cardiovascular diseases (Bramley et al., 2000).

2.4.7. Health Claims on Olive Oil

Protection of LDL particles from oxidative damage: Physiologically, a good number of diseases targeting proteins, lipids and deoxyribonucleic acid (DNA) are formed as by-product effects of reactive oxygen species (ROS) (Acín et al., 2007). Polyphenolic compounds are capable of reducing oxidative stress during lipid metabolism, thereby facilitating the protection of LDL from damages. There is a well-established cause and benefit balance between consumption of certain type or group of polyphenolic compounds at a specified amount (standardized by the content of

hydroxytyrosol and it derivatives) and protection of LDL particles from oxidative damage (EFSA, 2011)

Maintenance of normal blood HDL-cholesterol concentrations: Marrugat et al., (2004) showed a significant increase in HDL-cholesterol concentrations after consumption of extra virgin olive having a very high polyphenol content compared to refined olive oil. There is an inverse relationship between HDL-cholesterol and LDL-cholesterol in the risk of coronary heart disease. High density lipoproteins (HDL) act as a good cholesterol scavenger (while LDL acts opposite) and are involved in the reverse transport of cholesterol from the body (peripheral tissue) back to the liver for detoxification, as opposite to the activity of LDL. However, because of the insufficient evidence to establish cause and effect relationship between the consumption of olive oil polyphenol and maintenance of HDL-cholesterol then the claim has not been sufficiently substantiated.

Blood pressure normalizing effect: Several investigations both in men and women pointed out that replacing SFAs by MUFAs in the diet can lead to a decline in blood pressure (Ruiz-Gutiérrez, Muriana, Guerrero, Cert, & Villar, 1997). High blood pressure and hypertensive patients experienced greater relieve when fed on MUFAs-rich diets of olive oil and other high-oleic sunflower oil, then those placed on PUFAs-rich diets (Covas, 2007). The significant improvement in the health of these subjects when high-oleic acid oil is consumed may have been a synergistic influence of MUFAs and other minor constituents (Fitó et al., 2005). The beneficial impacts of olive oil phenolic compounds in alleviating blood pressure and other related ailments may be one of many of its protective effects on delicate vascular endothelial function (Gilani, Khan, & Ghayur, 2006).

Anti-inflammatory effects: Polyphenolic compounds have been found suitable for the modulation of enzymes responsible for inflammation (Ahmadvand, 2014). For example, hydroxytyrosol was reported to inhibit arachidonate lipoxygenase activity (Fernandez-Bolaños, Lopez, Lopez-Garcia, & Marset, 2012)

2.5. Oxidative Stability of Olive Oil

The most intriguing property of olive oil is its ability to exhibit longer shelf-life compared to other vegetable oils. Fatty acid compositions, phenolic compounds and

some other minor constituents synergistically determine the oxidative stability of olive oil. Immediately after extraction, the process of oxidation begins leading to deterioration that become more easily noticeable during storage. The primary oxidative product of lipid is hydroperoxide, which are tasteless, odorless and does not contribute to the sensory changes in olive oil (Sanchez-Ortiz, Perez, & Sanz, 2013). A subsequent decomposition of hydroperoxide result into various volatile compounds known as secondary oxidation products which are largely if not mostly free radicals. These products are responsible for the repulsive sensory characteristics of low quality olive oil. The traditional methods of quality evaluation in olive oil includes analysis for free fatty acid, peroxide value and K values. Other important measurable oxidative degradation property of olive oil is the Rancimat test, which is an accelerated, high temperature oxidative stress determination.

There are many factors influencing the acceleration of oxidative changes in olive oil: light, oxygen, temperature, pigments, metals, degree of saturation as well as the potency or nature of antioxidant present (Pristouri, Badeka & Kontominas, 2010). Phenolic compounds act as oxidative process chain breakers. They interrupt a continuous propagation lipid peroxidation and free radicals generations by donating hydrogen atoms to the lipid radicals formed. This reaction renders free radicals unreactive by delocalizing its unpaired electron (Rastrelli et al., 2002; Carrasco-Pancorbo et al., 2005). There was a positive correlation of stability with polyphenol, orthodiphenol, oleic/linoleic ratio, tocopherol, chlorophyll and carotenoids whereas a lower, negative correlation was observed with peroxide value, acidity index and K values (Gutierrez, Arnaud, & Garrido, 2001). Triglycerides compositions of olive oils are reported to act against oxidation, too. Studies showed that the most consistent and reproducible positive-contributors to the overall oxidative stability of olive oils are: tyrosol, hydroxytyrosol, oleic acids, and oleic/linoleic. Variable that are inversely proportional to olive oil stability are FFA, PUFA, PV, ethyl, methyl and wax esters, and K values (Uncu & Ozen, 2015; Jolayemi, Tokatli & Ozen, 2016). The study of Brenes et al. (2001) indicates the interactive effects of both fatty acids and minor components in ensuring olive oil stability.

2.6. Olive Oil Authenticity and Traceability

Olive oil is a highly controlled and regulated food commodity because of its high price and reputation. A number of regulatory limits have been established for certain analytical parameters against adulteration. The analytical methods required to determine these parameters are scrutinized, approved and published by the IOC. In general terms, European Union (EEC) regulatory organization and Codex Alimentarius Commission adopted these purity criteria as standard requirements. The purpose is to protect consumers by riding the market off adulterated olive oils.

Advancements in instrumental analysis have led to greater success in combatting adulteration and misrepresentation of olive oils. In the same vein, the perpetrators also adopt these advanced analytical resources to annul the usefulness of them. In order to be a step ahead of these fraudsters, the pace of authentication-based research activities should be rather more rapid to countervail fraudulent practices. A lot still need to be done to perfect these techniques and constantly develop new ones to resolve any future authenticity issues (Aparicio et al., 2007).

Detection of adulteration is not the only purpose of authentication but to determine the genuineness and conformity of olive oil to geographical origin and botanical variety. Each authenticity issue can be dealt with selected chemical variables using chromatographic and/or spectroscopic techniques. Still, there are many types of adulterants that are difficult to detect. For example, there exist industrial methods of mild deodorization to remove undesirable odor that can lower the acceptability of VOO. The resultant oil usually known as "deodorato," can be added to VOO without any sensory perceptible defect. Deodorato has been banned because VOO is not expected to undergo any thermal treatment (IOC, 2009). Scientist came up with the ratio of pheophytin A (a natural chlorophyll) and pyropheophytin A (a thermally degraded compound) content of olive oil to unravel the mystery of this kind of adulteration. The main drawback of this technique is that, pyropheophytin A increases over VOO shelf life, which nullify this method and invalidates this ratio as a formidable method of detecting this kind of adulteration. However, the method is still sufficient to increase consumer's confidence about VOO freshness. Determination of alkyl esters (methyl and ethyl ester) content of olive was proposed for the same purpose (Pérez-Camino, et al., 2008). The amounts of these compounds are inversely proportional to the quality of

olive oil. There is a high correlation between these compounds and low sensory quality (Bierdermann et al., 2008). Thus, a ratio above 1.5 between ethyl and methyl esters is an indication of adulteration, as VOO is low in ethyl ester content. The possibility of using mildly defective deodorized oil as adulterant could negate the detection of ethyl esters beyond the threshold level (Aparicio-ruiz, Romero, García-gonzález, & Aparicio, 2016).

Traceability and geographical characterization of VOO might just be the most difficult area that cannot be affirmed unequivocally. The reason is that, chemical compounds of VOO that are liable to variations during the shelf life cannot be used as yardsticks for identification of geographical peculiarity. Parameters such as phenols, chlorophylls, and carotenoids, or purity-determining physico-chemical properties such as free fatty acids, alkyl esters, peroxide value, K_{232} and diacylglycerides among others, are not reliable for this purpose. Several authors have tried to characterize geographical origin of VOO based on one or more of these variables. However, the result of traceability cannot be plausible, if any of the variables that are quality-induced, adulteration control, freshness testing or shelf life-testing are used. The most hopeful alternative is the quantification and characterization of major and minor compounds (e.g., fatty acids, alcohols, hydrocarbons, sterols) of which concentrations do not vary over time, but depend on variety and, to certain degree, the climate, soil types and other pedioclimatic nature of the orchards. The information obtained from the measurements of these parameters should be merged to develop a large database that can ensure highly representative geographical origin of VOOs with more exactness (Garcıa-Gonzalez et al., 2009). The advent of chemometric and multivariate analytical techniques, have broaden the scope of data managements (Casale et al., 2010). PDO-based VOO have increased lately, raising concerns of consumers and producers about the genuineness of the oils produced from the acclaimed PDOs. Consumer constant quest for assurance has resulted in EEC regulation that establishes labeling control of food products with respect to their geographical indications. The intention of PDOs implementation is to meet consumers' expectations economically and as well protect VOOs from any falsification or misleading labeling. Highly recognized and genuine VOOs produced under a stated PDO usually command higher market price; making them susceptible to fraudulent practices (Cosio, et al., 2006). However, none of these regulations provides a specific analytical approach to affirm the acclaimed information on the label. Consequently, VOO geographical origin is basically controlled administratively, rather

than by any specified physico-chemical parameters. Therefore, attention will be paid in the future, on creating an olive oil map, where already PDOs approved and most productive cultivars are chromatographically, spectroscopically and isotopically characterized. The resulting data bank in conjunction with other means of classification, would allow assessment of the efficiency of individual and combined techniques. These will no doubt enhanced efficient traceability.

2.7. Factors Influencing Olive Oil Quality

There are quite a number of factors on which the characteristics properties of olive oil depend on. A line of continuum exists from the cultivation of olive groves to the point of final product olive oils. Every point on this period contributes differently to the inherent characteristics of the final product. The two broad categories representing pre and postharvest factors are agronomical and technological variables.

2.7.1. Agronomical Factors

Genotype/cultivar: PDO Olive oil is based on the assumption that olive oil chemical and sensory profile is dependent on the interaction between the genetic potential of the cultivar and the growing conditions, which should result in a typical and consistent phenotypic expression. Fatty acids, phenolic, volatile fraction and color of olive oil are clearly influenced by genotype (Angerosa et al., 2004; Servili et al., 2004). The seasonal variation in olive oil composition of a given cultivar depends on its phenotypic stability. (Salvador et al., 2001). Authors have used fatty acid profiles of olive to differentiate olive oils from different variety (Gurdeniz, Ozen & Tokatli, 2008). Olive oil phenolic distribution also varies within and between cultivars and it has been a useful tool for varietal discrimination of oils (Bajoub, Ajal, Fernández-Gutiérrez, & Carrasco-Pancorbo, 2016). Due to its variability in terms of analytical techniques, phenolic fractions are largely ignored by PDO protocols, which usually do indicate not or indicate very low thresholds (Inglese et al., 2011). Nevertheless, phenolic fractions, secoiridoids and lignans relationship have been proposed as potential markers to establish genetic origin of olive oil (Servili et al., 2004). .

Geographical area and seasonal conditions: The purpose of linking agricultural product to its geographical provenance is to establish a clear discrepancy between the product and conventional one based on some peculiar properties (Bajoub et al., 2016). Environmental or geographical location of olive tree influence the overall physicochemical properties of the oil produced from it. This has necessitated adaptation of cultivars to climate parameters such as temperature, rainfall, humidity and other regional conditions that are different from the olive autochthonous regions (Borges et al., 2017). Earlier investigation indicated the relevance of latitude and altitude in modifying fatty acid distribution of olive oil. Lombardo et al. (2008) observed differences in the relative proportions of unsaturated and saturated fatty acids in olive oil with respect to altitudinal variation. Fluctuation in temperature or other climatic condition may result in different physicochemical characteristics of olive oil obtained from a single genotype (Romero et al., 2003). Cumulative amount of rainfall during summer season has been reported to determine the pattern of distribution of phenolic compound in olive oil (Yorulmaz, Poyrazoglu, Ozcan, & Tekin, 2012). In a study conducted at different regions in Italy, it was concluded regions of high temperature produced oil of lower polyphenol content and lower degree region produced oil of higher amount (Ripa et al., 2008). Other minor components of olive oil such as: tocopherol, sterol, chlorophyll, carotenoids, triterpenic alcohols etc. modulate mainly with combined effects of interrelated variables (genotype, ripening stage, pest and disease) rather than individual factors.

Maturation/Ripening stage: The physicochemical transformations that take place in olive fruit during ripening are result of enzymatic degradation processes. Chlorophyllase causes the depletion of chlorophyll and pronouncement of carotenoids as maturation advances. Unripe fruit has intact pectin that is firmly bound to cellulose enhancing the rigidity of the structure. As ripening increases, endogenous enzymes (pectinases) change the textural properties of the fruit into soft and easily crushable form (Clodoveo et al., 2014). This process continues until anthocyanin becomes apparent (turning color stage). Activity of other enzymes simultaneously increased during ripening (Panzanaro et al., 2010). There is a tendency for oleic acid and sometimes linoleic acid to increase or remain constant during ripening, while saturated fatty acids (palmitic and stearic) decrease. Generally, fatty acid composition hardly shows variations with respect to maturation or harvest time. An increase in the content of oleic acid with olive fruit ripening was reported (Roca & Minguez-Mosquera, 2001;

Jolayemi, Tokatli & Ozen, 2016); Beltran et al. (2010) observed a gradual increase in ethanol (an important reactant in alkyl ester formation) content with ripening of olive fruit. Giuffre (2014) also observed variation in wax esters during ripening. During fruit ripening, enzyme activity responsible for the olive oil aromatic characteristics may change (Kalua et al., 2007).

Harvest time: Oils produced from early harvest olives are characterized by their greenish color and intensive green fruity, bitter and spicy flavors. Harvest time has been used as a differential parameter because of the progressive changes that occur in olive fruit at different seasons. Early harvest olives are expected to give higher quality olive oil than the late harvest. Peres et al., (2016) observed high content of important phenolic compound in olive obtained at early stage of maturation. Early harvest olives have low free fatty acidity (FFA) and peroxide value (PV) which are relevant in determining high quality olive oils (Dıraman & Dibeklioğlu, 2009). Oils obtained from early harvest olive fruits are expected to be more stable to oxidation than those from mature olives. This is due to the fact that, earlier stage of fruit maturity is characterized by highly important bioactive compounds i.e. non-polar phenolic compounds, chlorophyll, aromatic components. Their antioxidative capacities before hydrolysis are considerably higher than when depolymerized as harvest time becomes prolonged (Gomez-Alonso et al., 2007). Another important aspect of harvest time is the strategic identification of maximum oil yield time of olive oils. It is a well-known fact that maximum oil yield is obtained toward the late harvest, which is less desirable in terms of quality (Inglese et al., 2011).

2.7.2. Technological Factors

Mechanically obtained olive oil devoid of any external inclusion is called virgin olive oil (Ranalli et al., 2003; Servili, et al., 2012). The processes involves a quality-minded, stepwise collection of fresh olive, leaf removal, washing, olive crushing, malaxation of olive paste, centrifugation with or without water in a continuous three-phase or two-phase system, filtration (optional), bottling and storage (Fig. 2.3).

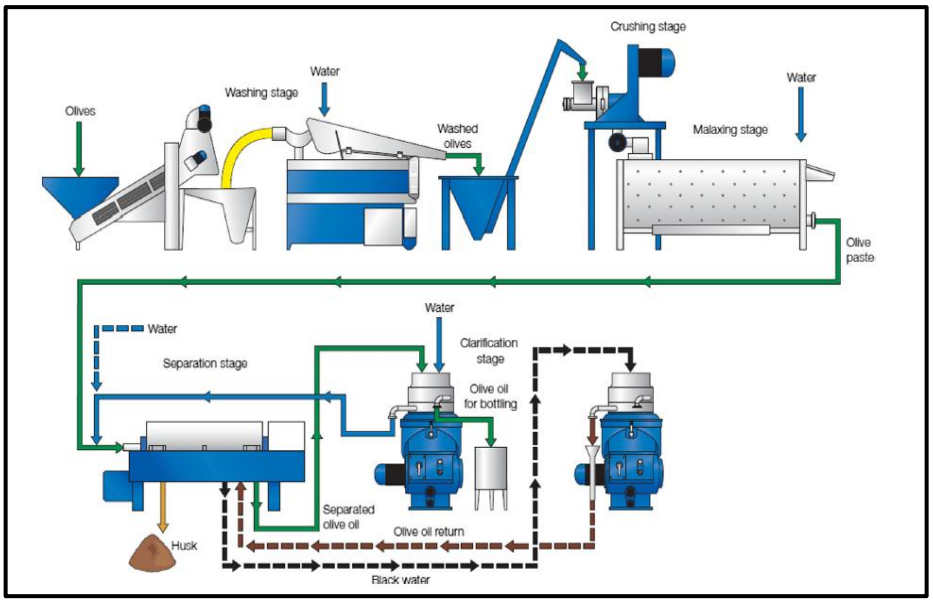

Figure 2.3. Schematic flow chart of olive oil extraction operation
(Source: Clodoveo, et al., 2013)

Leaf removal and washing: Apart from ensuring the purity of the olive, cleaning process prevents the equipment from being damaged by stones or other extraneous objects. Some authors have evaluated the output of olive crushed with leaves in terms of chemical and general quality of the oil. Malheiro, Casal, Teixeira, Bento, & Pereira, (2013) observed higher FFA, peroxide value and K_{232} in olive oil when olive leaves are added. The authors asserted that, the presence of lipolytic enzymes in the olive leaves may have caused the outrageous values in those standard parameters. Addition of water can lower the extraction efficiency as a result of water/oil emulsions. Oils obtained from washed olive fruits may lose their bitterness and pungency to certain degree, making them less desirable. This may also cause reduction in fruitiness and flavor properties (Clodoveo et al., 2014).

Crushing: Crushing is the first unit operation during olive oil extraction and involved breaking of the drupes and release of endogenous enzymes (Clodoveo, 2012; Servili et al., 2012). The release of these biological catalysts influences the changes in hydrophilic phenols and volatile compounds of VOO and examples of the most active enzymes in this case are: polyphenoloxidase (PPO), peroxidase (POD), lipoxygenase (LOX) and β-glucosidases. Crushing begins the generation of C5 and C6 saturated and unsaturated aldehydes, alcohols and esters which are the primary sensory notes in VOO (Servili et al., 2003a).

Malaxation: One of the most widely studied aspects of continuous olive extraction is malaxation step and it constitutes the most critical point, where a failure may lead to the ruin of entire extraction process (Clodoveo et al., 2014; Allouche et al., 2010). Malaxer machine is made up of stainless steel tank, equipped with a rotating central-screw stirring the crushed paste slowly and continuously, at a well-controlled temperature. During mixing of crushed paste, the wall-degrading endogenous enzymes are released from their localized cellular environment and come in close contact with their substrates i.e. enzymes such as: pectase, cellulase, hemicellulase and oxidoreductases (POP, POD & LOX) are released into the mix. These enzymes help in the rheological properties of the paste, break emulsions and enhance coalescence of oil-droplets to form large fat globules (Ranalli et al., 2003). During malaxation of olive paste, a complex biochemical changes occur resulting into significant modification of quality and compositional characteristics of the final product, especially phenolic and volatile profiles (Clodoveo, 2012). One or more malaxation conditions can be varied to influence the quality of VOO. Temperature and time have been widely studied (Stefanoudaki, Koutsaftakis, & Harwood, 2011). High malaxation temperature has been linked to increase in extraction yield, but in low phenolic compounds, and burnt-flavor (Inarejos-García, et al., 2009). C6 and C5 volatile carbonyl compounds increased when the duration of malaxation become extended (Kalua et al., 2006). Apart from time and temperature control during malaxation, strategic modulation of other important parameters such as head space of the tank is very important. The configuration of early designs of malaxers was characterized with remote shape and non-hermetic closing system. There were constant risks of exposing olive paste to atmospheric oxygen which could promote oxidative reactions; with unavoidable loss of volatile compounds (Raffo, Bucci, D'Aloise, & Pastore, 2015). Later designs now include hermetic seal to keep out atmospheric gases; some has an auxiliary inbuilt system that can incorporate inert gases such as nitrogen and argon. The presence of inert gas in the headspace reduces oxidase enzymes activity, thereby protecting polyphenolic compounds, but at the same time inhibiting the production of volatiles. Therefore, it is very important to balance the control of the oxygen with the synthesis of volatile compounds (Servili et al., 2012).

Storage: There are some changes that occur in the composition of olive oil during storage. A good example is the modification of phenolic compounds by endogenous enzyme activities. These enzymes are capable of lowering the pungency or bitter taste attributes of fresh olive oils. They are also the most potent antioxidants

preventing or interrupting oxidative stress in olive oil. Oxidation-resistance capacity of olive oil does not only stem from the present of phenols alone, but fatty acid distribution in olive oil as well as other minor compounds including tocopherol, chlorophyll and carotenoids (Servili et al., 2012, Clodoveo et al., 2014). During storage there is a tendency for these compounds to decline in concentration except for the lignans that are less prone to changes (Morello et al., 2003). On the contrary, the secoiridoid derivatives, (3,4-DHPEA-EDA, p-HPEA-EDA and 3,4-DHPEA-EA) increased as a result of enzymatic hydrolysis of secoiridoids (Gómez-Alonso et al., 2007). Chlorophylls and carotenoids pigments deplete gradually with time. Similarly, unpleasant odor or undesirable tastes can develop from oxidative or hydrolytic rancidity, if all the reaction requirements (light, temperature, and oxygen) are available (Morello et al., 2003). Stripping nitrogen to replace oxygen in the headspace is a recently adopted method of maintaining an inert storage environment for olive oil. Filtration prior to dark room storage can be advantageous because, water droplet is removed from the oil matrix reducing the breathing ground for lipase enzymes (Masella et al., 2009).

CHAPTER 3

MATERIALS AND METHODS

Experiments with mid-infrared and near-infrared spectroscopies, electronic nose analysis, and antioxidant content of oils were performed in the Department of Food, Environmental and Nutritional Science at University of Milan, Italy. The rest of the analyses were performed in the Department of Food Engineering at Izmir Institute of Technology (IYTE).

3.1. Olive Oil Samples

3.1.1. 2012-2014 Samples

In the harvest year of 2012, thirty six olive oil samples from Ayvalik (Edremit) and Memecik varieties, obtained at three levels of maturation stages designated as: early, mid and late harvest extracted at three malaxation temperatures (27, 37 and 47 ^{o}C), were analysed for their chemical characteristics. The experiments were designed based on general full factorial scheme, using three factors (olive type, harvest time and malaxation temperature) at different levels [2(olive types) x 3(harvest times) x 3(malaxation temperatures) x 2(replicates) = 36]. A two-phase continuous olive oil extraction system (Polat Machinery, Aydin, Turkey) located in the campus area of Izmir Institute of Technology, was used for the extraction. Ayvalik olives from west coast of Anatolia (north of Izmir), and Memecik olives from Aydin region (south of Izmir) were kindly provided by Taris Olive and Olive Oil Cooperatives Union. The agronomical and extraction details of the oil samples are presented in Table 3.1. Olive oil samples were put into clean airtight bottles and they were kept at refrigeration temperature (4^{o}C) until analyzed. A constant replacement of headspace of the sample with nitrogen was ensured throughout the course of the analysis.

In the harvest year of 2014, additional four samples from early harvest Ayvalik olive variety, processed at 27-47°C malaxation temperature were also obtained.

Table 3.1. Agronomical and extraction details of olive oil samples of 2012-2014

Origin	Variety	Harvest	Temp (°C)	Code
2012 samples				
Burhaniye	Ayv	Early	27	A27e
Burhaniye	Ayv	Early	37	A37e
Havran	Ayv	Early	47	A47e
Ayvalık+Ezine	Ayv	Mid	27	A27m
Ayvalik+Ezine	Ayv	Mid	37	A37m
Ayvalik + Ezine	Ayv	Mid	47	A47m
Edremit+Küçükkuyu	Ayv	Late	27	A27f
Edremit+Küçükkuyu	Ayv	Late	37	A37f
Edremit+Küçükkuyu	Ayv	Late	47	A47f
Bozdoğan+Horsunlu	Mem	Early	27	M27e
Bozdoğan+Horsunlu	Mem	Early	37	M37e
Bozdoğan+Horsunlu	Mem	Early	47	M47e
Bayındır + Selçuk	Mem	Mid	27	M27m
Bayındır + Selçuk	Mem	Mid	37	M37m
Bayındır + Selçuk	Mem	Mid	47	M47m
Erbeyli+Ortaklar	Mem	Late	27	M27f
Erbeyli+Ortaklar	Mem	Late	37	M37f
Erbeyli+Ortaklar	Mem	Late	47	M47f
Havran	Ayv	Early	27	A27e-2
Altinoluk	Ayv	Early	37	A37e-2
Altinoluk	Ayv	Early	47	A47e-2
Ayvalik + Ezine	Ayv	Mid	27	A27m-2
Ayvalik + Ezine	Ayv	Mid	37	A37m-2
Ayvalik + Ezine	Ayv	Mid	47	A47m-2
Edremit+Küçükkuyu	Ayv	Late	27	A27f-2
Edremit+Küçükkuyu	Ayv	Late	37	A37f-2
Edremit+Küçükkuyu	Ayv	Late	47	A47f-2
Bozdoğan+Horsunlu	Mem	Early	27	M27e-2
Bozdoğan+Horsunlu	Mem	Early	37	M37e-2
Bozdoğan+Horsunlu	Mem	Early	47	M47e-2
Bayındır + Selçuk	Mem	Mid	27	M27m-2
Bayındır + Selçuk	Mem	Mid	37	M37m-2
Bayındır + Selçuk	Mem	Mid	47	M47m-2
Erbeyli+Ortaklar	Mem	Late	27	M27f-2
Erbeyli+Ortaklar	Mem	Late	37	M37f-2
Erbeyli+Ortaklar	Mem	Late	47	M47f-2
2014 samples				
Edremit	Ayv	Early	37	A37-e
Edremit	Ayv	Early	27	A27e2
Edremit	Ayv	Early	37	A37e2
Edremit	Ayv	Early	47	A47e2

3.1.2. Maturation Index

Samples of the olive fruits were taken on arrival and their maturity indexes were determined prior to extraction according to IOC method (International Olive Council, 2011) using the equation 3.1. A hundred olive fruits were randomly selected and separated into eight color categories based on the color chart below with *a* to *h* representing the number of fruits in each category.

$$MI = \frac{a*0 + b*1 + c*2 + d*3 + e*4 + f*5 + g*6 + h*7}{100} \quad (3.1)$$

a = olives with intense green or dark green epidermis
b = olives with yellow or yellowish green epidermis;
c = olives with yellowish epidermis but with reddish spots or areas over less than half of the fruit;
d = olives with reddish or light violet epidermis over more than half of the fruit;
e = olives with black epidermis and totally white pulp;
f = olives with black epidermis and less than 50% purple pulp;
g = olives with black epidermis and violet (more than 50%) or purple pulp;
h = olives with black epidermis and totally dark pulp.

Early, mid and late harvest maturation index ranged between 1.08-2.45, 3.23-3.57, and 4.21-6.43, respectively for both varieties. The olive fruits were frozen using liquid nitrogen to prevent oxidation and lyophilized with freeze-dryer (Labconco, USA), stored in airtight glass jar until analyzed.

3.1.3. 2015-2016 Samples

In the harvest year of 2015, thirty olive oil samples of Ayvalik (A), Memecik (M) and Erkence (E) and some local varieties from Karaburun peninsula, Urla, Godence and Uzunkuyu-Cesme region of Izmir and Manisa were obtained from local producers (Table 3.2). Ayvalik and Memecik oils were provided by Taris Olive and Olive Oil Cooperatives Union. Samples were split into two 100 mL dark bottles, head-space

flushed with nitrogen flow, air-tight corked and kept in a completely dark room at room temperature for 15 months. Additional six olive oil samples of Ayvalik and Memecik were supplied by from Taris Union Cooperatives in 2016.

Table 3.2. Varietal and regional details of olive oil samples of 2015-2016

Date	Region	Olive	Harvest	Code	Number of Samples
November	Edremit Bay area	Ayvalik	Early	Ae	1
December	Edremit Bay area	Ayval?k	Mid	Am	1
January	Edremit Bay area	Ayval?k	Late	Af	1
December	Aydin	Memecik	Early	Me	1
December	Aydin	Memecik	Mid	Me	1
January	Ayd?n	Memecik	Late	Mf	1
December	Manisa-East of Izmir	Local varieties-mixture	Mid	Mn	4
November	Uzunkuyu-Cesme-Izmir	Memecik	Mid	Mu	3
December	Eglenhoca-Karaburun penisula	Erkence		E	12
November	Godence-Izmir	Erkence	Mid	GE	1
November	Godence-Izmir	Local varieties-mixture	Mid	Go	1
November	Urla-Izmir-Kemal Bey	Ayvalik	Early	AeU	1
November	Urla-Izmir-Kemal Bey	Ayvalik	Mid	AmU	1
	Urla	Local variety-mixture		M	1
2016	Edremit Bay area	Ayvalik		At	1
2016	Aydin-South Aegean	Memecik		Mt	1

3.1.4. Chemicals

The chemical reagents used for the qualitative and quantitative chemical characterization of the oil samples are given in Table 3.3. Ultrapure water used as both cleaning agent as well as universal solvent throughout the analysis was obtained from Sartorius Arium 611 VF system (Sartorius AG, Gottingen, Germany).

Table 3.3. Analytical grade chemical reagents and their various brand names

Chemical	Brand
Acetic acid	Riedel-deHaen 27225
Chloroform	Riedel-deHaen 24216
Sodium thiosulphate Na2O3S2	Fluka 72049
Potassium iodate KIO3	Fluka 60390
Potassium iodide KI	Riedel-deHaen 03124
Potassium hydroxide	Riedel-deHaen 30614
Starch	Carlo Erba 417587
Sulfuric acid H2SO4	Merck 1.00713.2500-UN1830
Phenolphthalein	Riedel-deHaen 33518
Potessium Hydrogen phthalate	EMSURE A0238674
Cyclohexane	Sigma-Aldrich C100307
Diethyl ether	Riedel-deHaen 24005
Methanol	Sigma-Aldrich 34885
Sodium carbonate	Merck A0268192 139
Ethanol	Sigma-Aldrich 32221
Folin_Ciocalteau phenol reagent	Sigma-Aldrich F9252
Gallic Acid	Sigma G7384
Tween20	Sigma-Aldrich P1379
Ethylacetate	Sigma-Aldrich 270989
Trolox (6-hydroxyl-2,5,7,8-tetramethylchroman-2-carboxylic acid)	Sigma-Aldrich 238813
DPPH (2,2-diphenyl-1-picrylhydrazyl)	Sigma-Aldrich 1846081
Acetonitrile	Sigma-Aldrich 34888
Gallic Acid	Sigma G7384
n-Hexane	Sigma-Aldrich 34859
Heptane	Sigma-Aldrich 34873
Methanol	Sigma-Aldrich 34885
Sudan 1	Sigma-Aldrich 103624
Silica Gel	Sigma-Aldrich 391484
Apigenin	Fluka 10798
Caffeic acid	Fluka 60020
Chlorogenic acid	Fluka 25700
Cinnamic acid	Fluka 96340
2,3 dihydroxybenzoic acid	Fluka37528
Ferulic acid	Fluka 46278
Hydroxytyrosol	Sigma H4291
Luteolin	Fluka 62696
m-Coumaric acid	Fluka 28180
o-Coumaric acid	Fluka 28170
Oleuropein	Extrasynthese 0204
p-Coumaric acid	Fluka 28200
p-hydroxyphenylacetic acid	Fluka 56140
3-hydroxyphenylacetic acid	Fluka 56130
Syringic acid	Fluka 86230
Tyrosol	Fluka 56105

(Cont. on the next page)

Table 3.3 (Cont.)

Chemical	Brand
Vanillic acid	Fluka 94770
Vanillin	Fluka 94750
Pinoresinol	Sigma L9283
Rutin (Rutin hydrate)	Sima R5143
Quercetin hydrate	Aldrich 337951
Apigenin-7-glucoside	Fluka 44692
Methyl-heptadecanoate	Sigma-Aldrich 51633
Dodecylarachidate	Sigma-A8671
FAME-Mix	Superco37

3.2. Methods

3.2.1. Oxidative Stability Index (OSI)

An automated Rancimat system (Model 873 Biodiesel, Metrohm, Switzerland) was used to estimate the oxidative stability of the oil samples. The system contains reaction vessels into which about 3.0 g of olive oil was measured. The vessels are covered and connected to conductivity cells containing ultrapure water as volatile absorbent. Airflow at 20 L/h and temperature of 120 ^{o}C are the two input reaction parameters, which facilitate rapid oxidation of the oil. The system is allowed to attain the required temperature and airflow before starting the oxidation process. The air serves as a carrier of the primary oxidative products from the vessel to the absorbent, while the rate of oxidation is monitored by the conductivity as shown in the curve (Fig. 3.1). The time (h) at which conductivity measurement shows a sharp increase (Induction time) is recorded. Measurements were replicated twice.

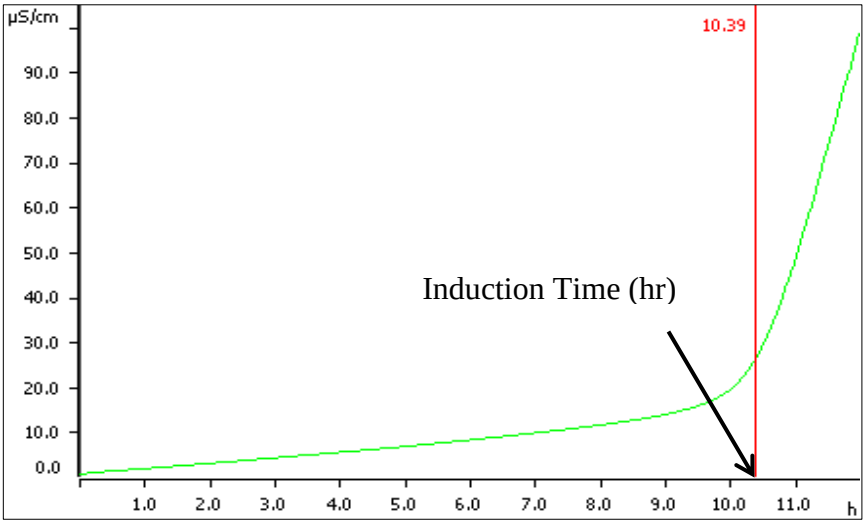

Figure 3.1. Rancimat oxidative stability curve

3.2.2. Peroxide Value (PV)

European Official Method of Analysis (EEC, 2013) was used in determining peroxide value of the olive oil (meq O_2/kg). The 2 g of olive oil sample with 10 mL of chloroform, 15 mL of acetic acid and 1 mL of freshly prepared saturated solution of potassium iodide (KI) (144g of KI in 100 mL of deionized water) is mixed rapidly for 1 min. The mixture is kept away from light at room temperature for 5 minutes, and after which 75 mL of deionized water and 0.5 mL starch indicator (1 g of starch dissolved in 100 mL of boiling deionized water) are added. The mixture is titrated with initially standardized 0.01 M sodium thiosulphate solution until the blue color turned colorless and volume of sodium thiosulphate spent is recorded. Peroxide values in terms of meq O_2/kg oil is calculated using eqn (3.2).

$$PV = \frac{V(mL) \times M(molL^{-1}) \times 100}{m(g)} \qquad (3.2)$$

V= mL of sodium thiosulphate solution required to titrate the sample

M = molarity of sodium thiosulphate solution

M = weight in g of the sample

Standardization of Sodium Thiosulphate

2 g of potassium iodate (KIO_3) is dried in an oven (Memmert) at 100 ^oC for 1hr and allowed to cool in a desiccator. 0.01 M of KIO_3 solution (0.107 g of KIO_3 in 500 mL deionized water) is prepared and the actual weight is recorded. 0.5M of H_2SO_4 (2.8 ml of 96% pure H_2SO_4 dissolved in 10 mL of deionized water) is prepared. For the purpose of standardization, 0.2 g of potassium iodide (KI) is weighted into Erlenmeyer flasks in triplicate, 1 mL of 0.5M H_2SO_4, 50 mL of KIO_3 and few drops of starch indicator are added. The reddish color of the mixture is titrated with the initially prepared 0.01 sodium thiosulphate until the solution turned colorless and the act. The volume of sodium thiosulphate spent is recorded and its actual molarity is calculated using eqn (3.3 and 3.4).

$$M_{KIO3} = \frac{m_{KIO3}\,(g)/MW_{KIO3}(gmol^{-1})}{V_{KIO3}\,(mL)\ solution} \tag{3.3}$$

$$M_{sodiumthiosulphate} = \frac{6 \times M_{KIO3}\,(mol^{-1}) \times V_{KIO3}\,(mL))}{V_{sodiumthiosulphate}\,(mL)\ spent} \tag{3.4}$$

m_{KIO3} = weight of KIO_3 (0.107 g)

MW_{KIO3} = molecular weight of KIO_3 (214 $gmol^{-1}$)

V_{KIO3} = total volume of KIO_3 solution (500 mL)

$V_{sodiumthiosulphate}$ = Amount of sodium thiosulphate spent during titration

3.2.3. Free Fatty Acid (FFA)

The free fatty acid content of the oil sample was determined using European Official Methods of Analysis (EEC 1991) as % oleic acid. The 0.1 M of potassium hydroxide (2.85g of KOH in 500 mL deionized water) to be used for neutralization of the oil acidity is standardized using potassium hydrogen phthalate ($KHC_8H_4O_4$). About 1.0 g $KHC_8H_4O_4$ is oven-dried for 2 hrs at 110 ^oC. Then, 75mL deionized water is added to 0.4 g of dried $KHC_8H_4O_4$ and this was prepared in triplicate. Few drops of phenolphthalein (0.5 g phenolphthalein in 50 mL 95% ethanol (v/v) is added as an

indicator. The mixture is titrated with 0.1 M KOH until the pink color of phenolphthalein persists for few seconds and volume of KOH spent is recorded and actual concentation of KOH is calculated (Eqn. 3.6).

The quantity of the sample used depends on the expected acidity. Therefore, 10 g of the oil sample is dissolved in 75 mL of diethyl ether-ethanol (1:1) mixture. The resultant mixture is neutralized with the standardized 0.1 M KOH. Few drop of phenolphthalein is added as an indicator until color change is observed. The volume of KOH spent is recorded and % FFA is calculated using eqn (3.5).

$$\% \text{ FFA} = \frac{V \times c \times M \times 100}{1000 \times m} \quad (3.5)$$

V = the volume of titrated KOH solution used (mL)
c = the actual concentration in mol/L of the titrated solution of KOH
M= the molar weight in g/mol of the acid used (Oleic acid: 282g/mol)
m = the weight in g of oil sample

$$c = \frac{m(KHC_8H_4O_4) \times 1000}{204.22 \times Vs} \quad (3.6)$$

m(KHC₈H₄O₄) = the weight in g of $KHC_8H_4O_4$ (0.4)
204.22 = Molecular weight of ($KHC_8H_4O_4$)
Vs = Volume of KOH spent during standardization

3.2.4. K values

European Official Method of Analysis (EEC, 1991) was used in determination of K_{232} and K_{270} extinction coefficients of the oil samples. Olive oil (0.25g) is weighed into a graduated falcon tube; dissolved and made up to 25 mL with cyclohexane. The mixture is homogenized using vortex (Velp Scientifika, Europe). Absorbance of the oil-cyclohexane mixture at wavelengths 270 nm and 232 nm corresponding to triens and diens bonds are taken spectrophotometrically in a UV-Spectrometer (Shimadzu UV-

2450, Kyoto Japan). The measurement is done in quartz cuvette and pure cyclohexane is used as blank.

3.2.5. Color

The CIELab color parameters (L*, a* and b*), chroma and hue (C and H) were calculated from the recorded transmission spectra of the olive oil samples as described by (Sikorska, Caponio, Bilancia, Summo, & Pasqualone, 2007) with a UV spectrophotometer (Shimadzu UV-2450 Kyoto, Japan) in a plastic cell with 1.0 cm optical path length. Transmittance was taken over the 380–780 nm range at 120 nm/min scan speed. Color parameters were calculated from the transmittance spectrum of each sample using the standard illuminant D65 and 10° observation angle. Values for each sample were calculated as the average of triplicates, with L* indicating lightness, a* indicating hue on the green (-) to red (+) axis, b* indicating hue on the blue (-) to yellow (+) axis.

3.2.6. Chlorophyll and Carotenoid

Total chlorophyll and carotenoid contents of olive oils were estimated according to a procedure by Mínguez-Mosquera, et al., (1991). Olive oil sample (7.5 g) is weighed into a graduated falcon tube and made up to 25 mL with cyclohexane. The absorbance corresponding to chlorophyll and carotenoid (A_{670} and A_{470} respectively) are measured by a UV spectrophotometer (Shimadzu UV-2450 Kyoto, Japan) at 1 cm optical path (d). Chlorophyll and carotenoid contents are expressed as mg/kg of oil using the extinction equations 3.7 and 3.8, respectively:

$$\text{Chlorophyll (mg/kg)} = \frac{A_{670} \times 10^6}{(613 \times 100 \times d)} \quad (3.7)$$

$$\text{Carotenoid (mg/kg)} = \frac{A_{470} \times 10^6}{(2000 \times 100 \times d)} \quad (3.8)$$

3.2.7. Antioxidant Activity (AA)

The antioxidant capacity of the oil samples were evaluated in terms of DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging capacity of the oil phenols in comparison with that of Trolox (6-hydroxyl-2,5,7,8-tetramethylchroman-2-carboxylic acid), following the modified methods of Carrasco-Pancorbo et al., (2005) and Buyuktuncel et al., (2014). DPPH (3.5 mg/100 mL) is freshly prepared in ethyl acetate and the initial absorbance adjusted between 0.60 and 0.62. The olive oil sample (0.5 g) is dissolved and made up to 5 mL with ethyl acetate and 1 mL of this mixture is added into 5 mL DPPH and incubated in a water bath at 35 ^{o}C for 5 min. UV-vis absorbance at 515 nm using a V-650 spectrophotometer (Jasco Europe, Cremella, Italy), of the mixture is taken within 1 min between successive samples. Trolox percentage inhibition calibration curve prepared between 100 – 1000 μmol of Trolox, with $R^2 > 0.99$, is used to extrapolate antioxidant capacity of the oil samples in μmol trolox equivalent/kg olive oil.

3.2.8. Total Phenol Content (TPC)

Total phenol content of olive oil extract was determined according to Folin–Ciocalteau spectrophotometric method at 765 nm and expressed in terms of Gallic acid (mg GA/kg oil) according to the method of Montedoro, et al., (1992). A 10 mL of methanol/water mixture (80:20v/v) and 2 to 3 drops of Tween 20 is added to 2g of olive oil sample. The mixture is homogenized (Heidoph-Silent Crusher M, Germany) at 25,000 rpm for 1 min, and centrifuged at 9000 rpm for 10 min (Nüve NF 615, Ankara, Turkey). The supernatant is collected and the extraction repeated two more times (only with addition of 10 mL methanol/water). The total volume of extract is recorded.

A 1mL of the extract is diluted to 6 mL with deionized water. Folin-Ciocalteau reagent (0.5 mL) is added and it is waited for 1 min. Then, 2 mL of Na_2CO_3 solution (15% g/mL) is added and finally diluted with 1.5 mL of deionized water. The mixture is mixed with using a vortex (Velp Scientifika, Europe) for 30 sec and allowed to incubate for 2 hr in the dark. Total phenol content of extracts is determined by the absorbance values obtained at 765 nm using spectrophotometer (Shimadzu UV-2450 Kyoto, Japan).

The absorbance value is converted to its equivalent gallic acid concentration in mg/kg, using gallic acid calibration curve.

Ten points calibration curve is prepared using gallic acid standard solution of concentrations between 0.01 mg/ml and 1.0mg/mL. Three parallel replicates are prepared for each concentration.6.5 mL of deionized water, 0.5 mL of Folin reagent and 2 mL of Na_2CO_3 solution (15% g/mL) are added to 1 mL of each standard concentration and the mixture is mixed and incubated for 2 hr in the dark. Absorbances are taken and a linear curve starting from the origin is obtained with relatively high R^2 (around 0.99). The equivalent gallic acid concentrations (total phenol content) of the olive oil extracts are extrapolated (eqn. 3.9) from the curve and the value is expressed as TPC in mg/kg as:

$$\text{TPC (mg/kg)} = \frac{\text{GA (mg/mL)} \times V_{extracts}\text{ (mL)} \times 1000}{W_{sample}\text{ (g)}} \qquad (3.9)$$

$V_{extracts}$ = the volume of phenolic extracts

GA= Gallic acid concentration equivalent in the sample

W_{sample} = weight of the olive oil sample

3.2.9. HPLC of Phenolic Profile

Extraction of phenolic compounds

Phenolic profiles of the oil samples were determined following the procedure of Alkan, Tokatli & Ozen (2012). 14 mL of methanol/water mixture (80:20) and 1 mL of gallic acid internal standard (25 μg/mL = 25ppm) are added to 14 g of oil sample and the mixture is homogenized (Heidoph-Silent Crusher M, Germany) at 25,000rpm for 1 min. The mixture is centrifuged at 9000 rpm for 10 min (Nüve NF 615, Ankara, Turkey) and the supernatant (phenolic extract) is collected. These procedures are repeated 3 more times and the combined extract obtained is transferred into round bottom flask suitable for rotatory vacuum evaporator (Heidolph Laborota-4000, Germany). Evaporation of the extract is done at 35 °C for 22 min to remove methanol. The residue is dissolved in 15 mL acetonitrile and washed with n-hexane three times

(3x20 mL) to remove the residual oil present. The resulting acetonitrile solution is evaporated under vacuum for about 20 min at 35°C. The final residue is flushed with nitrogen flow for approximately 5 min and dissolved in 1 mL of methanol/water. This final dissolved extract is filtered using 0.45 μm pore-size membrane (Minisart, Sartorius, and Gottingen, Germany) into clean vials. A 20 μL of it is injected into HPLC using Diode Array Detector (DAD).

HPLC operational procedures

HPLC system with a Perkin Elmer (PE) series 200 pump (Norwalk CT 06859, USA), PE series 200 diode array detector, PE-Nelson 900 series interface, Meta Therm HPLC column heater (series no:9540, Torrance) and a 5 μm, 25 cm ×4.6 mm, C18 column (Ace, Aberdeen, Scotland) is used to analyze phenolic compounds. Separation is achieved by elution gradient using an initial composition of 90% water (pH adjusted to 3.1 with 0.2% acetic acid) and 10% methanol. The concentration of the methanol is increased to 30% in 10 min and maintained for 20 min. Subsequently, the methanol percentage is raised to 40% in 10 min, maintained for 5 min, increased to 50% in 5 min, and maintained for another 5 min. Finally, methanol percentage is increased to 60, 70, and 100% in 5 min periods. Initial conditions are reached in 15 min. The mobile phase flow rate is 1 mL/min and column temperature is kept at 35 °C. In order to obtain effective separation of individual phenolic compounds, degassing of mobile phase is applied by using a sonication system.

Identification and quantification

Identifications of olive oil phenolic compounds were done by comparing the retention times of the commercial standards with that of the oil samples at 280 and 320nm. GA acid is used as internal standard (ISTD). As stated earlier, a known concentration of ISTD is added to the olive oil at the beginning of extraction and the same amount is added into each concentration of the prepared standard. This is to account for any possible loss during extraction procedure. The ratio of the peak area of standard and peak area of ISTD (Response ratio) versus the ratio of concentration of standard and concentration of ISTD (Amount ratio) are used to prepare 5-point calibration curves of R^2 range between 0.98 and 0.99. The amount of each phenolic compound is calculated (eqn. 3.10).

$$\text{Amount (mg/kg OO)} = \frac{\text{Response ratio x ISTD (ppm)}}{\text{Slope x volume (1mL) x weight (g)}} \quad (3.10)$$

3.2.10. GC Analysis of Fatty Acids

Sample preparation

According to the European Official Method of Analysis (EEC, 1991), 100 mg of oil sample is weighed into 20 mL test tube. The sample is dissolved in 10 mL of n-hexane and 100 µL 2N potassium hydroxide in methanol is added (2.8 g KOH in 25 mL methanol). Potassium hydroxide is added to esterify the fatty acids and make them available for quantification. The mixture is vortexed for 30 sec and centrifuged at 9000 rpm for 15 minutes. After centrifugation, supernatant phase is micro-filtered (0.45 µm filter) into 2 ml vial for chromatographic analysis.

GC-operational conditions

The analysis is performed on an Agilent 6890A GC system (Agilent Technologies, Santa Clara, USA) equipped with a Flame Ionization Detector (FID). The configuration of the instrument is summarized Table 3.4.

Table 3.4. Chromatographic conditions of analysis of fatty acids

Chromatographic system	Agilent 6890 GC System
Inlet	Split/splitless
Detector	FID
Automatic sampler	Agilent 7683
Liner	Split liner (p/n 5183-4647)
Column	100 m x 0.25 mm ID, 0.2µm HP-88 (J&W 112-88A7)
Inlet temperature	260^{o}C
Injection volume	1µL
Split ratio	Jan-50
Carrier gas	Helium
Head pressure	1.5mL/min constant flow
Oven temperature	1400C (5min), 30C/min. 220 ^{o}C, 5min
Detector temperature	260 ^{o}C
Detector gas	Hydrogen: 40 mL/min; Air: 450 mL/min Helium make-up gas: 30 mL/min

Esters of these following fatty acids are: Palmitic acid (C16:0), Palmitoleic acid (C16:1), Margaric acid (C17:0), Cis-10-heptadecanoic acid (C17:1), Stearic acid (C18:0), Oleic acid (C18:1), Linoleic (C18:2), Linolenic acid (C18:3), Arachidic acid

(C20:0), Cis-11-Eicosenoic acid (C20:1) and Behenic acid (C22:0). Each sample is analyzed at least two times. Nine major fatty acids in olive oil are evaluated with their retention times relative to the reference standard (FAME mix). The peak area of each fatty acid is integrated using Chem-station software. The integrated area is converted to % concentration by dividing the calculated area of each acid to total area of all the fatty acids composed of the oil sample.

3.2.11. GC of Alkyl Ester and Wax

Sample preparation

Alkyl esters and wax contents of oil samples are determined simultaneously according to the method described by International olive council IOC (IOC, COI/T.20/Doc. No 28/ Rev.1, 2010). 15 g of initially muffle oven dried (550 ^{o}C, 4 hrs) and 2% rehydrated silica gel, is carefully introduced into a clean dried glass column and tea-spoon full of Na_2SO_4 is added on top of Silica gel packed column and clamped. Being a good absorbent, Na_2SO_4 prevents the column from drying out during elution.

About 100 mL of mobile phase (1% diethyl ether-hexane mixture) is added to the column and elution continued for a while to condition the stationary phase. Two internal standards are prepared: 0.02% methyl heptadecanoate in hexane for alkyl esters and 0.1 % dodecyl/lauryl arachidate in hexane for wax contents. 200 µL and 100 µL of alkyl and wax internal standards are added to 0.5 g of oil sample. Few drops of Sudan 1 indicator (1 g of 1- phenylazo-2-naphthol in 100 mL of hexane) is added to the mixture. The mixture is then introduced into the conditioned column completely, and elution continues until the indicator is about an inch to the base of the column. The total eluent collected is dried in a rotary evaporator (Laborato 4000 Heidolph; Germany) for 10 min at 45 ^{o}C and the residue is dissolved in 2 ml heptane. About 1.6 µL of the final extract is injected into a GC system.

GC-FID operational conditions

Agilent 7890A Gas chromatography (Agilent Technologies, Santa Clara, California, USA) equipped with Agilent G4513A GC auto-sampler controller and flame ionization detector (FID) was used with the operating conditions of: HP-5 fused silica capillary column (30 m*0.25 mm ID*0.25 µm film), 1.0 µL injection volume, helium

carrier gas with a flow rate of 1 mL/min through the column, and split ratio of 1:50. The oven temperature is set at 80 ^{o}C for 1 min and increased at the rate of 20 ^{o}C /min to 140 ^{o}C, and finally increased with 5.0 ^{o}C/min to 335 ^{o}C and maintained for 20 min. Injection interval is set from 70 ^{o}C to 300 ^{o}C; and maximum temperature (detector temperature) is 350 ^{o}C .

Fatty acid methyl and ethyl esters elutes earlier than wax because of the lower molecular weights. The orders of their elution are as follows: methyl palmitate (C16:0 ME), ethyl palmitate (C16:0 EE), methyl heptadecanoate (Internal standard), methyl linoleate (C18:2 ME), methyl oleate (C18:1 ME), methyl stearate (C18:0 ME), ethyl linoleate (C18:2 EE), ethyl oleate (C18:1 EE) and ethyl stearate (C18:0 EE). The second half of the chromatograms constitutes wax components starting with the internal standard (Lauryl arachidate) and C42, C44, C46 and C48 in that order. Peak evaluation, data acquisition and processing are performed using Agilent ChemStation GC program.

GC-MS Identification of peak

Prior to quantification, structural confirmation of the chemical compounds corresponding to the identified methyl esters, ethyl esters and wax peaks were determined using Agilent 6890 Gas chromatography (Agilent Technologies, Santa Clara, California, USA) equipped with split/splitless front inlet and back inlet, Agilent 7683 series injector auto-sampler, Agilent 5973 (Network Mass Selective Detector), fused silica column HP-5MS, (30 m*0.25mm ID*0.25 μm Agilent 19091S-433) and split ratio of 10:1.

1.0 μL injection volume was used and helium carrier gas was at a flow rate of 13.9 mL/min in the front inlet and 102.2 mL/min in the back inlet. The oven temperature program was: 80 ^{o}C, 1 min, it was the raised at 20 ^{o}C/min to 140 ^{o}C, and finally at 5 ^{o}C/min to 335 ^{o}C in 20 min. The detector and maximum temperature was 350 ^{o}C with 20 Hz data generation rate. Peak evaluation, data acquisition and processing were performed using Agilent ChemStation GC program.

FAEE and wax quantification

The principal fatty acids methyl and ethyl esters of importance present in olive oil (C16 ME, C16 EE, C18 ME and C18 EE) and wax peaks corresponding to C42, C44, C46 and C48 were identified following the method of Perez-Camino, et al., (2008). The quantification of each peak was carried out on the basis of the area

corresponding to the C17:0 and C32:0 internal standards of alkyl esters and wax respectively.

Eqn. 3.11 and 3.12 as described in the European Union Official Journal (EEC, 2011) were used to calculate individual esters and total wax contents. The results were reported as both individual and sum of methyl and ethyl esters from C16 and C18. Also total wax contents were reported as sum of C42, C44, C46 and C48 in mg/kg.

$$\text{Esters (mg/kg)} = \frac{A_x * m_s * 1000}{A_s * m} \quad (3.11)$$

A_x = area corresponding to the peak for the individual C16 and C18 ester.

A_s = area corresponding to the peak for the methyl heptadecanoate internal standard

m_s = mass of the methyl heptadecanoate internal standard added in milligrams

m = mass of the sample taken for the determination, in grams

$$\text{Wax (mg/kg)} = \frac{(\sum A_x) * m_s * 1000}{A_s * m} \quad (3.12)$$

Where:

A_x = area corresponding to the peak for the individual ester

A_s = area corresponding to the peak for the lauryl arachidate internal standard

m_s = mass of the lauryl arachidate internal standard added in milligrams

m = mass of the sample taken for the determination, in grams

3.2.12. E-nose Determination

A PEN2 portable electronic nose (Win Muster Air sense (WMA) Analytics Inc., Schwerin, Germany) was used to evaluate aromatic compounds present in olive oil. The system consists of a sampling/injection compartment, sensors-embedded detector unit, and pattern recognition analytical software (Win Muster v.1.6) for initial data acquisition. There are 10 Metal Oxide Semiconductors (MOS), chemically specific and responsive sensor arrays available in the system. The sensors are described as: W1C (aromatic), W5S (broad-range), W3C (aromatic), W6S (hydrogen), W5C (aromatic-

aliphatic), W1S (Sulphur-organic), W2S (broad-alcohol), W2W (Sulphur-chloride), and W3S (methane-aliphatic) (Hai & Wang, 2006). The numerical signal outputs of the system with response to volatiles measured are expressed as resistivity Ω (ohms). The oil sample (2 g) is placed in 10 mL Pyrex ® vials fitted with pierceable Silicon/Teflon disk in the cap. After 10 min equilibration at 40 ^{o}C, the headspace is pumped over the sensor surfaces for 60 s (injection time) at a flow rate of 300 mL/min. During this time the sensor signal is purged for 180 s with filtered air prior to the next sample injection to allow reestablishment of the instrument baseline. Each sample is analyzed in duplicate and there is no sensor drift throughout the measurement.

3.2.13. FT-NIR Spectroscopy

Fourier Transform (FT)-NIR spectrometer (MPA, Bruker Optics, Ettlingen, Germany) fitted with an integrating sphere, was used to generate the NIR spectral data of the olive oil samples. The data are collected in transmission mode using a flow cell of 1 mm path length. Spectral range of 12,500 – 4000 cm^{-1} is collected with the following operational conditions: resolution, 8 cm^{-1}; scanner velocity, 10 kHz; background 16 scans and 16 scans per sample, at controlled temperature (23±1 ^{o}C). The two replicated spectra of each sample are averaged during elaboration. OPUS software (v. 6.5 Bruker Optics Ettlingen, Germany) is applied as instrument control as well as initial data processor.

3.2.14. FT-IR Spectroscopy

(a) The instrument used for the acquisition of MIR spectra at University of Milan was a FT-IR spectrometer (VERTEX 70, Bruker Optics, Ettlingen, Germany), equipped with a deuterated triglycine sulphate (DTGS) detector. The spectral data were collected over the range of 4000 - 700 cm^{-1} (resolution, 16 cm^{-1}; scanner velocity, 7.5 kHz; background 32 scans; and 32 scans per sample,) at controlled temperature (23±1 ^{o}C). The samples are positioned on a germanium crystal ATR (Attenuated Total Reflectance) accessory with multiple reflections is used as a sample holder through which the radiation passed. Measurements are replicated twice and data acquisition is done using Opus software (v. 6.5, Bruker Optics, Ettlingen, Germany).

(b) The instrument used for the acquisition of MIR spectra at IYTE: Perkin Elmer Spectrum 100 FTIR spectrometer (Perkin Elmer Inc, USA) embedded with a deuterated triglycine sulphate (DTGS) detector was used to obtain MIR spectra within the range of 4000-650 cm^{-1}. The spectrometer is equipped with a horizontal attenuated total reflectance (HATR) accessory with ZnSe crystal. Operational conditions are: 64 scans for each spectrum, at 4 cm^{-1} resolution and scan speed of 1 cm/s. Prior to measurement of each sample, initial background scans are taken. Measurements are repeated twice and averaged.

3.2.15. UV-vis Spectroscopy

UV-visible spectrophotometer (Shimadzu UV-2450 Kyoto, Japan) equipped with deuterium-discharge lamp as a source of ultraviolet wavelength range and a tungsten lamp for the visible range (200-700 nm), and a resolution of 2.0 nm,was used for the UV-vis spectrum of the samples. There are two rectangular cells one for sample and the other for blank. Plastic cuvettes of 10 mm path length are used for sample and blank holder. The UV-vis spectra of the oil samples are taken alone and in cyclohexane (as described in chlorophyll and carotenoids sections).

3.3. Data Matrices and Statistical Analysis

Analysis of Variance (ANOVA) was used to evaluate the significance of factors and their interactions effects on the chemical characteristics of the oil samples (Minitab 16.0, Minitab Inc., State College, USA). Tukey multiple means comparison test was used to establish degree of significance among observations.

In the multivariate statistical data analysis, data matrices were created in two categories (chemical and spectroscopic data matrices) using SIMCA (v. 13, Umetrics, Umea, Sweden). Depending on the variables, predictors and other model parameters, the following operations were performed on the matrices: pattern recognition, class modelling or discriminant analysis, variable prediction by regression analysis.

Chemical data matrices

a. 2012-2014 data matrix **X** of size (36 x 40) containing 36 olive oil samples (n observations) and 40 measured variables (k variables). The variables include eleven individual phenols, total phenolic acids (TPA), summation of hydroxytyrosol and tyrosol as oleuropein derivatives (O-der), total phenol content (TPC), five quality parameters, five color parameters, eleven fatty acids, saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), MUFA/PUFA.and oleic to linoleic acid ratio (C18:1/C18:2).
b. 2012 olive fruits data matrix **X** of (18 x 17) containing 18 olives samples (n observations) and 17 measured variables (k variables). The variables include 14 individual phenols, total phenolic acids (TPA), summation of hydroxytyrosol and tyrosol as oleuropein derivatives (O-der) and total phenol content (TPC).
c. 2015-2016 data matrix **X** of size (54 x 54) containing 54 olive oil samples (n observations) after removing oil samples with no precise olive varieties, and 54 measured parameters (k variables). The models were built to evaluate the effect of 15 months dark room storage on the chemical properties of olive oils. Chemical properties are eleven individual phenols, total phenolic acids (TPA), summation of hydroxytyrosol and tyrsosol as oleuropein derivatives (O-der), total phenol content (TPC), five quality parameters, five color parameters, eleven fatty acids, SFA, MUFA, PUFA, MUFA/PUFA and oleic to linolenic acid ratio (C18:1/C18:2), four ethyl esters, four methyl esters, FAEE, FAME, FAAE, FAEE/FAME, and total wax content.

Spectroscopic classification

The following data matrices were built for the purpose of classification of olive oil with respect to variety, harvest time and harvest year:

a. FT-IR data matrix includes the spectral ranges of 3090-2750 cm^{-1} and 1874 -700 cm^{-1} (103 samples of 2012-2016).
b. UV-vis data matrix includes the spectral range of 200-800 nm involving 103 samples.
c. Fused spectra matrix such as: UV-IR, are considered on olive oil varietal and harvest year classification

d. FT-NIR data matrix includes the spectral range of 9000 and 5400 cm^{-1} (63 samples of 2012-2015).
e. E-nose data matrix: This is composed of 10 Metal Oxide Semi-conductor (MOS) sensors of 70 olive oils samples (70 x 10). Each sensor corresponds to a specific volatile compound or group of compounds expressed in resistivity Ω. i.e. W1C (aromatic) W5S (broad-range) W3C (aromatic) W6S (hydrogen) W5C (aromatic-aliphatic) W1S (Sulphur-organic) W2S (broad-alcohol) W2W (Sulphur-chloride) and W3S (methane-aliphatic).

Spectroscopic prediction of chemical parameters

a. FT-IR data matrixes were considered in the multivariate models (63 samples).
b. UV-vis (ordinary) data matrix involves 63 samples
c. Combined spectra matrix UV-IR of 63 samples

SNV (Standard Normal Variate) is a mathematical treatment operation applied to all the spectroscopic data prior to chemometric multivariate modelling. It was applied individually and coupled with second derivatives, to eliminate spectral background noise effects, and baseline shifting or tilting (Alamprese et al., 2013). These spectral defects are brought about by non-specific scattering of radiation at the surface of the samples, spectral path-length variation, and chemical composition of the samples. The algorithm is designed to work on individual sample spectra, by mean centering it and scales it by its own standard deviation. Second derivative spectra transformation was done using Savitzky-Golay method with third-order fifteen point smoothing polynomials was employed to create the second derivative models (Alciaturi et al., 2001).

PCA (Principal Component Analysis) was applied to examine the natural clustering pattern in each of the data matrices, by factorizing the **X** matrix into two matrices: score (**T**), loading (**P**) and residual error (**E**) as shown in equation (3.13) and Fig. 3.2. It is an unsupervised multivariate data elaboration technique with the objective of obtaining a linear transformed and dimensionally reduced data output, that keeps reasonable amount of variance in the original data matrix (Worley & Powers, 2012). The vectors of measured variances are called principal components and they are arranged based on the amount of variance explained. PC 1 is the axis that lies in the direction containing most explained variation. The subsequent principal component (PC

2) is statistical unrelated (orthogonal) to the previous, but described lesser amount of variance and the other continues.

$$\mathbf{X = T*P + E} \qquad (3.13)$$

The number of columns in the matrix **T** and the number of rows in matrix **P** are equal.

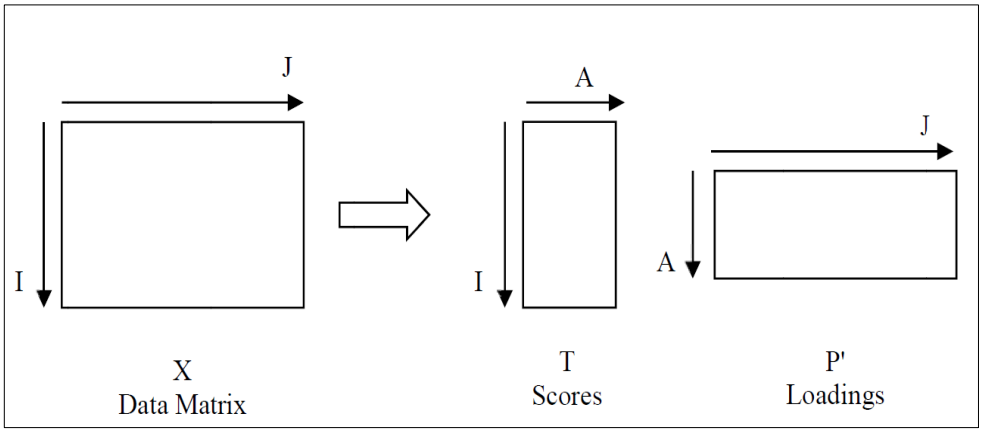

Figure 3.2. Graphical representation of PCA modeling (Source: Eriksson et al., 2001)

As a supervised technique, discriminant analysis by orthogonal projections to latent structures (OPLS-DA) was used in the classification of oil samples, in which **Y** variable is a user-defined variable representing the classes of samples. OPLS is an advancement on the conventional PLS model (Fig 3.3)

$$\mathbf{Y = T*C + F} \qquad (3.14)$$

T and **C** matrices are the scores and loadings for **Y**, and residual errors unaccounted for are contained in **F** matrix.

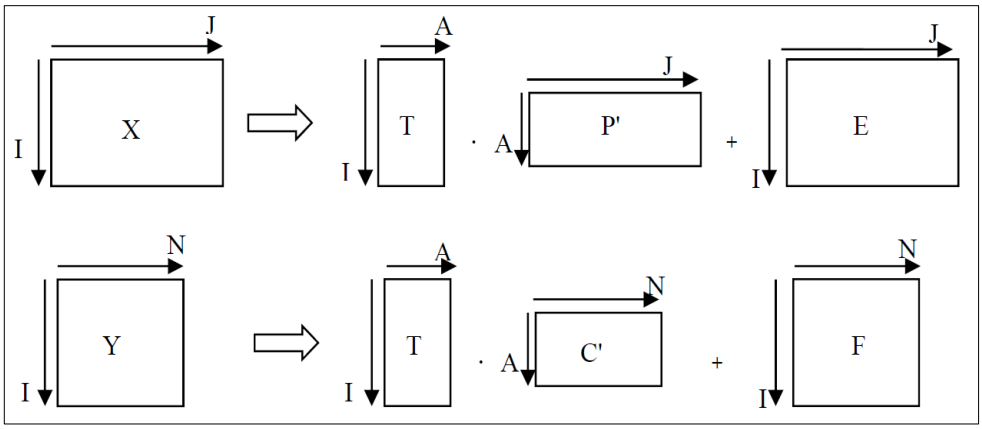

Figure 3.3. Graphical representation of PLS modelling (Source: Eriksson et al., 2001)

OPLS has the ability to resolve systematic variations in **X** matrix into two parts; a predictive part ($\mathbf{T_pP_p}$) correlated to **Y** variable (class information in this case), an orthogonal part ($\mathbf{T_oP_o}$) uncorrelated to **Y** as shown in equation (3.15) (Galindo-Prieto, Eriksson, & Trygg, 2015).

$$\mathbf{X = T_pP_p + T_oP_o + E} \quad (3.15)$$

The multivariate models were described with their number of components (PC), R^2 as the total variation explained and R^2_{CV} as the leave-one-out cross validation. The principal components of OPLS models were given as $P_p + P_o$, where p and o stand for the number of predictive and orthogonal components, respectively. In the case of olive oil classification using spectral and e-nose data, the observations are divided into training and validation sets. The results are further expressed in terms of percent correct classification in both calibration and validation models.

OPLS regression technique was applied to evaluate and compare the predictive capacities of different spectra (FT-IR and UV-vis) and their combination over some important chemical parameters. 30 % of the observation was used as validation set and the rest as calibration (training set). The selection of these parameters was based on the previous knowledge of their various contributions to olive oil overall quality characteristics such as oxidative stability index (OSI), fatty acid ethyl and alkyl esters, FAEE/FAME, wax, MUFA, PUFA, MUFA/PUFA, total phenol content, free fatty acid and peroxide values. Apart from the significance of these parameters in variable influence projection (VIP) of our previous studies, they were also common to all the 63 recent samples (2015-2016) considered. Models of pretreated spectra were built separately for each group of parameter. Leave-one-out cross-validation (CV) method was applied as the inner-validation. The same independent validation sets were used for the two spectra in order to justify the comparison of their predictive abilities. Details of the computed statistical outputs were reported in terms of root mean square error of calibration (RMSEcal), validation (RMSEval), regression coefficients for calibration, R^2_{cal}, and cross-validation R^2_{CV}, as criterion that define the predictive abilities of the models and their applicability to independent data sets. Overfitting of the models was avoided by using autofit (automatic fitting) embedded in SIMCA 13.3.0 software that automatically generates the significant number of principle components.

CHAPTER 4

RESULTS AND DISCUSSION

EFFECTS OF MALAXATION TEMPERATURE, OLIVE VARIETY AND HARVEST TIME ON THE CHEMICAL CHARACTERISTICS OF OLIVE OIL

The combined effects of olive variety, harvest time and malaxation temperature on the chemical characteristics of olive oils are discussed in this chapter. There are few other important malaxation conditions that could be considered such as: time, headspace etc. In other to have a manageable number of experiments, only temperature was considered in conjuction with the two agronomical factors (harvest time and olive variety). The results of phenolic and fatty acid profiles and quality parameters are presented as means of two replicate outputs of ANOVA. In the multivariate analytical section, unsupervised Principle Component Analysis (PCA) and supervised Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) were applied. OPLS multiple regression of oxidative stability index (OSI) of the oil samples, using some predictors was determined and the contributions of the significant variables (negative or positive) were assessed. Finally, phenolic contents of the olive fruits (from which the oil samples were produced) were evaluated. Variations in phenolic content of the fruits, with respect to variety and harvest times were discussed as well.

4.1. Chemical Parameters

ANOVA results of the experimental data showed the significance of olive variety, harvest time (degree of maturation) and their interaction on the quality and chemical properties of olive oil at 95% confidence interval ($p < 0.05$). The results implied variation of the sample chemical compositions with respect to these three factors (harvest time, olive variety and malaxation temperature) (Table 4.1-4.3). Temperature of malaxation and its interaction with other factors were only influential on some important phenolic compounds such as hydroxytyrosol, tyrosol, pinoresinol, pCoumaric acid, PV but not with fatty acid profiles of the oil. Quantitatively, OSI, TPC

and linoleic acid were different among the oil varieties, but they are statistically not significant with respects to the factors. The p-values results of the univariate analysis of all the chemical variables determined (Table A.1-A.3).

The FFA is one of the conventional parameters for establishing grades and quality classes of olive oil. Official grading system allotted 0.8, 2.0 and 3.3% for extra virgin, virgin and olive oil, respectively. Therefore almost all the samples are within virgin and olive oils categories. There was a slight increase in FFA values of Ayvalik oils with respect to malaxation temperature compared to Memecik oils. Hydrolysis of TAG (Triacylglycerol) by endogenous lipase enzyme is one of the factors leading to the generation of free fatty acids and a slight increase in temperatures do not automatically increase this hydrolytic process. Some author observed a decrease in FFA of olive oils at temperature above 35 ^{o}C (Clodoveo et al., 2014; Panzanaro et al., 2010; Boselli et al., 2009).

PV values of almost all the samples are within the legal threshold limit (20 meq O_2kg^{-1}) of EU regulation (Commission Regulation EEC No 2568/91) except oils of late Memecik obtained at 47^{o}C. There is a statistically significant difference between olive oils of the two varieties with Ayvalik having lower peroxide values range (7.74-17.06) than Memecik (13.36-25.33). There is no significant correlation between PV and FFA values. However, Memecik oils generally exhibited higher values of these two parameters. Temperature is one of many notable factors influencing olive oil peroxidation as indicated in the increased trend of PV with malaxation temperature.

Chlorophyll and carotenoid pigments are the major coloring agents influencing the visual appearance of olive oil ranging between green-yellow to golden, depending on varietal differences and olive maturation stage. Apart from the coloring properties, these pigments are appreciated also for their bioactivities such as: provitamin A function (beta-carotene and beta-cryptoxanthin), antioxidant property, and antimutagenic activities (Achat, Rakotomanomana, Madani, & Dangles, 2016). There were significant differences with respect to these paramters; between olive oils of the two varieties and their harvest periods. This observation is supported by earlier reports about decrease in olive oil color pigment with olive maturation (Criado et al., 2007). Chlorophyll range of 1.50– 4.65 mg/kg and carotenoids of 1.20 –2.86mg/kg were observed in Memecik oil. This is approximately 50% higher than that of Ayvalik. There was a sharp decrease in the chlorophyll and carotenoids content of Memecik oil as against the progressive decrease in that of Ayvalik, with harvest time. The same

observations were true for the color parameters with respect to olive variety and harvest time. Memecik oil increased in lightness (L*) with harvest time probably due to chromophore degradation as maturation advances. A mere visual observation of Memecik oils appeared to be more yellowish than Ayvalik. This explained the reason why chromatic parameter b* value reduced with maturity.

Table 4.1 Quality parameters (mean± SD) of Ayvalik and Memecik olive oils at three harvest times (early, mid and late) and malaxation temperatures (27^0C, 37^0C and 47^0C)

	Early Harvest			Mid Harvest			Late Harvest		
Responses	27^0C	37^0C	47^0C	27^0C	37^0C	47^0C	27^0C	37^0C	47^0C
Ayvalik olive oils									
TPC	116.7±23.0	101.3±29.3	84.12±0.35	94.65±14.6	98.96±8.3	189.8±96.6	191.3±111	181±104	83.4±3.80
FFA	1.0±0.01	0.83±0.0	1.60±0.08	0.63±0.0	0.56±0.01	1.04±0.31	1.96±0.23	0.98±0.01	1.15±0.36
PV	7.74±2.57	6.50±0.13	15.19±1.99	12.72±3.01	11.35±4.3	13.36±3.9	17.06±4.8	9.16±0.01	11.93±3.31
OSI	5.75±0.71	6.96±0.21	4.25±0.57	4.60±0.04	5.20±0.24	5.89±1.08	5.37±0.25	5.20±0.01	5.52±0.01
Chl	2.12±0.76	2.57±0.31	2.39±0.14	1.67±0.91	1.31±0.06	1.51±0.11	1.30±0.35	1.28±0.11	1.34±0.34
Car	1.43±0.35	1.61±0.42	1.45±0.33	1.17±0.31	1.01±0.21	1.29±0.03	1.04±0.08	1.33±0.31	1.05±0.12
L*	87.5±2.57	88.4±3.08	91.1±2.64	92.34±0.92	94.97±3.53	90.84±2.09	87.14±0.74	87.68±0.46	87.77±5.77
a*	-1.88±1.24	-2.53±1.05	-3.02±0.11	-3.14±1.12	-4.30±0.18	-3.20±0.24	-0.82±0.61	-3.76±0.24	-3.56±0.98
b*	71.51±12.8	72.82±0.27	65.36±1.77	63.17±23.1	49.4±0.83	59.9±5.76	77.05±6.02	50.32±1.35	54.27±2.61
C	71.54±12.7	72.82±0.27	65.36±1.77	63.26±23.0	49.58±0.81	60.0±5.73	77.14±5.96	50.32±1.34	54.39±2.67
H	91.63±1.28	92.0±0.83	92.66±0.16	93.25±2.21	94.99±0.30	93.09±0.52	91.75±1.12	94.30±0.30	93.74±0.85
Memecik olive oils									
TPC	154.5±30.2	206.1±81.8	141.0±54.6	117.1±18.7	124±13.7	194.3±10.2	115.03±74.8	103.9±16.3	78.45±5.13
FFA	3.77±1.53	1.78±0.30	1.53±0.14	0.87±0.13	2.17±1.65	1.09±0.22	1.41±0.53	0.79±0.06	0.84±0.06
PV	18.6±1.41	16.86±1.10	15.18±0.95	13.64±1.29	15.10±0.59	18.81±9.26	13.36±0.05	18.07±3.10	25.33±11.9
OSI	4.29±1.07	5.40±0.08	6.22±0.30	5.89±0.46	5.25±2.06	7.06±0.55	5.35±0.59	5.63±0.63	5.50±1.44
Chl	4.10±0.63	3.31±0.23	4.55±0.73	2.72±0.66	2.21±0.63	2.74±0.86	1.67±0.06	1.50±0.13	1.78±0.02
Car	2.35±0.53	2.38±0.52	2.86±0.59	2.07±0.02	1.77±0.64	2.14±0.43	1.30±0.18	1.11±0.35	1.20±0.17
L*	82.3±2.73	82.6±5.34	79.21±0.34	81.1±5.25	86.13±1.40	83.26±1.40	89.95±1.32	92.33±3.84	89.97±4.65
a*	0.64±2.40	1.28±1.10	2.05±0.36	-0.51±0.40	-1.24±1.56	1.13±0.69	-3.56±0.13	-3.21±0.43	-2.87±0.25
b*	91.36±21.1	91.28±0.77	99.86±1.99	83.88±10.6	82.13±15.1	91.64±6.38	53.84±3.47	55.66±0.75	57.99±0.60
C	91.38±21.2	91.29±0.76	99.89±2.00	83.21±10.61	82.89±15.1	91.65±6.38	53.96±3.47	55.76±0.77	58.06±0.59
H	89.77±1.47	89.19±0.70	88.82±0.18	90.39±0.32	90.98±1.25	89.27±0.48	93.37±0.49	93.30±0.40	92.84±0.28

SD: Standard deviation of two replicate TPC: total phenol content (mg/kg), FFA: Free fatty acid (% Oleic acid), PV: Peroxide value (meq O2/kg), OSI: Oxidative stability index (hr), Chl: Chlorophylls (mg/kg), Car: Carotenoids (mg/kg), CIELAB color paramters: L* (lightness-Darkness), a* (greenness-redness), b*(blueness – yellowness), C (Chroma), H (Hue angle^o)

Table 4.2. Phenolic and fatty acid profiles (± SD) of Ayvalik olive oils at three harvest times (early, mid and late) and malaxation temperatures (27^0C, 37^0C and 47^0C)

	Early Harvest			Mid Harvest			Late Harvest		
Responses	27^0C	37^0C	47^0C	27^0C	37^0C	47^0C	27^0C	37^0C	47^0C
Phenolic profile (mg/kg)									
Hyt	0.16±0.10	0.13±0.02	0.08±0.01	0.56±0.20	0.29±0.11	2.29±0.49	0.89±0.14	0.28±0.08	0.23±0.06
Tyr	8.00±0.87	6.64±2.58	9.26±1.53	3.46±1.59	2.48±0.76	5.63±1.70	2.22±0.42	1.19±0.34	1.57±0.22
4Hpa	15.4±3.35	16.4±3.44	11.9±2.86	1.48±0.28	2.66±0.08	2.02±0.50	3.40±0.29	1.44±0.13	1.34±0.40
3Hpa	1.55±0.87	0.86±1.03	0.88±0.93	0.30±0.10	0.55±0.40	0.65±0.62	0.27±0.08	0.16±0.08	0.19±0.12
Caf	0.81±0.37	0.76±0.13	0.46±0.05	0.17±0.03	0.11±0.13	0.11±0.10	0.12±0.04	0.07±0.02	0.07±0.01
Pin	1.86±0.08	0.86±0.17	1.59±0.23	7.30±6.06	2.04±0.42	4.45±1.43	1.82±0.58	0.82±0.30	0.49±0.01
Dba	0.36±0.16	0.24±0.01	0.19±0.08	0.11±0.05	0.15±0.04	0.18±0.00	0.12±0.06	0.07±0.00	0.10±0.01
Vnl	0.19±0.06	0.17±0.06	0.19±0.21	0.18±0.12	0.14±0.06	0.15±0.06	0.08±0.05	0.07±0.03	0.06±0.01
pCu	0.77±0.05	0.49±0.19	0.40±0.06	0.41±0.21	0.18±0.02	0.54±0.09	0.36±0.16	0.10±0.02	0.14±0.01
Fer	0.21±0.08	0.18±0.01	0.13±0.02	0.06±0.01	0.05±0.01	0.07±0.03	0.05±0.01	0.02±0.00	0.03±0.01
Lut	4.75±0.93	3.65±0.27	4.00±0.51	5.24±0.88	4.08±1.07	3.47±0.67	2.74±0.95	2.28±0.26	1.39±0.04
O-der	8.16±0.97	6.76±2.60	9.34±1.53	4.02±1.79	2.77±0.87	7.91±1.22	3.11±0.28	1.47±0.25	1.80±0.28
TPA	19.13±3.13	18.94±4.55	13.94±1.76	2.52±0.52	3.69±0.16	3.57±1.14	4.30±0.52	1.84±0.10	1.85±0.57
Fatty acid profile (%)									
C16:0	13.63±0.17	13.74±0.35	14.87±0.74	12.68±0.42	13.06±0.04	12.08±0.24	12.46±0.29	12.10±0.03	11.87±0.41
C16:1	0.81±0.11	0.82±0.06	0.82±0.03	0.72±0.06	0.75±0.03	0.58±0.10	0.64±0.02	0.66±0.03	0.67±0.01
C17:0	0.11±0.03	0.11±0.04	0.12±0.02	0.14±0.01	0.14±0.02	0.11±0.07	0.15±0.02	0.15±0.02	0.14±0.02
C17:1	0.19±0.04	0.19±0.06	0.20±0.03	0.22±0.01	0.21±0.02	0.16±0.07	0.20±0.02	0.20±0.02	0.20±0.02
C18:0	2.17±0.30	2.17±0.24	2.31±0.11	2.60±0.03	2.55±0.13	2.41±0.98	3.08±0.03	3.02±0.11	2.96±0.26
C18:1	72.75±1.46	72.81±0.69	71.99±0.85	71.87±0.54	72.34±0.25	72.80±1.53	73.35±2.30	71.99±0.47	72.29±0.80
C18:2	9.08±0.41	8.78±0.28	8.36±0.29	10.41±1.31	9.55±0.05	10.58±0.48	10.41±0.23	10.27±0.04	10.23±0.08
C18:3	0.61±0.03	0.62±0.07	0.59±0.03	0.61±0.02	0.59±0.02	0.57±0.17	0.69±0.01	0.72±0.04	0.72±0.02
C20:0	0.38±0.11	0.38±0.10	0.44±0.04	0.44±0.05	0.45±0.03	0.39±0.20	0.52±0.01	0.51±0.03	0.50±0.04
C20:1	0.22±0.12	0.26±0.09	0.27±0.05	0.27±0.05	0.29±0.03	0.20±0.17	0.30±0.02	0.31±0.04	0.32±0.03
C22:0	0.07±0.10	0.16±0.03	0.07±0.10	0.07±0.10	0.09±0.07	0.16±0.02	0.13±0.02	0.08±0.11	0.11±0.06
SFA	16.36±0.72	16.56±0.70	17.81±1.01	15.93±0.62	16.28±0.22	15.15±1.47	16.33±0.20	15.86±0.29	15.59±0.80
MUFA	73.97±1.18	74.08±0.49	73.27±0.73	73.08±0.67	73.59±0.17	73.74±1.19	74.49±2.24	73.16±0.37	73.48±0.73
PUFA	9.69±0.44	9.39±0.21	8.95±0.26	11.02±1.32	10.15±0.02	11.16±0.30	11.09±0.21	10.99±0.08	10.95±0.06
18:1/18:2	8.02±0.52	8.30±0.19	8.61±0.20	6.96±0.92	7.57±0.01	6.88±0.16	7.05±0.07	7.01±0.07	7.07±0.02
MU/PU	7.64±0.47	7.89±0.12	8.18±0.16	6.68±0.86	7.25±0.00	6.61±0.07	6.71±0.07	6.66±0.08	6.71±0.03

SD: Standard deviation of two replicates, Hyt: Hydroxytyrosol, Tyr: Tyrosol, 4Hpa: 4-hydroxyphenyl acetic acid, 3Hpa: 3-hydroxyphenyl acetic acid, Pin: Pinoresinol, Dba: 2,3dihydroxylbenzoic acid, Vnl; vanillin, pCu: p-Coumaric acid, Fer: Ferulic acid, Lut; Luteolin, TPA: Total phenolic acids, C16:0: Palmitic acid, C16:1: Palmitoleic acid, C17:0: Heptadecanoic acid, C17:1: Cis-10-Heptadecanoic acid, C18:0: Stearic acid, C18:1: Oleic acid, C18:2: Linoleic acid, C18:3: Linolenic acid, C20:0: Arachidic acid, C20:1: Cis-11-Eicosenoic acid, SFA: Saturated fatty acids, MUFA: Monounsaturated fatty acids, PUFA: Polyunsaturated fatty acids, OA/LnO: Oleic:Linoleic acids ratio

Table 4.3. Phenolic and fatty acid profiles (mean± SD) of Memecik olive oils at three harvest times (early, mid and late) and malaxation temperatures (27^0C, 37^0C and 47^0C)

	Early Harvest			Mid Harvest			Late Harvest		
Responses	27^0C	37^0C	47^0C	27^0C	37^0C	47^0C	27^0C	37^0C	47^0C
Phenolic profile (mg/kg)									
Hyt	0.28±0.01	0.18±0.18	0.72±0.40	0.77±0.53	1.16±1.01	2.65±0.88	0.24±0.13	0.38±0.15	0.36±0.12
Tyr	2.90±0.18	10.17±0.62	14.0±1.73	12.4±5.92	6.64±2.60	18.0±2.01	2.49±1.24	3.61±1.40	3.50±0.85
4Hpa	4.88±2.78	2.94±1.44	6.24±0.82	3.39±1.54	6.32±3.70	3.13±0.64	2.89±2.99	0.91±0.05	1.01±0.26
3Hpa	0.21±0.00	0.21±0.10	0.20±0.04	0.15±0.04	0.16±0.02	0.14±0.10	0.22±0.03	0.17±0.11	0.48±0.54
Caf	0.33±0.18	0.30±0.08	0.40±0.06	0.17±0.06	0.26±0.07	0.13±0.03	0.13±0.11	0.08±0.03	0.09±0.04
Pin	12.54±1.48	10.89±2.18	22.3±2.45	4.74±0.67	8.98±2.11	7.75±1.14	4.29±1.24	4.74±0.12	4.35±0.67
Dba	0.12±0.08	0.12±0.00	0.31±0.06	0.10±0.01	0.16±0.01	0.12±0.04	0.08±0.03	0.09±0.01	0.07±0.09
Vnl	0.06±0.02	0.08±0.01	0.12±0.00	0.20±0.18	0.16±0.06	0.11±0.01	0.04±0.03	0.05±0.01	0.08±0.01
pCu	0.75±0.25	2.05±0.75	2.89±1.17	0.42±0.02	0.53±0.16	0.87±0.23	0.11±0.13	0.10±0.01	0.19±0.06
Fer	0.17±0.13	0.19±0.10	0.27±0.12	0.06±0.01	0.10±0.01	0.08±0.03	0.03±0.01	0.01±0.01	0.01±0.01
Lut	4.55±0.87	6.64±1.47	5.57±0.49	0.72±0.66	2.86±0.16	2.88±0.26	3.05±1.44	3.08±0.40	2.43±0.25
O-der	3.18±0.16	10.35±0.43	14.72±1.20	13.13±5.52	7.79±3.61	20.65±1.12	2.73±1.38	3.99±1.25	3.86±0.97
TPA	6.45±3.42	5.81±2.47	10.29±2.28	4.28±1.61	7.51±3.61	4.47±1.07	3.45±3.24	1.35±0.22	1.84±0.49
Fatty acid profile (%)									
C16:0	12.96±0.18	13.47±1.10	13.32±0.52	12.23±0.37	11.50±0.41	11.60±0.08	11.80±0.25	11.97±0.04	11.79±0.09
C16:1	0.76±0.05	0.84±0.06	0.88±0.00	0.81±0.00	0.54±0.29	0.74±0.02	0.85±0.05	0.88±0.01	0.87±0.04
C17:0	0.10±0.00	0.09±0.01	0.09±0.00	0.07±0.00	0.07±0.00	0.05±0.00	0.05±0.01	0.04±0.00	0.04±0.00
C17:1	0.15±0.02	0.13±0.02	0.12±0.01	0.09±0.03	0.11±0.05	0.08±0.01	0.08±0.01	0.06±0.00	0.06±0.00
C18:0	2.35±0.10	2.28±0.02	2.50±0.07	2.69±0.06	2.40±0.11	2.36±0.04	2.45±0.10	2.41±0.04	2.38±0.02
C18:1	71.63±0.36	70.97±0.18	71.55±0.00	74.37±0.11	72.73±3.53	75.16±0.38	72.89±0.38	73.13±0.13	73.88±0.31
C18:2	10.51±0.92	10.47±1.43	9.72±0.57	8.16±0.46	11.26±4.38	8.57±0.56	10.17±0.09	9.78±0.03	9.26±0.03
C18:3	0.76±0.17	0.91±0.04	0.94±0.01	0.75±0.02	0.78±0.06	0.72±0.02	0.87±0.04	0.90±0.02	0.89±0.06
C20:0	0.42±0.03	0.43±0.02	0.48±0.01	0.44±0.00	0.36±0.06	0.39±0.01	0.44±0.02	0.44±0.01	0.43±0.02
C20:1	0.29±0.02	0.31±0.00	0.31±0.01	0.29±0.00	0.26±0.03	0.28±0.02	0.33±0.02	0.34±0.02	0.33±0.03
C22:0	0.11±0.01	0.12±0.00	0.12±0.01	0.11±0.00	0.05±0.07	0.09±0.01	0.12±0.01	0.12±0.02	0.12±0.00
SFA	15.94±0.32	16.39±1.15	16.51±0.59	15.54±0.32	14.38±0.62	14.49±0.13	14.86±0.37	14.97±0.11	14.76±0.09
MUFA	72.84±0.41	72.25±0.22	72.86±0.00	75.56±0.14	73.65±3.80	76.25±0.41	74.15±0.32	74.41±0.09	75.15±0.24
PUFA	11.27±0.75	11.38±1.38	10.66±0.55	8.90±0.44	12.03±4.44	9.30±0.54	11.04±0.05	10.68±0.01	10.15±0.11
C18:1/C18:2	6.84±0.63	6.84±0.95	7.37±0.43	9.13±0.53	7.06±3.06	8.79±0.62	7.17±0.03	7.48±0.01	7.98±0.08
MUFA/PUFA	6.48±0.47	6.40±0.79	6.84±0.36	8.50±0.43	6.63±2.76	8.22±0.52	6.72±0.00	6.96±0.00	7.41±0.11

SD: Standard deviation of two replicates, Hyt: Hydroxytyrosol, Tyr: Tyrosol, 4Hpa: 4-hydroxyphenyl acetic acid, 3Hpa: 3-hydroxyphenyl acetic acid, Caf: caffeic acid, Pin: Pinoresinol, Dba: 2,3dihydroxylbenzoic acid, Vnl; Vanillin, pCu: p-Coumaric acid, Fer: Ferulic acid, Lut; Luteolin, TPA: Total phenolic acids, C16:0: Palmitic acid, C16:1: Palmitoleic acid, C17:0: Heptadecanoic acid, C17:1: Cis-10-Heptadecanoic acid, C18:0: Stearic acid, C18:1: Oleic acid, C18:2: Linoleic acid, C18:3: Linolenic acid, C20:0: Arachidic acid, C20:1: Cis-11-Eicosenoic acid, SFA: Saturated fatty acids, MUFA: Monounsaturated fatty acids, PUFA: Polyunsaturated fatty acids, OA/LnO: Oleic:Linoleic acids ratio

The fatty acid profiles of the oil samples were significantly influenced by olive variety and harvest time. Olive oil is a mixture of triacylglycerols, which are esters of mono, di and polyunsaturated fatty acids; but predominantly monounsaturated ones (oleic acid). Oleic acid is believed to be one of the main inherent natural defensive mechanisms of olive oil against oxidation and extended shelf-life. Its single bond increases the activation energy needed to overcome for oxidation reaction to be initiated (Bendini et al., 2006). The higher the number of multiple bonds in the fatty acids composition of the oil, its susceptibility to oxidation becomes the greater. Oleic acid; the principle fatty acid in olive oil, was observed within the range of 70.97-75.16 %. The degree of maturation has been reported to affect oleic acid content of olive oil (Beltrán, Del Rio, Sanchez & Martinez, 2004) and this study indicated an increase in oleic acid content with fruit maturity. Memecik oil exhibited a similar trend however, Ayvalik oils showed more stability with respect to oleic acid. The changes in linolenic acid (C18:3) content were found significant with respect to olive variety and harvest time. Linolenic acid content of Ayvalik oils was in the range of 0.57 - 0.72 %, with the late harvest having higher values. Conversely, Memecik oils showed no specific difference in linolenic acid among harvest times, but the content is higher (0.72-0.94%) compared to Ayvalik. Linolenic acid is one of the most preferred substrate by lipoxygenase (LOX) enzyme during hydroperoxide formation (Tamborrino et al., 2014), which means that polyunsaturated fatty acid-rich olive oil samples may likely have higher peroxide values (PV). This probably explained why Memecik oils have higher PV values.

Palmitic acid (C16:0) constitutes over 90% of the total saturated fatty acid of olive oil. It ranged between 11.50-14.87% in Ayvalik oils which is significantly higher than that of Memecik. This result is similar to earlier observations (Gurdeniz, Ozen & Tokatli, 2010). The susceptibility of oils to oxidation is a function of the nature and the degree of saturation of its constituent fatty acids. As reported by Manai-Djebali et al., (2012), olive oils of high SFA contents are expected to be less prone to oxidation than those high in PUFA. However, in spite of the higher SFA content of Ayvalik oils, there was no significant difference in the OSI values of olive oils, even though FFA and PV of Ayvalik oils were lower than Memecik oils.

Phenolic compounds' involvement in resisting lipid oxidation makes the qualitative and quantitative evaluations of olive oil phenols very important. The most important phenolic compounds identified in olive oil are grouped into five classes

based on their structural and functional differences (Bendini et al., 2007). These include phenolic acids (3-hydroxylphenyl acetic acid, caffeic acid, p-coumaric acid, 4-hdroxylacetic acid, vanillic acid, 2,3 dihydroxyl benzoic acid, cinnamic acid and ferulic acid), phenolic alcohols (hydroxytyrosol and tyrosol/oleuropein derivatives), phenolic aldehyde (vanillin), lignans (pinoresinol) and flavones (apigenin and luteolin) Table 4.3 showed that the most abundant phenolic compounds are hydroxytyrosol, tyrosol, apigenin, luteolin and pinoresinol. The summation of all the phenolic acids was given as total phenolic acid (TPA) and summation of tyrosol and hydroxytyrosol as oleuropein derivative (O-der), and total phenol content (TPC) represents a rough estimate of total phenols present in the oil.

The changes in oleuropein derivatives (hydroxytyrosol and tyrosol) and TPC were compared in Fig. 4.1. One of the most important oxidative stability determinants in olive oil is the TPC. The oxidative stability conferred on olive oil by these compounds is based on their ability to neutralize or prevent the initiation of free radicals and chelate catalytic metals in the oil matrix (Ben-Hassine et al., 2013). Memecik oils experienced reductions in TPC with respect to harvest time. Quantitatively, there is no statistically significant difference between hydroxytyrosol contents from both varieties (0.08-2.29 mg/kg Ayvalik and 0.24-2.65 mg/kg Memecik as shown in Table 4.2 and 4.3, but the difference is highly significant with respect to harvest time and malaxation temperature. During olive malaxation, oleuropein hydrolysis to hydroxytyrosol and tyrosol is facilitated because; hydrolytic enzymes (β-glucosidase and esterase) are released (Taticchi, Esposto, Veneziani, Urbani, Selvaggini & Servili, 2013).

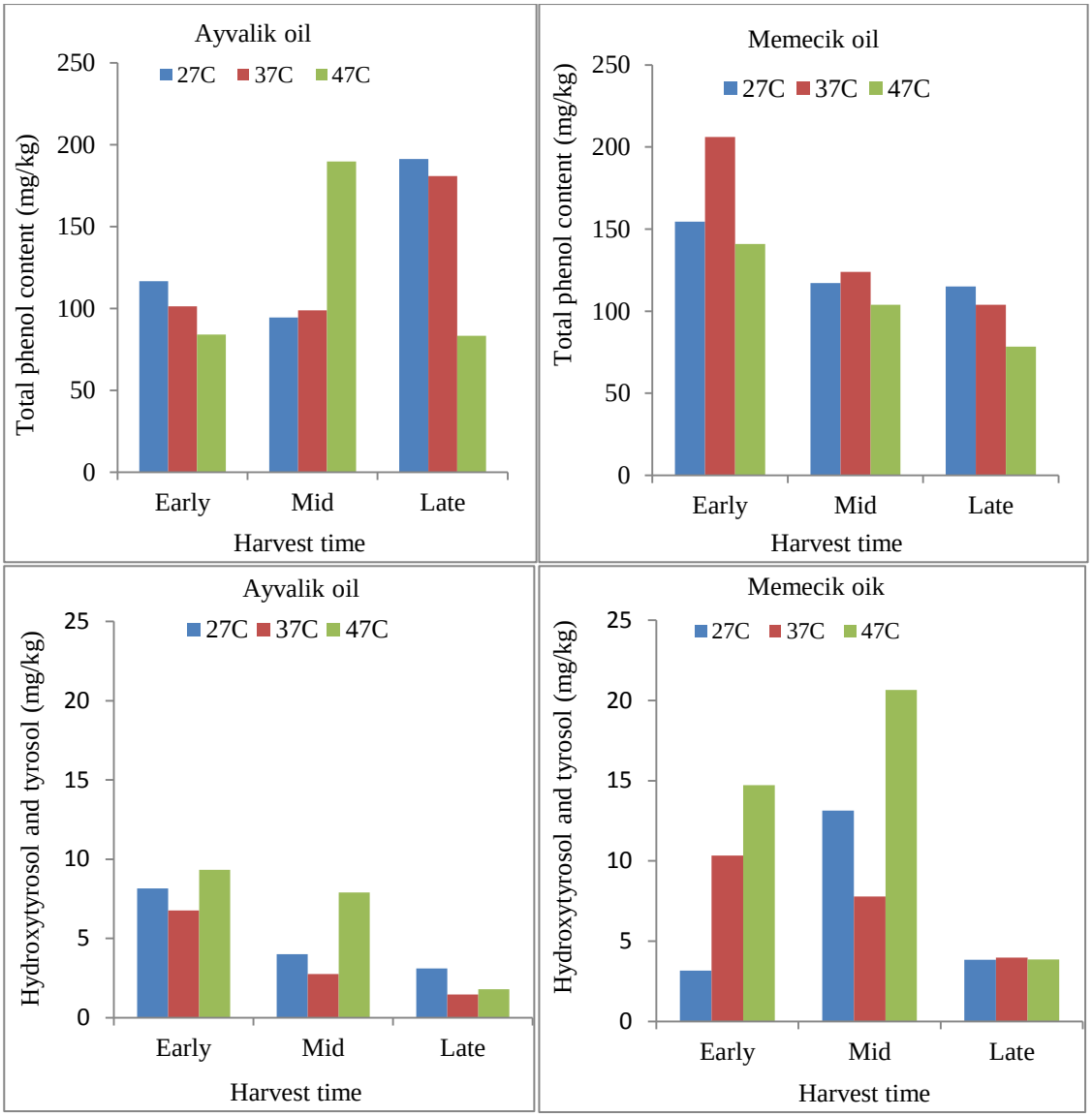

Figure 4.1. Changes in the total phenol content (TPC) and the sum of hydroxytyrosol and tyrosol (O-der) of Ayvalik and Memecik olive oils with respect to malaxation temperatures

Therefore, high amount of hydroxytyrosol in oils from both varieties at 47 ^{o}C malaxation temperature was probably due to an increase in hydrolytic activities of the endogenous enzymes. A recent report from European Food Safety Authority (EFSA, 2012) proposed a daily intake of 5 mg hydroxytyrosol or/and its derivatives per 20 g of olive oil as being capable of protecting oxidative damage to LDL particles as well as regulating blood HDL-cholesterol. Oils from early and mid-harvest of both varieties have comparatively higher amount of oleuropein derivatives (sum of hydroxytyrosol and tyrosol) and reasonable consumption of them may confer some health benefits. Higher amounts of oleuropein derivatives (sum of hydroxytyrosol and tyrosol) in oils

were obtained from early and mid-harvest of both varieties (Table 4.1 and 4.3). Tyrosol was more abundant than hydroxytyrosol in the samples. There was a linear decrease in tyrosol of Ayvalik oils with harvest time and the late harvest having less than 20% of the initial value. Early and mid-harvest Memecik oils were higher in tyrosol content compared to the late harvest, with its value ranging between 2.49-18.0 mg/kg; almost twice that of Ayvalik (1.19-9.26 mg/kg). This finding implies that, Ayvalik and Memecik olives obtained between early and mid-harvest and processed at 47 ^{o}C malaxation temperatures are expected to contain reasonably high amount of tyrosol.

Pinoresinol is an important phenolic compound belonging to the group called lignans. According to Rigane, Ayadi, Boukhris, Sayadi, & Bouaziz (2013), lignans and its derivatives are naturally localized within olive seeds. Their presence in olive oil might be due to breaking of olive pits during crushing (Cecchi et al., 2013). Pinoresinol is the most abundant phenolic compound in the Memecik olive oil samples (Table 4.1). There was a significant decrease in the amount of pinoresinol in Memecik oils from early harvest to late, whereas no substantial changes were observed in the Ayvalik oils. Pinoresinol was found significant with respect to the main factors and their interactions, including malaxation temperature, as against the observation of Taticchi et al. (2013), who could not find a significant difference in pinoresinol of olive oil between 20 ^{o}C and 35 ^{o}C. Pinoresinol content of early harvest Memecik oils obtained at 47^{o}C were more than twice of those obtained at lower temperature.

Studies showed that olive drupe is rich in flavonoids and luteolin is one of the most widely distributed one. It exists in the fruit as luteolin and luteolin-7-glucosides derivatives. The amphiphilic nature of luteolin derivatives explains why it can be found in highly hydrophobic phase like olive oil (Bendini et al., 2007). Luteolin content was significantly affected by all factors and their interactions. The highest values of luteolin were observed at mid-harvest for Ayvalik and early harvest for Memecik oils. Over 100% increase in luteolin occurred between early to late harvest Memecik oils. Contrary to the report about the relative thermal stability of flavones and lignans (Boselli et al., 2009), Ayvalik oils obtained at 27 ^{o}C was significantly higher in luteolin compared to oils obtained at malaxation temperatures of 37 and 47 ^{o}C. In the same vein, Memecik oils obtained at 37 ^{o}C have more luteolin content in all the harvest seasons. The roles of phenolic acids in the overall quality desirability of olive oil are basically synergistic with other components. Summation of all individual phenolic acids given as total phenolic acids (TPA), was significantly influenced by variety,

harvest time and combined effect of both. Ayvalik oils had higher (1.86-19.13 mg/kg) TPA than Memecik oils (1.43-10.38 mg/kg).

4.2. Multivariate Analysis

The multivariate statistical models were built with the combination of quality parameters, phenolic compounds and fatty acids, to evaluate the differences/similarities among oils samples with respect to the significant factors. In order to observe any inherent homogenous or heterogeneous groupings or clusters of among the oil samples, a PCA model with 5 PCs, having 81% explained variance and R^2_{CV} of 0.53 was built. The model projection patterns of oils as observed in the score plot were traceable to harvest time and olive variety rather than malaxation temperature (Fig. 4.2A). The most recognizable pattern in this unsupervised model is that the early harvest Ayvalik oils are clearly different from mid and late harvest oils, clustering at lower right part of the control eclipse. These clusters of early harvest Ayvalik oils are mainly defined by their comparatively higher amount of phenolic acids and saturated fatty acids according to loading plot (Fig. 4.2B). There was no difference in the characteristic properties of Memecik and Ayvalik oil with regards to early harvest; however mid harvest Memecik oils are completely different from others. They clearly separated out and clustered at the upper right quarter in the score plot (Fig. 4.2A).

Ayvalik oils of early harvest have high amounts of simple phenols and phenolic acids such as vanillin, caffeic, ferulic, 3Hpa, Dba and 4Hpa. Olive oil oxidative stabilizing potency of these phenolic acids may not be as pronounced as that of oleuropein derivatives (hydroxytyrosol, tyrosol etc.) and secoiridoids compounds in olive oil, but they are also capable of forming protective action against oxidation (Servili, et al., 2013). Another contributive factor to early and mid-harvest Ayvalik oils is the low linolenic acid content. The right part of the loading plot was filled with all the important parameters such as oxidative stability index, highly potent phenolic compounds, pigments and degree of saturation. Interestingly, this part of the plot corresponds to early or mid-harvest of both olive varieties. The properties characterizing the later harvest oil samples as shown in the upper part of the control ellipse includes: fatty acids such as linoleic, stearic and arachidic acids and PV.

In order to improve separation among oils of different cultivar, a more definitive OPLS-DA model with 1+2 PCs, R^2 of 0.95 and R^2_{CV} of 0.86 was built. As can be seen in the score plot of the model, clusters of Ayvalik and Memecik oils samples occupied two separate parts of the control ellipse (Fig. 4.2C). In case of Memecik oils, the early and mid-harvest oils separated themselves in the lower right quarter of the ellipse with their typical properties such as higher contents of tyrosol, pinoresinol, p-coumaric acid, color pigments and different color characteristics. The oxidative stability of Memecik oils, regardless of their high free fatty acids, peroxide values and unsaturated fatty acids, may be due to the protective impact of phenols against lipid oxidation.

With respect to harvest time classification of the oil samples, OPLS-DA model of 82% explained variance with 2+2 PCs and R^2cv of 0.62 was built. As shown in the score plot (Fig. 4.2D), early harvest oils were distantly projected away from other oils, whereas mid and late harvest oils formed their individual close clusters. The differentiations of the oil samples in the two models were not influenced by temperature of malaxation (Fig. 4.2C and 4.2D). Additionally, high phenolic content, pigments concentration and stability (high OSI, low PV and FFA) favored oils obtained from early harvest of both varieties. Also, Memecik oils from mid-harvest olives showed similar characteristics regardless of malaxation temperature between 27 - 47 °C. The comments on the high malaxation temperature of 47 °C will be limited to this statement, since sensory analysis was not performed on the olive oils. It may be hard to deduce the possible influence of high temperature on overall acceptability of the samples.

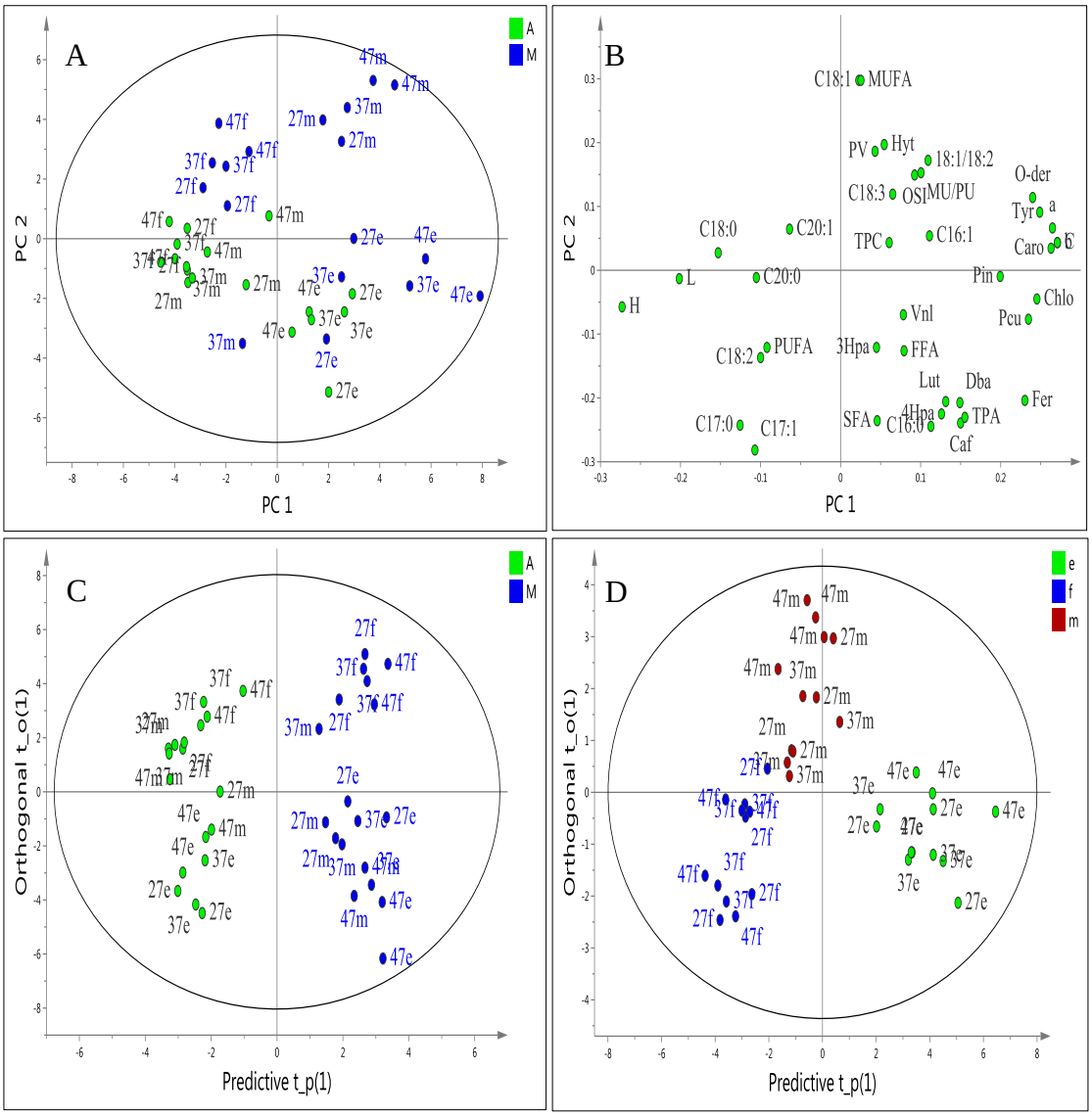

Figure 4.2. Results of multivariate models: Score plot of PCA models (A). Loading plot of PCA model (B); Score plot of OPLS-DA model of varietal (Ayvalik, Memecik & Erkence) classification (C) and Score plot of OPLS-DA model of harvest time (e: early, m: mid, f: late) classification (D)

OSI (Y) was modeled with a regression analysis (OPLS) in terms of chemical and quality variables (X) to evaluate the influence of these parameters on the oxidative stability of olive oil. Variable Influence on Projection (VIP) feature of the SIMCA software was used to identify the most influential variables describing OSI. VIP coefficients reflect the relative importance of each of X variable in the prediction model, thus represent the significance of each X variable in fitting both the X and Y-variates since Y variate (OSI) is predicted from X variables (chemical parameters). VIP allows classification of X variables according to their explanatory power of Y.

Therefore, predictors with large VIP than 1 are the most relevant for explaining Y variable. In this case, variables with VIP below 0.3 were excluded and the model rebuilt with the rest. According to VIP values of the model, chlorophyll content, pinoresinol, vanillin, 3Hpa, 4Hpa and C18:0 were not found significantly contributive in the modelling of OSI and thereby removed from the data set (VIP less than 0.3). The OPLS model with 1+2 PCs, R^2 of 0.76 and R^2_{CV} of 0.6 was rebuilt with the remaining variables. The loadings of predictive component of the model are given in Fig. 4.3 (Loadings bar plot and regression plot of OSI). With respect to their weights (contribution) in the regression model, total concentration of hydroxytyrosol and tyrosol (O-der), the ratio of oleic/linoleic acids and oleic acid have positive impacts on OSI of olive oils. The high concentrations of free fatty acid, peroxide value, linoleic acid, and total polyunsaturated fatty acids have adverse effects on the stability. It was also observed that high total phenol content did not necessarily cause stability, but rather the high concentrations of individual phenolic variables such as hydroxytyrosol and tyrosol, and carotenoids did. The significant effects of hydroxytyrosol and total hydroxytyrosol and tyrosol concentrations on the oxidative stability were reported by other researchers (Uncu & Ozen, 2015; Allalout et al., 2009).

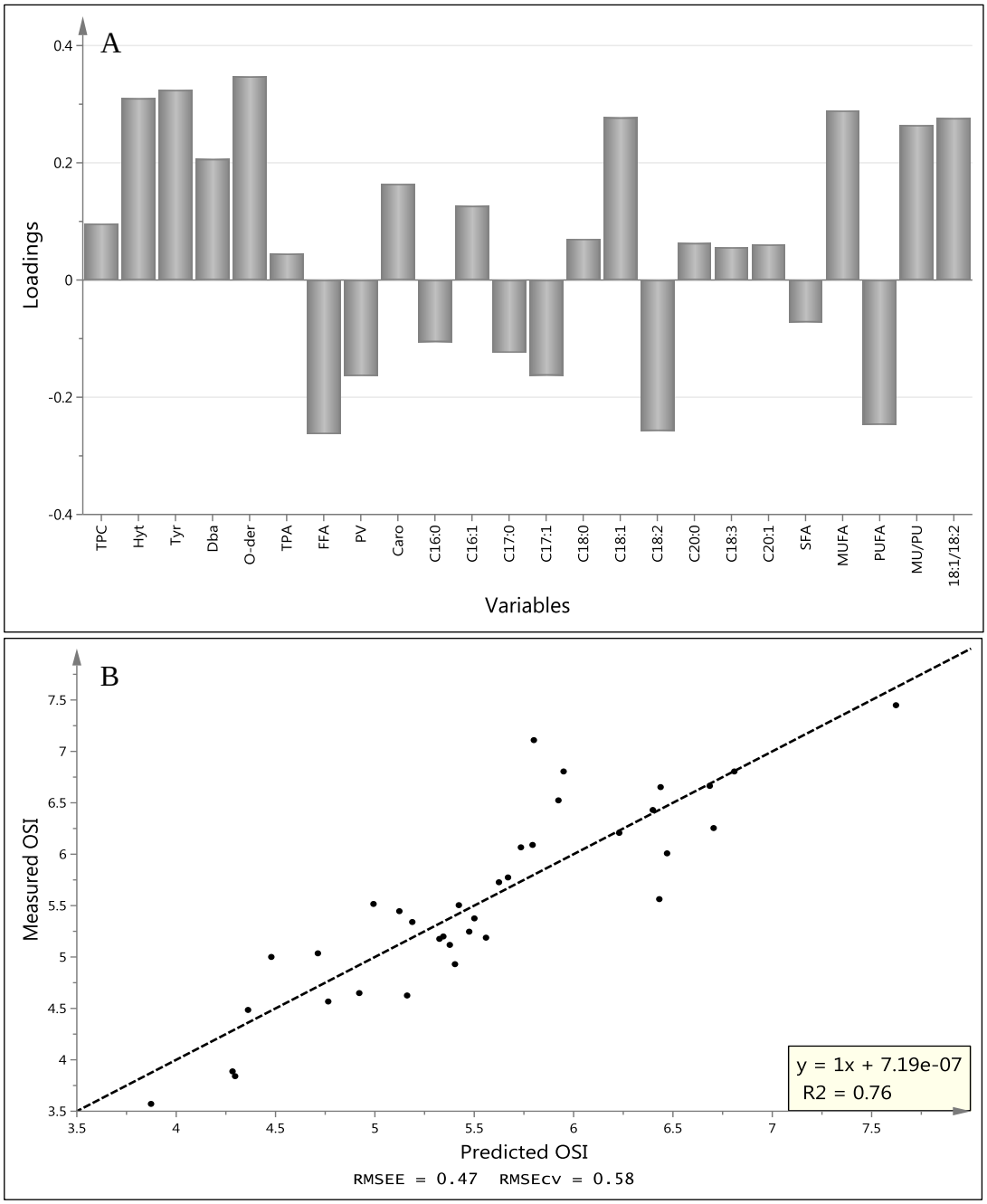

Figure 4.3.Prediction results of OSI with OPLS model. The loading weights of chemical variables of the regression model (A) and scatter plot of predicted to measured values (B).

4.3. Phenolic Contents of Olives

The concentration of phenolic compounds of olive fruits depends on many agronomical conditions such as cultivar and harvest seasons (Criado, et al., 2007). Total phenol content of Ayvalik olive variety ranges between 668.27-782.65 mg/100g with mid-harvest slightly higher than early and late harvest. On the other hand there

was a gradual increase in TPC of Memecik olive fruits with harvest time (804.95 to 895.05 mg/100g dry weight) (Table 4.4). There was no significant difference in TPC among different harvest times in both Ayvalik and Memecik olive fruits. The most important classes of phenolic compounds in olive fruit include phenolic acids, phenolic alcohols, flavonoids and secoiridoids (Lopez et al., 2014). Secoiridoids are the predominant phenolic compounds responsible for varying properties of olive fruits. Oleuropein, demethyoleuropein, ligstroside, and their hydrolytic derivatives, oleuropein aglycones, hydroxytyrosol and tyrosol are all derivatives of secoiridoids that are found at different stages of maturation in olive. (Ghanbari et al., 2012). Among the identified phenols in the olive fruit samples, oleuropein and hydroxytyrosol were the most abundant (Table 4.4). There was a gradual decline in oleuropein content of Ayvalik olive (3652.3 to 3001.48 mg/kg) compared to Memecik, in which the reverse was the case. Oleuropein is expected to decline with maturation as commonly reported in literature (Gomez-Rico, Fregapane & Salvador, 2008). The fate of oleuropein during olive fruit maturation has been correlated with considerable increase in tyrosol and hydroxytyrosol. There was no significant difference between hydroxytyrosol contents of the two olive varieties. However, early and mid-harvest samples are quantitatively high in hydroxytyrosol content compared to late harvest (Table 4.4). Tyrosol on the other hand is influenced by olive variety and harvest time interaction. Memecik olive possessed higher tyrosol content (77.7-237.3 mg/100g) than Ayvalik olive (18.6-68.3 mg/100g). The pattern of changes of these phenols with harvest time in Ayvalik olive (reduced from early to late) was relatively more consistent than Memecik (Fig. 4.4).

Table 4.4. Phenolic profiles (mean± SD) of Ayvalik and Memecik olive fruits at three harvest times

Phenolic compound	Ayvalik olive			Memecik olive		
	Early	Mid	Late	Early	Mid	Late
TPC	668.3±33.7	782.7±115	683.8±130	805.0±228	817.6±181	895.1±319
Hyt	436.6±97.9	303.3±274	44.46±40.0	416.7±348.7	420.9±193	399.4±128
Tyr	68.3±26.1	31.4±25.67	18.6±12.7	111.33±43.8	77.7±44.0	237.3±142.8
Ole	3409.5±2467	3377±1616	3001±1803	1431±1155	1814±1052	5235±4927
Gal	6.98±7.47	3.46±5.99	4.66±5.40	9.80±9.82	5.06±5.48	4.81±1.78
oCu	29.7±8.40	12.3±8.35	7.51±2.29	45.6±34.7	29.4±19.2	47.9±37.0
Vac	34.6±10.4	22.0±10.8	21.3±12.8	41.3±9.62	24.7±3.74	85.1±80.2
Vnl	37.3±7.87	12.2±7.86	6.53±3.03	36.7±23.5	26.1±11.8	32.1±20.32
pCu	47.6±57.8	15.14±8.5	10.7±6.58	39.8±34.3	29.9±25.7	29.23±31.8
Syr	16.7±17.3	7.21±5.58	6.65±2.84	47.9±27.7	17.9±12.6	60.7±32.5
Fer	113.7±72.1	44.3±15.9	44.4±39.6	129.2±113.5	93.2±33.8	302.9±95.3
Caf	14.3±8.20	17.5±13.6	10.3±7.62	45.4±44.4	28.6±24.1	71.8±80.8
Lut	52.6±6.67	48.95±25.3	30.45±26.5	70.2±45.7	261.6±303.5	26.86±11.5
Qer	30.1±13.8	32.3±10.3	25.2±12.8	72.8±8.0	103.7±108.9	64.6±39.6
Rut	597.7±216.6	278.7±163.1	258.9±184.9	353.2±46.8	525.9±357.5	883.1±534
O-der	505±123.9	334.8±299.8	63.0±52.7	528.0±343.1	498.7±195.1	636.7±18.4
TPA	184.2±58.9	121.8±59.1	105.6±74.8	359.0±183	228.8±72.1	602.4±196.4

SD: Standard deviation of two replicates, Hyt: Hydroxytyrosol, Tyr: Tyrosol, Ole: oleuropein, Gal: Gallic acid, oCu: o-Coumaric acid, Pin: Pinoresinol, Vac: Vanillic acid, Vnl; Vanillin, pCu: p-Coumaric acid, Fer: Ferulic acid, Syr: Syringic acid, Fer: Ferulic acid, Caf: Caffeic acid, Lut; Luteolin, Qer: Quercetin, Rut: Rutin, O-der: Oleuropein derivative (hydroxytyrosol+tyrosol), TPA: Total phenolic acids,

Only ferulic is significantly different with respect to olive variety and harvest time among all the phenolic acids. However, total phenolic acid (the sum of the phenolic acids) changed with olive type and olive-harvest time interaction. Memecik olive had greater amount of TPA (228.8-602.4 mg/100g) than Ayvalik (105.6-184.2 mg/100g). Interestingly, the reverse is the case in term of olive oil, where Ayvalik oils have higher TPA as shown previously. The reason is that, the process of extraction usually leads to over 60% loss of phenolic compounds and the fate of the remainder depends on many partition factors. Solubility of phenols between phase, olive type and many other factors affect the amount of phenols that eventually end up in the oil (Jerman & Mozetic, 2012). Quercetin, luteolin and rutin are the flavonoids quantified in the olive fruits. They usually occur as phenolic glucosides that are hydrolyzed enzymatically during olive extraction (Hachicha Hbaieb et al., 2015). The antioxidant efficiency of these flavonoid compounds especially quercetin has been equated to that of oleuropein

as a result of the ease at which it's H-atom is transferred (Achat, Rakotomanomana, Madani, & Danglas, 2016)

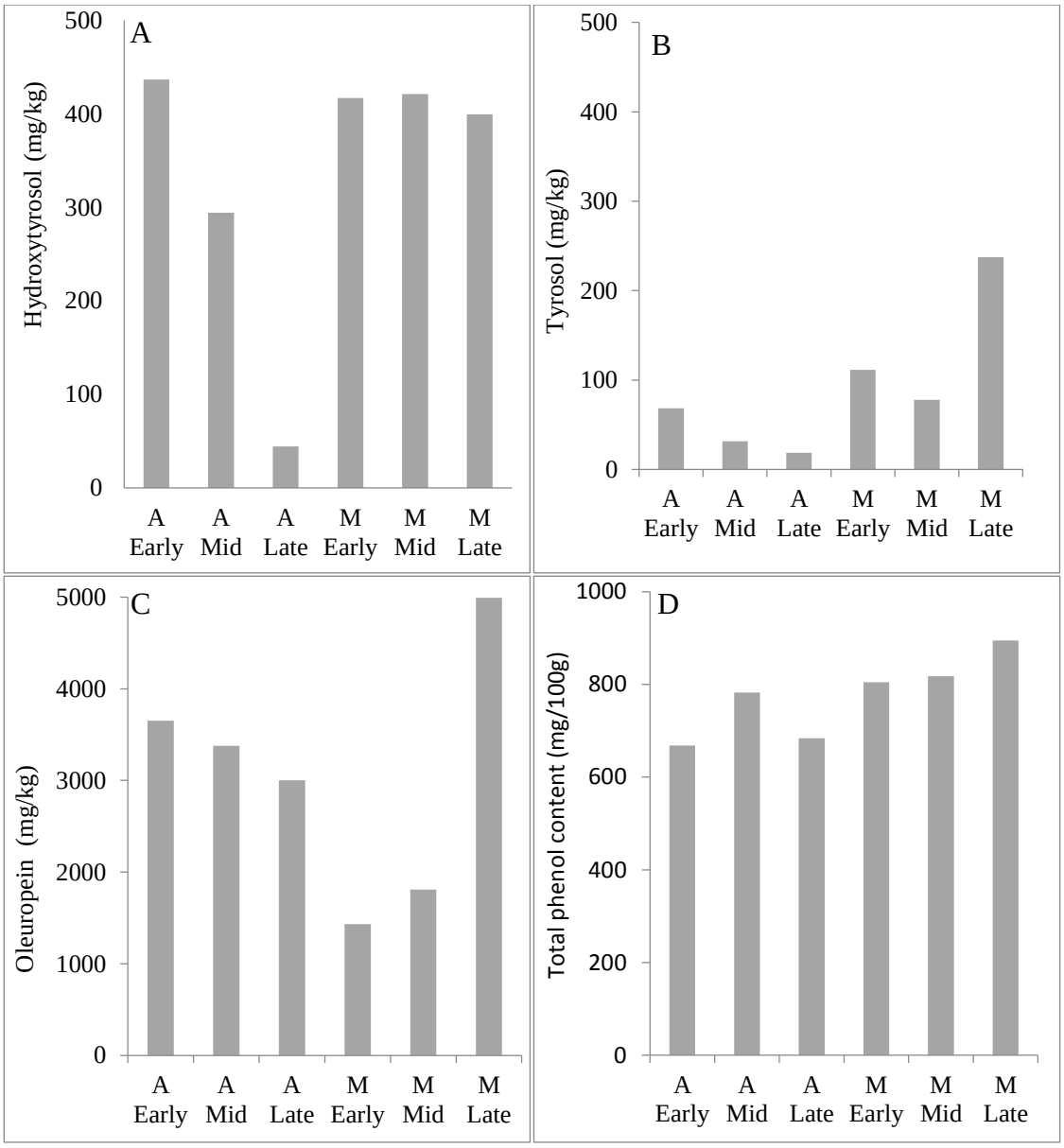

Figure 4.4 Changes in Hydroxytyrosol (A), Tyrosol (B), Oleuropein (C) and Total phenol content (D) of Ayvalik and Memecik olive fruits with respect to harvest time (Early, Mid and Late).

4.4. Multivariate Analysis

In order to visualize the clustering pattern of the olive fruits and variable (phenolic compounds) descriptors responsible for this pattern, PCA model was applied

to the data matrix. The matrix is made up of 18 observations from two olive varieties and 17 variables. These variables include 16 individual phenols and total phenol content (TPC). The PCA model explained about 63% of total variance, with relatively low coefficient of cross-validation (R^2cv of 0.25) using the first two principal components. However, the model is useful in projecting the intrinsic difference between the two olive varieties with respect to their phenolic distribution as shown in Fig. 4.5A and 4.5B. The first half of the score plot contained higher number of Ayvalik olive samples and the second half is predominantly made up of Memecik variety. The first half of the score plot is described by luteolin; which is close to the origin in the loading plot. This implies that either of Ayvalik or Memecik olives situated around ellipsoid center are responsible for the higher content of luteolin. On the other hand, the right side of the ellipse is explained by the majority of the phenols, indicating their significance in Memecik olives.

In order to be sure of this variables projection on observations, OPLS-DA discriminant model was built with the olive fruit phenolic profile. The model described 82 % of the total variance with 1+2 PCs and R^2_{CV} of 0.48 (Fig.4.5C). The control ellipse in the score plot is divided into two with each variety localized differently on the ellipse. The first half was occupied by Ayvalik olive fruits and Memecik the second half. However, the clustering pattern of Ayvalik olive samples was more compact and precise than Memecik. This shows that majority of Ayvalik olive phenols have little or no variation regardless of harvest time. Same cannot be said of Memecik, whose cluster was more dispersed (Fig. 4.5C). In the loading plot (Fig. 4.5D), it was obvious that virtually all the phenolic compounds only served as descriptors for Memecik olive fruit and none helped in defining Ayvalik. Luteolin and quercetin which are both flavonoid phenolic compounds, separated some mid and early harvest Memecik fruits, located at the upper right side of the ellipse. p-coumaric and gallic acid are located reasonably close to the centre of of ellipse describing Memecik fruit at the center of the ellipse. However, hydroxytyrosol, tyrosol, oleuropein, vanillin, rutin, TPC, TPC and some phenolic acids, separate the portion of Memecik fruits located at the right lower end of the score plot. In spite of the low number of observations, it can be shown that variations of phenolic compounds with respect to variety and maturation stage of olives are apparent in the model, which agreed with literatures (Sousa, Malheiro, Casal, Bento, & Pereira, 2014; Dagdelen, Tümen, Özcan, & Dündar, 2013).

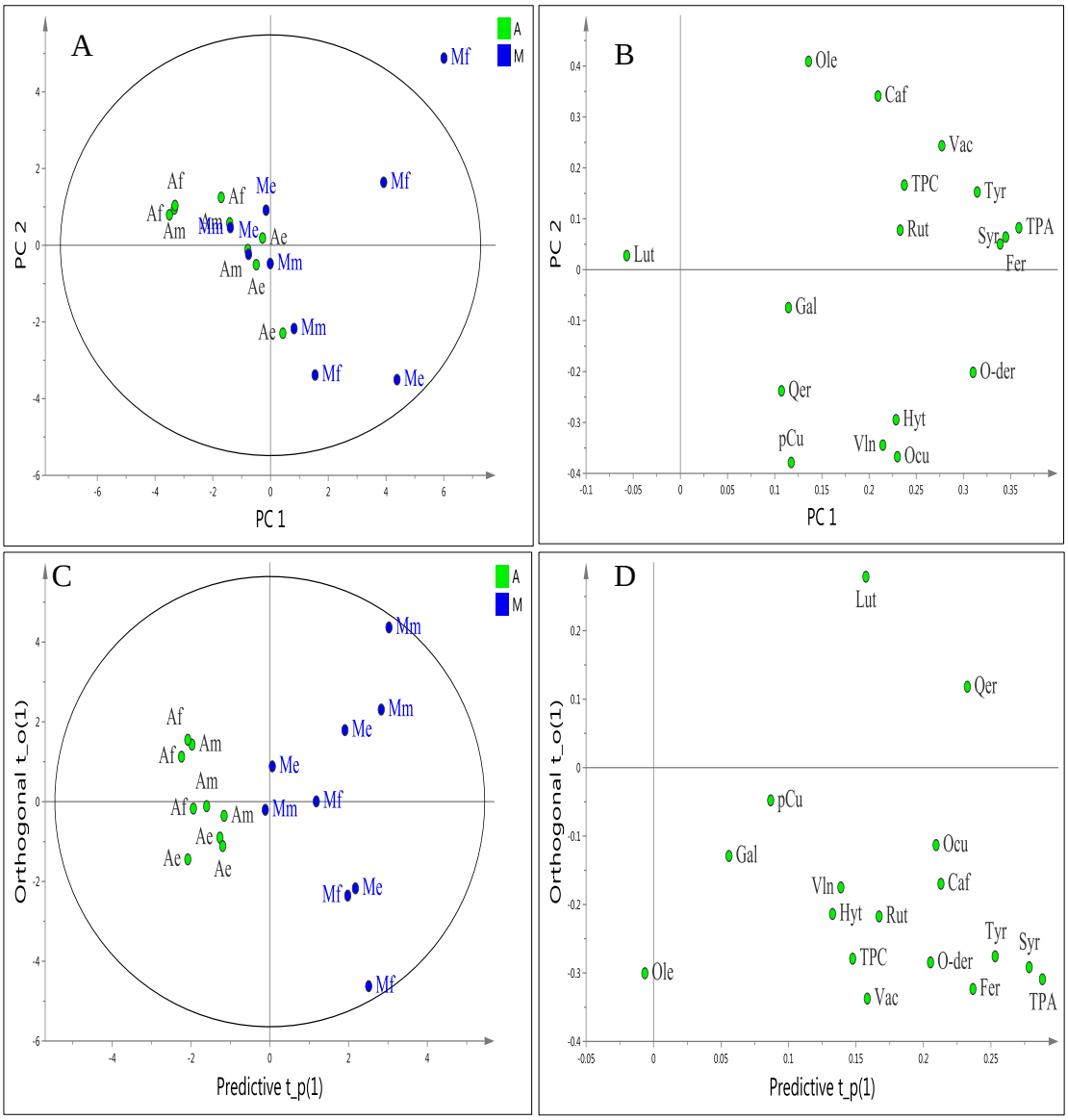

Figure 4.5. Results of PCA model Score plot (A), loading plot (B) and OPLS-DA model: Score plot (C) and Loading plot (D) of olive fruit varietal: Ayvalik and Memecik (e: early, m: mid, f: late hrvest) classification.

CHAPTER 5

EFFECTS OF STORAGE ON THE CHEMICAL CHARACTERISTICS OF OLIVE OIL

This chapter discusses the effects of 15 months of dark storage at room temperature, on the chemical characteristics of olive oils harvested in 2014/2015 harvest year. One-way ANOVA univariate analytical results of quality parameters, fatty acids, alkyl esters and phenolic profiles are presented in the tables. Means of the measured variables were compared for significant differences using Tukey's multiple comparison tests. Additionally, the extents of changes in the variables within the storage periods were presented as the percentages of increase or decrease. The results revealed the most susceptible olive oil properties to long term storage.

Principle Component Analysis (PCA) as a data-transformation tool, helped to disclose the inherent influences of storage on each group of chemical properties. Supervised Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) was applied to classify the samples with respect to variety/region and storage time. OPLS regression analysis of oxidative stability index (OSI) of the oils was performed to assess the contributions (negative or positive) of the predictors, such as fatty acids, alkyl esters, phenolic compounds and quality variables

5.1. Changes in Quality Parameters

Table 5.1 shows the initial and after-storage quality characteristics of the oil samples. Oils obtained from wholesome fruits under a well-controlled extraction condition are expected to have a minimum amount of free fatty acids (FFA) depending on the variety and harvest season (Clodoveo, et al., 2014). In terms of initial FFA contents, there were no significant differences among Ayvalik, Memecik and Manisa olive oils. These oil samples especially Ayvalik and Memecik maintained FFA quality index within the threshold standard acceptable limits, even after 15 months of storage. FFA is a good indicator of enzymatic hydrolysis of exposed oil triglycerides and oxidation of fatty acid double bonds at suitable environmental conditions such as light, oxygen and high temperature. Absence of neither of these catalyzing parameters in the

storage conditions, helped to keep FFA close to the initial value. This observation was equally shared by other authors (Kotsiou & Tasioula-Margari, 2016, Boselli et al., 2009). FFA values of oils from Erkence variety (2.91-3.31%) and oils from Uzunkuyu region (3.09-3.66%) exceeded the standard limit for extra and virgin olive oils in both cases.

The fragmented products of autoxidation of unsaturated fatty acids and hydroxyperoxide primary oxidative products are called conjugated dienes and trienes. Specific absorbances of olive oils at ultraviolet region (232 nm and 270 nm) are the analytical estimation of these dienes and trienes, which are the indicators of secondary phase oxidation (Terpinc, et al., 2012). K_{232} of Ayvalik, Erkence and Manisa oils are significantly different with respect to storage. The value of this parameter was within regulatory requirement among all the samples at their fresh stage, ranging between 2.29 -2.50. However, by the end of the storage time, all the samples have exceeded the upper limit for VOO (2.53-2.69) as shown in Table 5.1. This finding is supported by an earlier observation about K_{232} as storage monitoring parameter (Gomez-Alonso et al., 2007). Surprisingly, Memecik having the highest initial K_{232} value had the lowest % increase (6.9%) in K_{232} compared to 12% in Ayvalik oils (Table 5.2). According to K_{270} values, only Ayvalik and Manisa oils are within the acceptable limit (0.21 and 0.19 respectively). There was a significant difference in the K_{270} values of Erkence oils before (0.17) and after storage (0.28) which is equivalent to almost 65% increment. This somehow indicates the vulnerability of the oil to oxidative damage.

Oxidative stability index (OSI) result of Rancimat equipment is a non-standard means of estimating olive oil quality. The induction period determined above 100 ^{o}C may likely lead to over-prediction of oxidative stability for long term storage at room temperature (Mancebo-Campos, Salvador & Fregapane, 2007). This is because it does not generally correlate with the real-life storage condition of olive oil. However, the result is useful for a rapid assessment of initial characteristics of the oil samples and its response to maximum probable thermal treatment. Varietal difference was apparent in the OSI of the oil samples (Table 5.1). Oils of Ayvalik, Memecik, Manisa and Uzunkuyu were comparatively more stable than Erkence. Their OSI values are almost double that of Erkence before and after storage (Fig. 5.1).

Table 5.1 Changes in the quality parameters (mean±SD) of olive oil during 15 months at room temperature in dark

Quality parameters	Ayvalik	Erkence	Memecik	Manisa	Uzunkuyu
Fresh samples					
TPC	398.5±50.1^{ab}	336.68±26.2^{b}	436.4± 17.9^{a}	338.47± 63.4^{ab}	345.93± 84.3^{ab}
FFA	0.64±0.16^{b}	3.31±0.61^{a}	0.75± 0.08^{b}	0.90± 0.53^{b}	3.09± 1.65^{a}
OSI	5.87±0.26^{a}	3.04±0.48^{b}	7.15± 0.07^{a}	6.03± 1.68^{a}	5.28± 2.22^{a}
Chl	3.66±0.20^{ab}	2.28±0.67^{c}	5.18± 0.17^{a}	3.72± 1.35^{ab}	2.92± 1.01^{bc}
Car	2.12±0.40^{ab}	1.23±0.19^{c}	2.35±0.05^{a}	1.80±0.39^{ab}	1.61±0.48^{bc}
K_{232}	2.36±0.12^{ab}	2.47±0.04^{a}	2.50±0.06^{ab}	2.29±0.19^{b}	2.46±0.08^{ab}
K_{270}	0.14±0.03^{bc}	0.17±0.03^{bc}	0.22±0.04^{ab}	0.13±0.05^{c}	0.25±0.06^{a}
L*	89.11±2.37^{ab}	93.90±1.42^{a}	85.40±0.23^{b}	86.40±8.03^{b}	88.21±3.06^{ab}
a*	-1.57±0.92^{b}	-3.75±0.26^{c}	2.07±0.18^{a}	-0.47±3.17^{ab}	-1.48±1.25^{bc}
b*	83.53±7.58^{b}	56.76±6.21^{c}	108.61±1.24^{a}	73.15±2.28^{b}	78.35±16.8^{b}
C	83.55±7.57^{b}	56.89±6.19^{c}	108.62±1.25^{a}	73.20±2.24^{b}	78.37±16.8^{b}
H	91.12±0.70^{b}	93.84±0.61^{a}	88.90±0.08^{b}	90.32±2.55^{b}	91.08±0.94^{b}
Stored samples					
TPC	311.73±58.9^{a}	257.42±16.21^{a}	327.48±35.8^{a}	268.35±61.0^{a}	262.63±71.64^{a}
FFA	0.65±0.19^{b}	2.91±0.65^{a}	0.74±0.01^{b}	1.02±0.63^{b}	3.66±1.71^{a}
OSI	4.73±1.61^{a}	2.45±0.64^{b}	4.98±2.17^{a}	4.94±1.46^{a}	3.26±0.33^{ab}
Chl	2.98±0.64^{b}	1.29±0.29^{c}	4.67±0.06^{a}	2.28±0.33^{b}	2.40±0.39^{b}
Car	1.44±0.24^{b}	0.82±0.11^{c}	1.96±0.14^{a}	1.28±0.33^{b}	1.34±0.24^{b}
K_{232}	2.65±0.04^{a}	2.66±0.07^{a}	2.67±0.10^{a}	2.53±0.12^{a}	2.69±0.06^{a}
K_{270}	0.21±0.02^{ab}	0.28±0.05^{a}	0.30±0.06^{a}	0.19±0.03^{b}	0.29±0.05^{a}
L*	90.38±1.45^{ab}	94.73±0.94^{a}	86.35±0.74^{b}	87.91±7.05^{b}	89.87±0.60^{ab}
a*	-3.15±1.13^{b}	-5.40±0.17^{c}	-0.29±0.95^{a}	-1.84±2.77^{ab}	-3.76±0.36^{bc}
b*	77.10±10.4^{b}	50.37±4.94^{c}	97.05±4.65^{a}	68.66±2.47^{b}	69.22±15.51^{b}
C	77.18±10.4^{b}	50.66±4.91^{c}	98.05±2.92^{a}	68.73±2.46^{b}	69.32±15.49^{b}
H	92.50±1.15^{bc}	96.18±0.63^{a}	90.18±0.60^{c}	91.52±2.34^{bc}	93.21±0.67^{b}

SD: Standard deviation. TPC: total phenol content (mg/kg), FFA: Free fatty acid (% Oleic acid), OSI: Oxidative stability index (hr), Chl: Chlorophylls (mg/kg), Car: Carotenoids (mg/kg), K_{232} & K_{270}: Oil secondary oxidation UV-extinction values, CIELAB color parameters: L* (lightness-Darkness), a* (greenness-redness), b*(blueness – yellowness), C (Chroma), H (Hue angle^{o})
Note: Values that do not share one or more superscripts on the same row are significantly different (P<0.05)

Table 5.2 Percentage changes (increase/decrease) in quality parameters with storage

Parameters	Ayvalik	Memecik	Erkence	Manisa region	Uzunkuyu region
TPC	-22.16	-25.10	-23.40	-20.83	-24.45
FFA	1.53	-0.85	-9.93	10.54	21.14
OSI	-19.31	-30.26	-19.37	-18.43	-32.03
Chl	-18.73	-9.75	-40.69	-32.01	-14.15
Car	-31.35	-16.83	-32.45	-26.71	-14.99
K_{232}	12.70	6.90	7.54	11.32	9.21
K_{270}	58.34	39.65	64.99	64.62	18.73
L*	1.47	1.11	0.90	1.86	1.98
a*	127.12	-116.81	44.62	35.44	328.77
b*	-7.97	-10.66	-11.03	-6.11	-11.77
C	-7.90	-9.74	-10.72	-6.10	-11.65
H	1.51	1.45	2.49	1.34	2.35

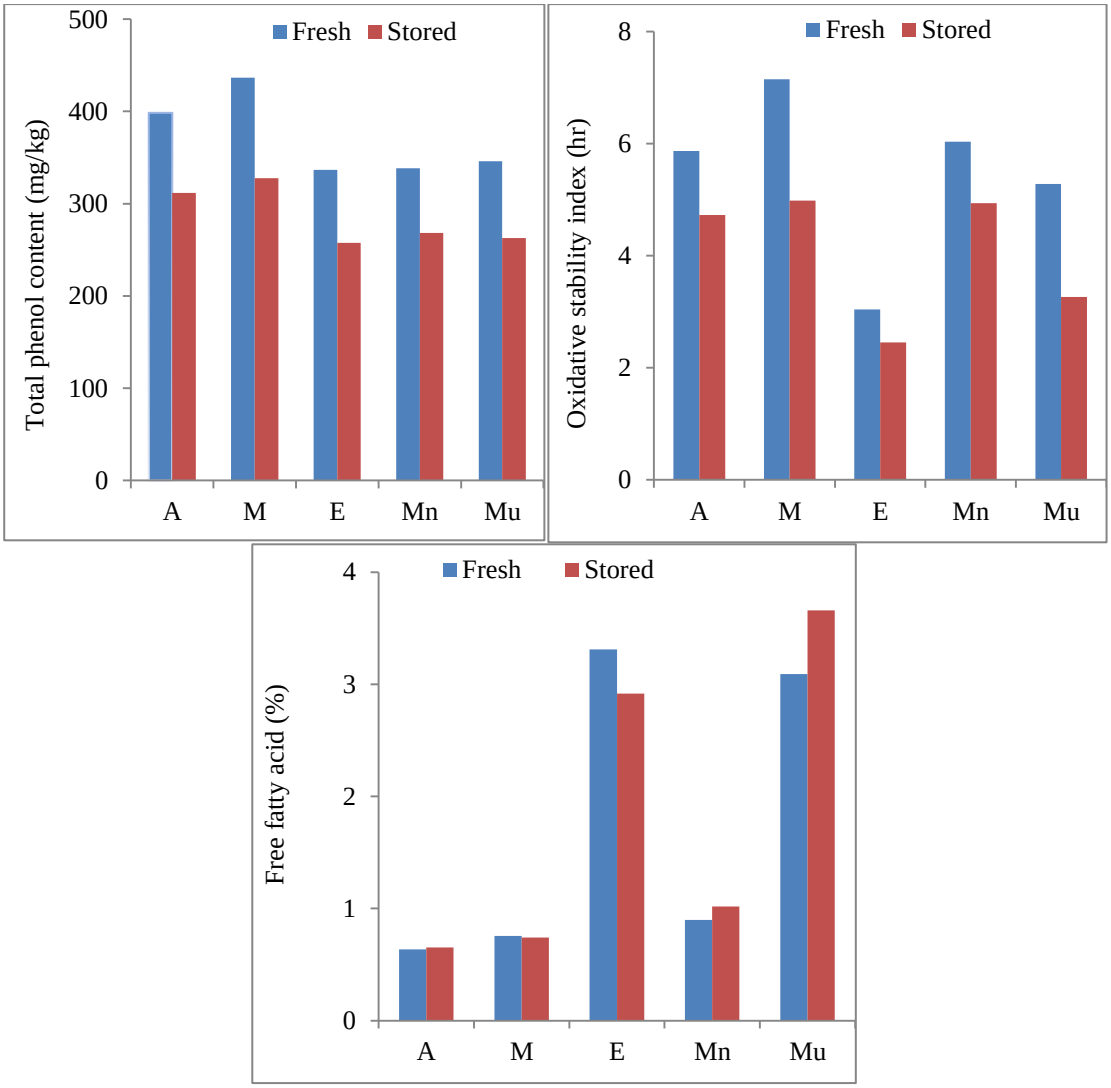

Figure 5.1 Changes in some quality parameters (TPC, OSI and FFA) of fresh and stored oils of different types (A: Ayvalik, M: Memecik, E: Erkence, Mn: Manisa region, Mu: Uzunkuyu region)

This implies that, there is a formation of secondary oxidative products (K_{270}) that need to be combated by phenolic compounds and other protective mechanism in the oils of Erkence. However, oils of Erkence, Manisa and Ayvalik experienced less than 20% decrease in their OSI values with storage time, compared to over 30% in Memecik and Uzunkuyu samples.

There was no significant difference in TPC values between oils of different varieties except Erkence. Quantitatively, Memecik and Ayvalik oils had the highest TPC ranged between 436.4 and 398.5 mg/kg respectively. TPC of the oil samples decreased almost uniformly across varieties (20.8-25.1%) with storage time. Kotsiou & Tasioula-Margari, (2016) and Gomez-Alonso et al., (2007) reported maximum of 31% and 43-73% decline in total phenolic compounds after 24 and 21 months storage, respectively. A much higher reduction was observed within a shorter period when oil was stored with some headspace, elsewhere (Okogeri & Tasioula-Margari, 2002). Therefore, absence of headspace and light and minimum temperature may have slowed down the rate of oxidation, thereby reducing the amount of phenols required for defensive mechanism against oxidation. Additionally, reduction in TPC was more prominent in the samples with high initial values (Memecik) as shown in (Table 5.2).

There was no significant difference in the chlorophyll contents of Memecik, Manisa and Ayvalik oils. Memecik had the highest chlorophyll and carotenoid content among the varieties. The values of these pigments were significantly low in Erkence oil before and after storage. This further agrees with the reducing trends of other quality parameters in Erkence olive oil compared to others. Storage caused substantial reductions in these pigments in all the varieties. However, Erkence oil experienced the highest percentage reduction (41 and 32%) in chlorophyll and carotenoids respectively (Table 5.2). This minimum amount of decline in the pigments content with storage was attributed to the absence of light in the storage condition. This could have used up chlorophyll as a photosensitizer in the prevention of oil photo-oxidation (Fadda et al., 2012, Psomiadou & Tsimidou, 2002). In Erkence and Manisa oils, percent losses of carotenoids were less than that of chlorophyll, while other samples had greater reduction. This is partly supported by Morelló, Motilva, Tovar, & Romero, (2004) who observed lower rate of loss in carotenoids compared to chlorophyll in 12 months stored olive oil. However, the reductions in both pigments were more pronounced in Erkence oil samples (Fig. 5.2).

The changes in chromatic ordinate L* and b* values with olive variety and storage time are presented in Table 5.2. Chromatic a* ordinate (greenness – redness) values were not presented because the previous knowledge of the samples' colors are best defined by b* and L* ordinates. Erkence oils luminosity (L*) is significantly higher (lightness) than others. This explains the low pigments content and higher rate of pigment loss in Erkence oils. Storage time had no significant effects on L* values among the varieties, and the slight decline in L* can be due to the degradation of oil chromophores as stated in literature (Sikorska et al., 2007). A slight reduction in the yellowness (reduced b* ordinate) of the oil after the storage was observed (Fig. 5.2).

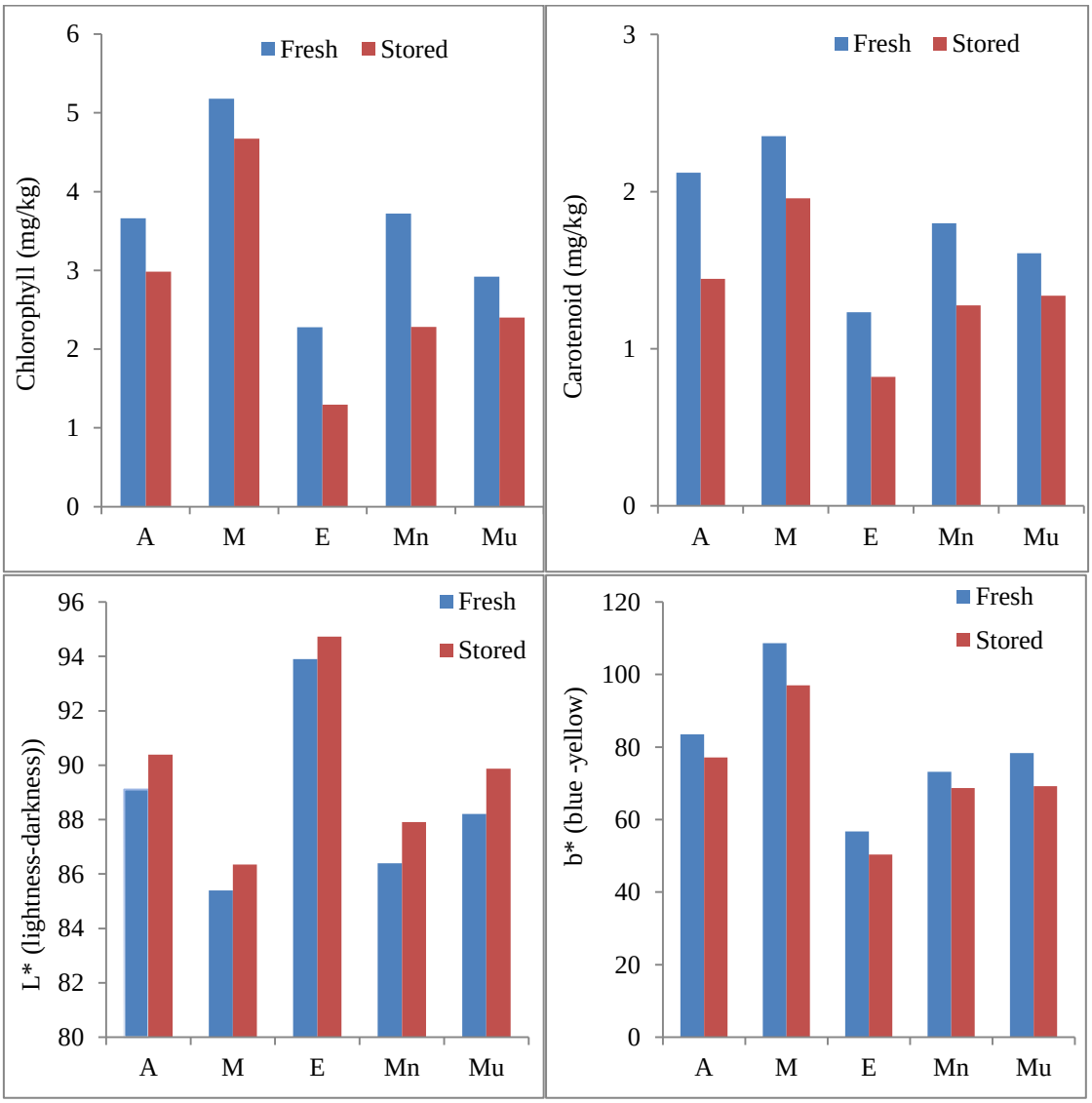

Figure 5.2. Changes in pigments and color chromatics (L* and b*) of fresh and stored oils of different olive types (A: Ayvalik, M: Memecik, E: Erkence, Mn: Manisa region, Mu: Uzunkuyu region)

Oils of Erkence variety and Uzunkuyu region experienced greater percentage losses in b* values at 11.03 and 11.77% and appeared more transparent than others. A similar finding about yellow zone chromatic b* is also reported by others (Romero, Crado, Tovar, & Motilva, 2002).

5.2. Changes in Fatty Acids Profile

There were no significant changes in oleic acid of the oil samples after 15 months of storage. This further explained the stability of the oils with respect to FFA over the storage period – as it's measured as % oleic acid. On the other hand, Erkence oils have the lowest oleic acid content. As a contributive factor to stability, low content of oleic acid in Erkence oils is part of it low induction value (OSI). However, linoleic and linolenic acids content of Erkence oils were substantially higher than oils of other varieties (Table 5.3 and Fig. 5.3). The health importance of these polyunsaturated fatty acids is well known, but they also increase the vulnerability of oils to oxidative rancidity and lipoxygenase hydroperoxide generation (Tomborrino et al., 2014). Linoleic acid has a negative correlation with OSI. It has been well proven that the ease of formation of fatty acid radicals increases with increasing unsaturation (Manai-Djebali et al., 2012). Oleic acid (C18:1) is said to be 10 to 40 times less susceptible to oxidation than linoleic acid, whereas linolenic acid oxidizes twice as fast as linoleic acid because of the decrease in bond dissociation energy associated with the addition of methylene-interrupted carbon (McClements & Decker, 2008). Therefore, Erkence oils are expected to have a shorter shelf life compared to other because of higher PUFA, lower MUFA, and lower MUFA/PUFA ratios (Fig 5.3). These shortcomings become evidenced in the comparative low OSI and high FFA characteristics of Erkence oils. In addition to that, Memecik oils showed the highest loss in PUFA (5.3%) and Erkence oils had the lowest fall (0.49%) after the storage (Table 5.4). This may be an advantage at the long run, because the rate of generation of unsaturated fatty acid in Erkence is lower than other varieties. Stability of PUFA in olive oil has been reported to remain constant over a long storage period until the oil antioxidant contents decline (Rastrelli et al., 2002).

Table 5.3. Changes in the fatty acid profiles (mean±SD) of olive oil during 15 months at room temperature in dark

Fatty acid profile (%)	Ayvalik	Erkence	Memecik	Manisa	Uzunkuyu
Fresh samples					
C16:0	13.49±1.13^{a}	12.10±0.31^{b}	11.19±0.60^{b}	13.44±0.49^{a}	13.27±0.36^{a}
C16:1	0.16±0.02^{ab}	0.16±0.02^{ab}	0.19± 0.00^{a}	0.14±0.01^{b}	0.16±0.01^{ab}
C17:0	0.15±0.02^{ab}	0.14±0.01^{b}	0.06±0.00^{c}	0.17±0.02^{a}	0.15±0.01^{ab}
C17:1	0.24±0.02^{a}	0.21±0.01^{b}	0.09±0.00^{c}	0.25±0.01^{a}	0.24±0.03^{a}
C18:0	2.70±0.22^{bc}	2.64±0.07^{c}	2.74±0.04b^{c}	3.35±0.30^{a}	3.00±0.03^{ab}
C18:1	69.10±0.85^{b}	66.89±1.08^{b}	73.01±0.47^{a}	68.54±3.30^{b}	68.72±1.18^{b}
C18:2	11.78±0.751^{b}	15.88±0.933^{a}	10.01±0.15^{b}	11.57±3.33^{b}	12.01±1.22^{b}
C18:3	0.68±0.01^{b}	0.76±0.073^{ab}	0.82±0.01^{a}	0.72±0.08^{ab}	0.73±0.03^{ab}
C20:0	0.46±0.03^{a}	0.41±0.01^{b}	0.47±0.01^{a}	0.48±0.05^{a}	0.44±0.02^{ab}
C20:1	0.33±0.01^{a}	0.32±0.01^{a}	0.34±0.00^{a}	0.27±0.01^{b}	0.29±0.01^{b}
C22:0	0.19±0.11^{a}	0.12±0.01^{a}	0.14±0.00^{a}	0.12±0.01^{a}	0.11±0.01^{a}
SFA	17.71±1.11^{a}	15.77±0.25^{b}	15.53±0.64^{b}	18.50±0.37^{a}	17.86±0.31^{a}
MUFA	69.83±0.89^{b}	67.58±1.07^{b}	73.63±0.48^{a}	69.21±3.30^{b}	69.40±1.17^{b}
PUFA	12.46±0.75^{b}	16.65±0.99^{a}	10.84±0.16^{b}	12.29±3.41^{b}	12.74±1.25^{b}
C18:1/C18:2	5.88±0.37^{a}	4.23±0.32^{b}	7.29±0.06^{a}	6.39±2.15^{a}	5.77±0.71^{ab}
MUFA/PUFA	5.62±0.34^{a}	4.08±0.31^{b}	6.80±0.06^{a}	6.04±1.97^{a}	5.49±0.66^{ab}
Stored samples					
C16:0	13.12±0.81^{a}	11.57±0.31^{b}	12.57±0.07^{a}	13.22±0.60^{a}	13.12±0.33^{a}
C16:1	0.14±0.02^{b}	0.16±0.01^{b}	0.19±0.03^{a}	0.15±0.01^{b}	0.15±0.00^{b}
C17:0	0.15±0.02^{ab}	0.15±0.01^{b}	0.06±0.00^{c}	0.17±0.02^{a}	0.16±0.01^{ab}
C17:1	0.24±0.01^{a}	0.21±0.01^{b}	0.09±0.00^{c}	0.26±0.02^{a}	0.24±0.01^{ab}
C18:0	2.79±0.22^{bc}	2.70±0.07^{c}	2.86±0.01b^{c}	3.48±0.36^{a}	3.10±0.01^{ab}
C18:1	70.13±0.63^{ab}	67.66±1.06^{b}	72.87±0.13^{a}	69.48±3.58^{ab}	69.76±1.42^{ab}
C18:2	11.75±0.72^{b}	15.85±0.94^{a}	9.57±0.18^{b}	11.56±3.25^{b}	11.82±1.09^{b}
C18:3	0.69±0.06^{a}	0.77±0.08^{a}	0.78±0.04^{a}	0.73±0.07^{a}	0.71±0.03^{a}
C20:0	0.49±0.03^{ab}	0.44±0.02^{b}	0.51±0.00^{ab}	0.52±0.08^{a}	0.49±0.02^{ab}
C20:1	0.33±0.01^{a}	0.34±0.01^{a}	0.34±0.01^{a}	0.29±0.01^{b}	0.30±0.01^{b}
C22:0	0.15±0.02^{a}	0.14±0.01^{a}	0.15±0.01^{a}	0.15±0.02^{a}	0.14±0.01^{a}
SFA	16.71±0.70^{b}	15.00±0.27^{c}	16.14±0.06^{b}	17.54±0.50^{a}	17.02±0.33^{ab}
MUFA	70.85±0.66^{ab}	68.37±1.04^{b}	73.50±0.16^{a}	70.17±3.60^{ab}	70.45±1.42^{ab}
PUFA	12.44±0.77^{b}	16.63±0.98^{a}	10.36±0.21^{b}	12.29±3.32^{b}	12.53±1.12^{b}
C18:1/C18:2	5.98±0.38^{a}	4.28±0.32^{b}	7.61±0.16^{a}	6.46±2.15^{a}	5.95±0.70^{ab}
MUFA/PUFA	5.71±0.36^{a}	4.13±0.30^{b}	7.10±0.16^{a}	6.11±1.96^{a}	5.66±0.65^{a}

SD: Standard deviation. C16:0: Palmitic acid, C16:1: Palmitoleic acid, C17:0: Heptadecanoic acid, C17:1: Cis-10-Heptadecanoic acid, C18:0: Stearic acid, C18:1: Oleic acid, C18:2: Linoleic acid, C18:3: Linolenic acid, C20:0: Arachidic acid, C20:1: Cis-11-Eicosenoic acid, SFA: Saturated fatty acids, MUFA: Monounsaturated fatty acids, PUFA: Polyunsaturated fatty acids, C18:1/C18:2: Oleic:Linoleic acids ratio Note: Values that do not share one or more superscripts on the same row are significantly different ($P<0.05$)

Table 5.4. Changes (increase/decrease) in fatty acid profiles parameters with storage

Fatty acid	Ayvalik	Memecik	Erkence	Manisa region	Uzunkuyu region
C16:0	-3.32	11.47	-4.74	-2.64	-2.00
C16:1	-9.21	1.68	-0.90	0.38	-6.72
C17:0	-1.07	-7.69	5.14	3.60	7.42
C17:1	-2.19	2.57	-0.19	0.43	-1.11
C18:0	2.42	3.36	2.12	2.79	2.45
C18:1	0.78	-1.11	0.79	0.41	0.62
C18:2	-0.91	-5.27	-0.56	-0.89	-2.37
C20:0	5.98	6.80	8.60	8.18	11.64
C18:3	0.81	-5.74	1.09	0.54	-3.43
C20:1	1.97	-1.29	5.85	5.63	5.44
C22:0	-5.03	6.40	12.90	17.92	22.38
SFA	-2.34	9.64	-2.99	-1.08	-0.63
MUFA	0.75	-1.10	0.80	0.43	0.61
PUFA	-0.82	-5.30	-0.49	-0.80	-2.44
C18:1/C18:2	1.72	4.39	1.36	1.31	3.07
MUFA/PUFA	1.61	4.45	1.31	1.24	3.13

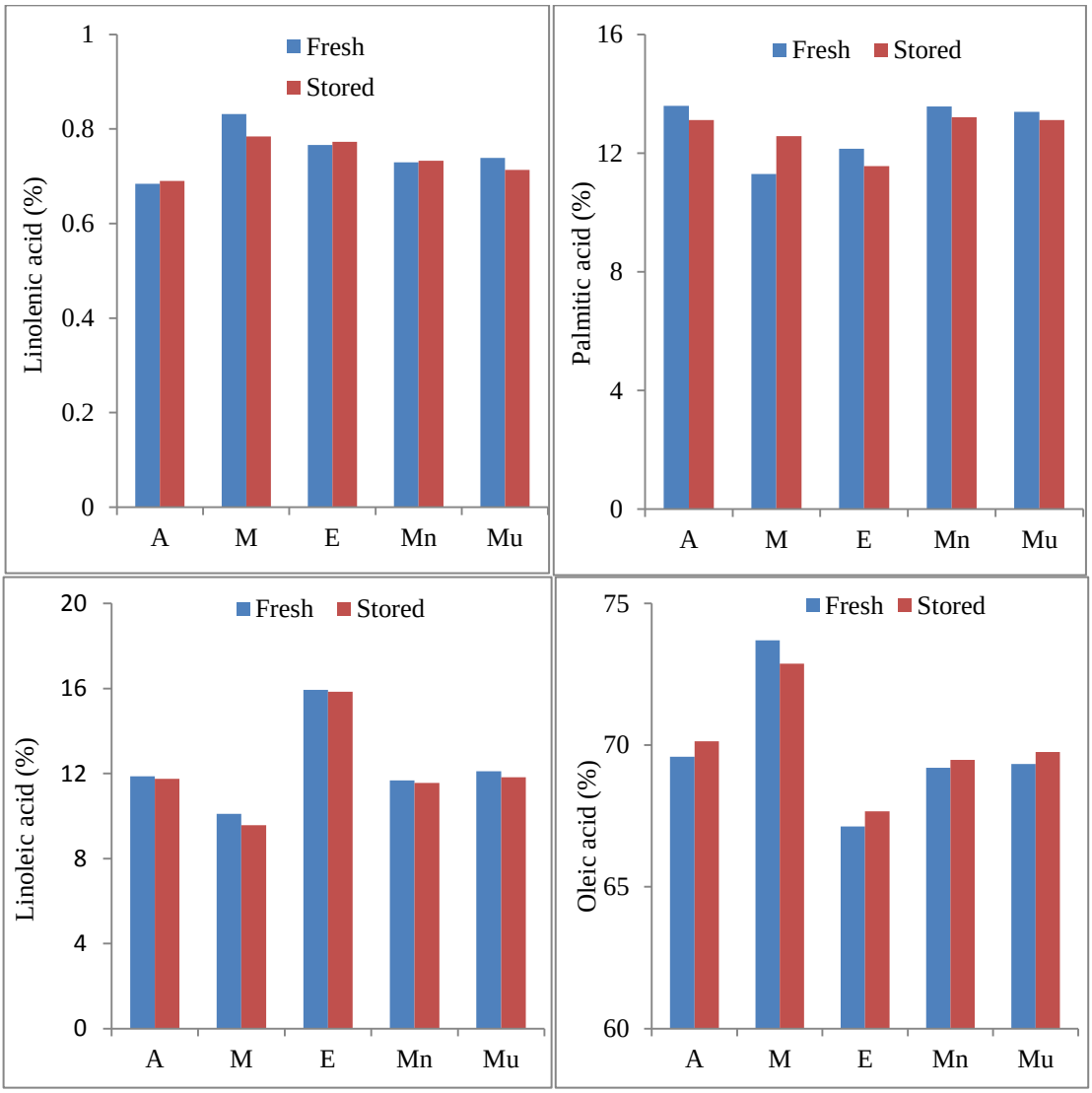

Figure 5.3. Changes in some fatty acids (linolenic, palmitic, linoleic and oleic) of fresh and stored oils of different olive types (A: Ayvalik, M: Memecik, E: Erkence, Mn: Manisa region, Mu: Uzunkuyu)

The oils of Ayvalik and Manisa were significantly more saturated than others. The rate of oxidation in SFA-rich olive oils will be lesser compared to PUFA-rich oil. Therefore, oils of Erkence variety having high amounts of PUFA (16-36-16-65%) may fall short of stability. Generally, it can be seen that the variation in fatty acid compositions of the oil samples are primarily a function of varietal or other possible agronomical differences among the oil samples. There was no significant difference between the initial values of any of the fatty acids after 15 months of storage. These results imply that fatty acids profile of olive oil is not a suitable tool for monitoring storage effects. They are fundamentally dependent on agronomic-based parameters such as variety/cultivar, climate, geographical differences, genetics and many more. These views were equally shared by other authors (Roboredo-Rodriguez et al., 2016; Mateos, et al., 2005).

5.3. Changes in Phenolic Profile

Hydroxytyrosol and tyrosol constitute the two most important phenolic alcohols derivatives of secoiridoids (3,4-DHPEA hydroxytyrosol and *p*-HPEA tyrosol), endowed with numerous health benefits to human (EFSA, 2011). The distribution of these essential phenols varies with olive varieties and storage duration as shown in Table 5.5. Although, there was no statistical difference among the samples with respect to the initial values of hydroxytyrosol, Ayvalik and Memecik oils had higher values (2.68 and 2.00 mg/kg respectively), while Erkence, Uzunkuyu and Manisa oils are lower in that order. The effectiveness of hydroxytyrosol as an active antioxidant polar phenolic compound, capable of scavenging free radicals and inhibiting peroxidation of lipid, has been affirmed in literature (Servili et al., 2013). This probably explained the comparative higher oxidative stabilities (OSI) of Ayvalik and Memecik olive oils. The same inferences can be made concerning other oil stability parameters such as FFA, K-values and unsaturated fatty acids. Erkence oils had the highest initial tyrosol and pinoresinol contents (5.37 and 11.56 mg/kg respectively) and its flavonoids (luteolin and apigenin) are relatively the same with Uzunkuyu samples. In spite of the considerable quantities of phenolic compounds in these oils (Erkence and Uzunkuyu), they have comparatively less desirable general quality characteristics.

Hydroxytyrosol and tyrosol concentrations experienced a significant increase in practically all varieties after 15 months of storage (Fig. 5.4). This observation has been

attributed to the non-oxidative enzyme-mediated hydrolysis of secoiridoid derivatives, which are the polymer of these two important phenolic alcohols (Brenes, García, García, & Garrido, 2001). The rate of increase of tyrosol is very similar to hydroxytyrosol and the amounts of both compounds after storage were proportionate with the initial contents in all varieties. A similar upward trend in the concentration of secoiridoid derivatives during storage was observed by others (Gómez-Alonso, Mancebo-Campos, Salvador, & Fregapane, 2007).

There was a significant increase in pinoresinol contents of Ayvalik, Memecik and Manisa oil samples. Erkence and Uzunkuyu oil did not increase substantially in pinoresinol after the storage. Literatures contain different findings on the impacts of storage on pinoresinol and its derivatives content of olive oil. Gambacorta et al., (2010) observed a slight increase after 6 months of storage and a decline afterward. In another study it was, found out that pinoresinol concentration did not change after one year of storage in the dark. However, the most plausible explanation on the pinoresinol is about its usual high concentration in olives as reported earlier (Bendini et al., 2007; Jolayemi, Tokatli & Ozen, 2016).

The differences in the concentrations of apigenin and luteolin during storage are given in Table 5.6. There was a significant decline in the content of luteolin with each variety having less than 50% initial luteolin concentration by the end of storage time. The most influenced samples were the oil obtained from Uzunkuyu and Erkence losing over 80% of their initial concentrations. The reverse was true for apigenin, which appeared to be relatively stable among the varieties. This is partly supported by a study in literature (Kotsiou & Tasioula-Margari, 2016), which found flavones and lignans significantly unchanged after 24 months of olive oil storage.

Table 5.5. Changes in the phenolic profiles (mean±SD) of olive oil during 15 months at room temperature in dark

Phenolic profile (mg/kg)	Ayvalik	Erkence	Memecik	Manisa	Uzunkuyu
Fresh samples					
Hyt	2.68± 0.43^{a}	1.56±0.74^{a}	2.00±0.33^{a}	0.95±0.27^{a}	1.70±1.57^{a}
Tyr	2.75 ± 1.74^{ab}	5.37±2.05a	3.12±0.50^{ab}	1.79±2.02^{b}	2.56±1.15^{ab}
4Hpa	0.98±0.84^{a}	0.50± 0.15a	0.21±0.06^{a}	0.24±0.21^{a}	0.24± 0.14^{a}
3Hpa	0.59±0.60^{a}	0.58±0.17a	0.21±0.11^{a}	0.27±0.31^{a}	0.22±0.11^{a}
Caf	1.20±0.77^{a}	0.60±0.15^{ab}	0.47±0.20^{ab}	0.30±0.28^{b}	0.60±0.46^{ab}
Pin	9.24± 5.54^{a}	11.56±2.76^{a}	8.28±1.28^{a}	6.23±2.94^{a}	6.97±2.70^{a}
Dba	0.37±0.35^{a}	0.10±0.09^{b}	0.23±0.08^{ab}	0.05±0.03^{ab}	0.19± 0.09^{ab}
Vnl	0.37±0.33^{a}	0.21±0.09^{a}	0.23± 0.03^{a}	0.08±0.08^{a}	0.15±0.11^{a}
Vac	0.12±0.13^{a}	0.15±0.08^{a}	0.05±0.02^{a}	0.04±0.05^{a}	0.08±0.05^{a}
pCu	1.23±0.93^{a}	0.84±0.42^{a}	1.25±0.18^{a}	0.38±0.31^{a}	0.79±0.69^{a}
Fer	0.06±0.03^{a}	0.03±0.01^{a}	0.05±0.01^{a}	0.06±0.08^{a}	0.06±0.05^{a}
Cin	0.01± 0.00^{b}	0.03±0.01^{a}	0.00±0.00^{b}	0.01±0.01^{b}	0.01±0.01^{ab}
Lut	9.76±4.08^{a}	4.23±1.43^{b}	6.37±1.02^{ab}	4.24±1.62^{b}	5.75±1.97^{ab}
Apg	3.38±1.14^{a}	3.02±0.64^{a}	2.80±0.42^{a}	2.47±0.27^{a}	3.28±1.05^{a}
O-der	5.43±3.01^{a}	6.94±2.76^{a}	5.12±0.79^{a}	2.74±2.21^{a}	4.26±2.64^{a}
TPA	4.56±3.29^{a}	2.84±0.81^{a}	2.47±0.64^{a}	1.35±1.26^{a}	2.19±1.57^{a}
Stored samples					
Hyt	25.25±7.00^{a}	12.70±4.11^{b}	20.74±8.04^{ab}	14.87±10.7^{ab}	13.20±13.84^{ab}
Tyr	25.76±11.48^{a}	24.44±3.65^{a}	25.00±11.25^{a}	12.92±11.37^{a}	11.80±8.00^{a}
4Hpa	0.48±0.23^{a}	0.49±0.26^{a}	0.49±0.34^{a}	1.06±1.40^{a}	0.50±0.53^{a}
3Hpa	2.23±1.76^{a}	1.72±0.95^{a}	0.97±0.58^{a}	0.58±0.43^{a}	0.62±0.34^{a}
Caf	0.86±0.28^{a}	0.36±0.17^{b}	0.27±0.02^{b}	0.32±0.39^{b}	0.23±0.28^{b}
Pin	23.47±2.97^{a}	21.85±6.01^{a}	25.87±17.83^{a}	17.23±14.64^{a}	8.01±4.34^{a}
Dba	0.61±0.33^{a}	0.19±0.06^{b}	0.23±0.17^{ab}	0.21±0.24^{b}	0.10±0.08^{b}
Vnl	0.99±0.56^{a}	0.36±0.21^{b}	0.57±0.52^{ab}	0.21±0.17^{b}	0.19±0.14^{b}
Vac	1.25±0.81^{a}	0.26±0.15^{b}	0.28±0.12^{b}	0.38±0.45^{b}	0.22±0.19^{b}
pCu	1.92±0.53^{a}	0.58±0.29^{b}	1.54±0.80^{a}	0.29±0.17^{b}	0.42±0.28^{b}
Fer	0.13±0.05^{a}	0.15±0.05^{a}	0.22±0.16^{a}	0.11±0.13^{a}	0.10±0.09^{a}
Cin	1.14±0.81^{a}	0.36±0.17^{c}	1.16±0.59^{abc}	0.57±0.46^{abc}	0.11±0.03^{bc}
Lut	4.24±2.03^{a}	0.79±0.32^{b}	2.71±1.38^{ab}	1.62±1.35b	0.50±0.15^{b}
Apg	4.99±1.49^{a}	2.58±1.23^{a}	3.72±2.52^{a}	3.93±2.89^{a}	1.47±1.12^{a}
O-der	51.01±14.4^{a}	37.15±6.35^{ab}	45.74±19.20^{a}	27.79±21.99^{b}	24.99±21.64^{b}
TPA	8.63±2.84^{a}	4.11±1.611^{b}	5.16±1.49^{ab}	3.52±3.59^{b}	2.29±1.35^{b}

SD: Standard deviation of two replicates, Hyt: Hydroxytyrosol, Tyr: Tyrosol, 4Hpa: 4-hydroxyphenyl acetic acid, 3Hpa: 3-hydroxyphenyl acetic acid, Pin: Pinoresinol, Dba: 2,3dihydroxylbenzoic acid, Vnl; vanillin, Vac: Vanilic acid, pCu: pCoumaric acid, Fer: Ferulic acid, Cin: Cinnamic acid, Lut; Luteolin, O-der: Oleuropein derivative, TPA: Total phenolic acids, Note: Values that do not share one or more superscripts on the same row are significantly different ($P<0.05$)

Table 5.6. Changes (increase/decrease) in phenolic compounds with storage

Phenols	Ayvalik	Memecik	Erkence	Manisa	Uzunkuyu
Hyt	842.50	938.00	711.48	1459.88	676.81
Tyr	835.85	699.97	354.75	623.65	360.49
O-der	839.13	792.79	435.21	914.67	486.64
Pin	154.07	212.57	88.99	176.45	14.84
Apg	47.80	32.83	-14.69	59.10	-55.15
Lut	-56.56	-57.48	-81.32	-61.82	-91.33
TPA	89.25	49.31	44.49	159.77	4.55

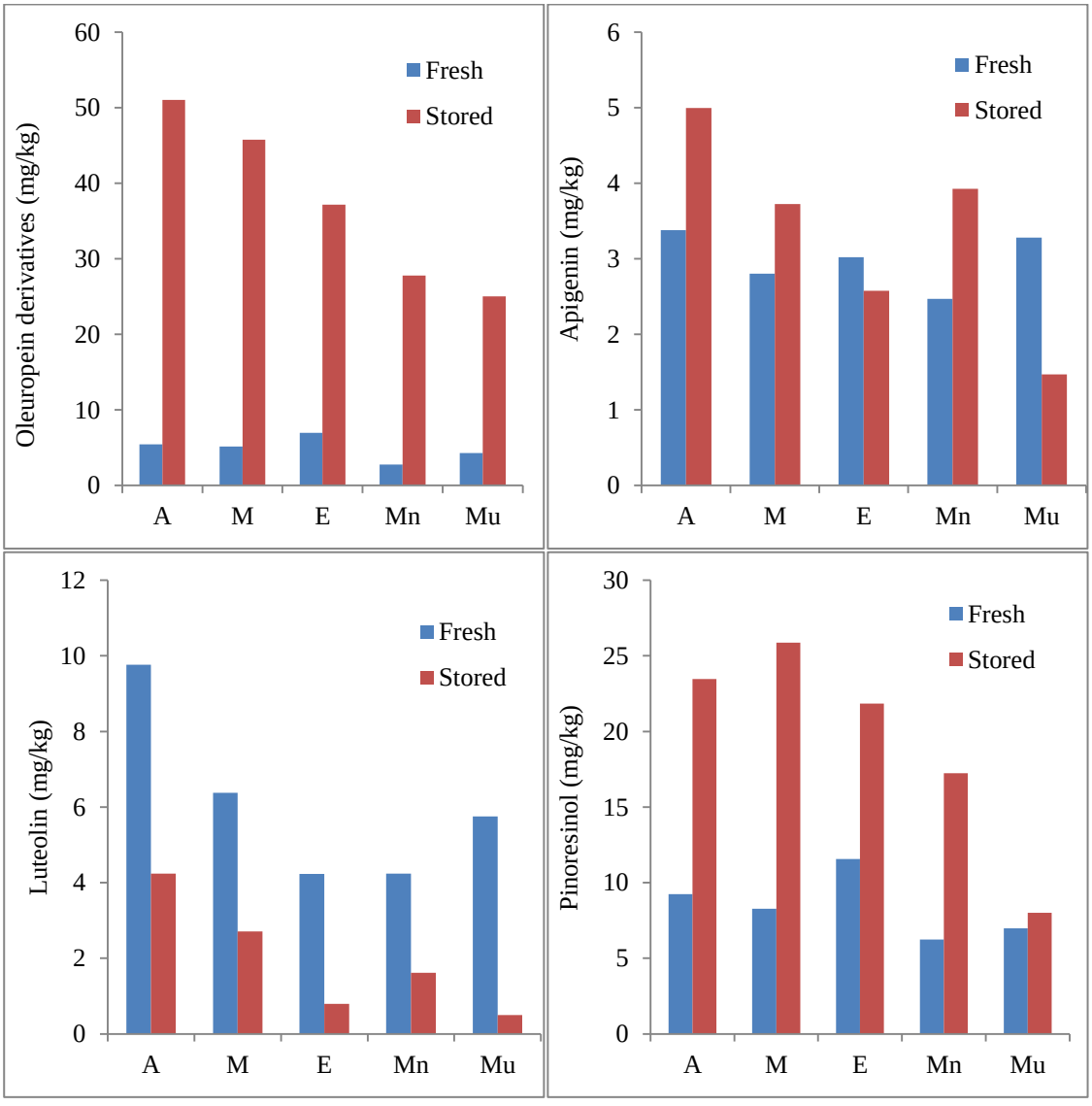

Figure 5.4.Changes in phenolic compounds (Oleuropein derivatives, Apigenin, Luteolin and Pinoresinol) of fresh and stored oils of different olive types (A: Ayvalik, M: Memecik, E: Erkence, Mn: Manisa region, Mu: Uzunkuyu region)

The initial concentration of total phenolic acids (TPA) was not statistically different among the varieties, but the highest concentration was found in Ayvalik oils (4.56 mg/kg). However, this concentration changed as storage period of the oil advanced and become significant in the case of Ayvalik olive variety (8.63 mg/kg). The increment is understandable, owing to the fact that, concentrations of some phenolic acids such as: cinnamic and vanillic became heightened with storage.

5.4. Changes in Alkyl Esters and Wax Content

A typical chromatogram of olive oil sample with identifiable peaks of methyl and ethyl esters in conjunction with the internal standard (methyl heptadecanoate) are presented in Appendix D. The wax esters characterized from the samples were C42, C44, and C48 as shown in the figure and were similarly well resolved and identified alongside their internal standard (lauryl arachidate). Fatty acid alkyl esters corresponding to palmitic (C16:0), oleic (C18:1) and linoleic (C18:2) acids are the chief primary alkyl esters found in olive oil according to International Olive Council (IOC, 2010) and European Commission Regulation (EEC, 2011). Retention times of alkyl esters and wax components were ascertained according to the study by Jabeur et al., (2015).

The concentration of fatty acid alkyl esters (FAAEs) in olive oil has been gaining special attention as one of the most significant quality and authentication parameters. It does not only reflect the wholesomeness (health conditions) of olive fruits prior to extraction, but also measure the extent of hydrolytic and fermentative processes in olive fruits (Gómez-Coca et al., 2012). Alkyl esters content of olive oil constitutes one of the newest methods of unravelling adulteration of extra virgin olive oil with mildly refined, low quality or defective olive oil. The resultant blend of extra virgin olive oil and refined oil is capable of circumventing the standard and conventional methods of quality regulation. Therefore, attention has been focused on parameters whose values are expected to be within certain limits in extra virgin olive such as: alkyl wax esters determination (Pérez-Camino et al., 2008). FAAEs are a family of natural lipids present in olive oils comprising of methyl and ethyl esters and are formed by esterification of FFA with low molecular weight alcohols such as ethanol and methanol (Beltrán, Bejaoui, Jimenez, & Sanchez-Ortiz, 2015).

Table 5.7 shows methyl, ethyl and wax esters content of fresh and stored olive oils. The initial contents of alkyl and wax esters of Ayvalik and Memecik oils were within the threshold limits of the most recently (2015/2016) established international standard for extra virgin olive oils (< 35 mg/kg) (EEC, 2013; IOC, 2015). Ayvalik and Memecik oil samples were obtained from sources, where postharvest and extraction conditions of the olive fruits were well controlled. The FAAEs content (of Manisa oils 66.51 mg/kg) was below the previous IOC standard (IOC, 2009) (75-150 mg/kg), while Erkence and Uzunkuyu oils were above allowable range (Fig. 5.5). Another observable distinction between the oil samples is the difference between FAEEs and FAMEs. In the case of Erkence and Uzunkuyu oil samples, as the total amounts of FAAEs increases, the amounts of FAEEs appear much higher than FAMEs, reaching in some cases, a ratio above 2.0 which is beyond the allowable level of 1.5 (Table 5.8). European Commission regulation (EEC, 2011) stated that FAAEs contents above permissible limit may be tolerated for extra virgin olive oil in some cases, provided that the ratio of FAEEs to FAMEs is below 1.5. This further confirmed the extra virgin status of oils of Ayvalik and Memecik varieties and some Manisa region oils before storage. Prolonged olive maturation leading to possible fermentation of olive fruits before and after harvest generates short chain ethanol (EtOH) and methanol (MeOH) as by products (Beltrán et al., 2015). These metabolites not only serve as aroma and volatile attributes of olive oils, they are also effective precursors of ethyl and methyl esters in olive oil during esterification (Perez-Camino, et al., 2008). As one of the rate determining factors, acidity increases this rate of esterification (Gómez-Coca, Fernandes, Pérez-Camino, & Moreda, 2016). However, as shown earlier Erkence and Uzunkuyu oils were significantly high in FFA contents beyond the level classified as extra-virgin oil (>1.0). This acidic condition of the oils may have facilitated accumulation of FAAE as reported in literature (Gómez-Coca, Moreda, & Pérez-Camino, 2012). The same trend was observed in the wax category, the values varied with olive varieties, but they all remained within the limits of extra virgin olive oils (< 250 mg/kg).

Table 5.7 Changes in the alkyl esters profile (mean±SD) of olive oil during 15 months at room temperature in dark

Alkyl esters (mg/kg)	Ayvalik	Erkence	Memecik	Manisa	Uzunkuyu
Fresh samples					
C16:0 ME	2.21±1.09^{b}	8.93±2.71^{a}	3.35±0.37^{b}	6.46±3.13^{ab}	12.81±5.70^{a}
C16:0 EE	2.61±1.77^{c}	16.92±4.20^{ab}	4.45±0.39^{c}	7.76±6.60^{bc}	26.76±14.6^{a}
C18:2 ME	0.95±1.04^{b}	6.30±1.75^{a}	1.14±0.17^{b}	2.43±1.84^{b}	7.86±3.20^{a}
C18:1 ME	6.64±3.74^{c}	32.91±11.5^{ab}	9.66±0.93^{c}	16.35±12.0^{bc}	42.29±18.5^{a}
C18:0 ME	0.25±0.39^{b}	1.44±0.44^{a}	0.67±0.15^{ab}	0.75±0.86^{ab}	1.81±1.02^{a}
C18:2 EE	1.13±0.72^{b}	10.90±2.89^{a}	1.10±0.31^{b}	3.72±3.29^{b}	14.61±7.33^{a}
C18:1 EE	7.10±5.76^{c}	56.63±16.2^{ab}	12.29±2.57^{c}	27.62±22.2^{bc}	74.86±34.2^{a}
C18:0 EE	0.36±0.37^{b}	2.48±0.62^{a}	0.63±0.10^{b}	1.41±1.08^{ab}	2.83±1.11^{a}
Wax	70.09±25.8^{b}	113.64±17.7^{a}	28.52±5.33^{b}	48.15±20.4^{b}	64.10±20.3^{b}
FAME	10.05±6.07^{c}	49.58±16.3^{ab}	14.82±1.60^{c}	25.99±16.8^{bc}	64.78±28.4^{a}
FAEE	11.20±8.32^{c}	86.92±23.5^{ab}	18.47±3.31^{c}	40.51±33.1^{bc}	119.05±57.2^{a}
FAAE	21.24±14.1^{c}	136.50±38.9^{ab}	33.29±4.82^{c}	66.51±49.8^{bc}	183.83±83.9^{a}
FAEE/FAME	1.11±0.29^{b}	1.81±0.26^{a}	1.24±0.11^{ab}	1.38±0.48^{ab}	1.86±0.35^{a}
Stored samples					
C16:0 ME	10.73±4.36^{b}	26.02±6.50^{a}	10.96±1.16^{b}	15.05±10.26^{ab}	29.77±6.31^{a}
C16:0 EE	5.41±3.33^{b}	42.68±11.4^{a}	10.48±0.35^{b}	20.93±15.92^{b}	58.32±23.8^{a}
C18:2 ME	4.72±1.63^{b}	17.27±4.24^{a}	4.93±0.65^{b}	7.86±6.38^{b}	24.15±9.04^{a}
C18:1 ME	33.71±12.3^{c}	96.11±28.1^{ab}	38.98±5.20^{c}	53.82±38.15^{bc}	122.94±34.9^{a}
C18:0 ME	2.41±1.31^{b}	3.93±0.88^{ab}	1.89±0.07^{b}	2.59±1.78^{b}	6.00±1.71^{a}
C18:2 EE	2.70±1.71^{b}	29.73±5.39^{a}	4.47±0.13^{b}	12.47±10.86^{b}	35.24±13.1^{a}
C18:1 EE	20.63±13.9^{b}	160.23±43.73^{a}	38.76±1.45^{b}	90.62±70.40^{ab}	189.99±61.5^{a}
C18:0 EE	0.85±0.42^{c}	6.22±1.74^{ab}	1.61±0.02^{c}	3.69±2.85^{bc}	8.86±4.07^{a}
Wax	42.01±18.8^{b}	117.48±16.4^{a}	42.65±6.81^{b}	41.37±14.99^{b}	77.83±33.4^{b}
FAME	51.57±18.9^{c}	143.33±39.3^{ab}	56.76±6.90^{c}	79.32±56.49^{bc}	182.86±51.7^{a}
FAEE	29.58±19.31^{c}	238.85±60.87^{ab}	55.32±1.64^{c}	127.70±99.9^{bc}	292.41±100.8^{a}
FAAE	81.20±32.2^{b}	382.20±96.9^{a}	112.1±8.30^{b}	207.0±156.3^{b}	475.3±152.0^{a}
FAEE/FAME	0.59±0.31^{b}	1.69±0.25^{a}	0.98±0.10^{b}	1.54±0.20^{a}	1.58±0.13^{a}

SD: Standard deviation C16:0 ME: methyl palmitate, C16:0 EE: ethyl palmitate, C18:2ME: methyl linoleate, C18:1ME: methyl oleate, C18:0ME: methyl stearate, C18:2EE: ethyl linoleate, C18:1EE: ethyl oleate, C18:0EE: ethyl stearate, Wax: Sum of C42, C44 and C48, FAME: (Sum of C16:0ME, C18:2ME, C18:1ME and C18:0ME), FAEE: (Sum of C16:0EE, C18:2EE, C18:1EE and C18:0EE). Note: values that do not share one or more superscripts on the same row are significantly different ($P<0.05$)

Table 5.8 Changes in alkyl esters profile of olive oil with storage

Esters	Ayvalik	Memecik	Erkence	Manisa region	Uzunkuyu region
C16:0 ME	433.79	228.01	199.73	160.26	156.91
C16:0 EE	120.27	136.11	150.96	205.26	129.58
C18:2 ME	156.50	336.10	183.72	211.97	209.78
C18:1 ME	467.73	303.83	203.82	236.38	209.55
C18:0 ME	50.37	194.74	192.18	86.90	285.94
C18:2 EE	156.98	325.80	190.52	245.46	150.70
C18:1 EE	208.35	222.92	185.99	252.22	164.38
C18:0 EE	24.95	159.20	151.44	171.06	206.45
wax	-38.47	50.24	5.48	-8.03	19.06
FAME	501.42	283.63	199.74	194.11	200.50
FAEE	170.78	204.68	177.93	235.51	155.90
FAAE	331.34	239.83	185.58	207.97	170.11
FE/FM	-47.91	-20.62	-6.19	28.90	-13.38

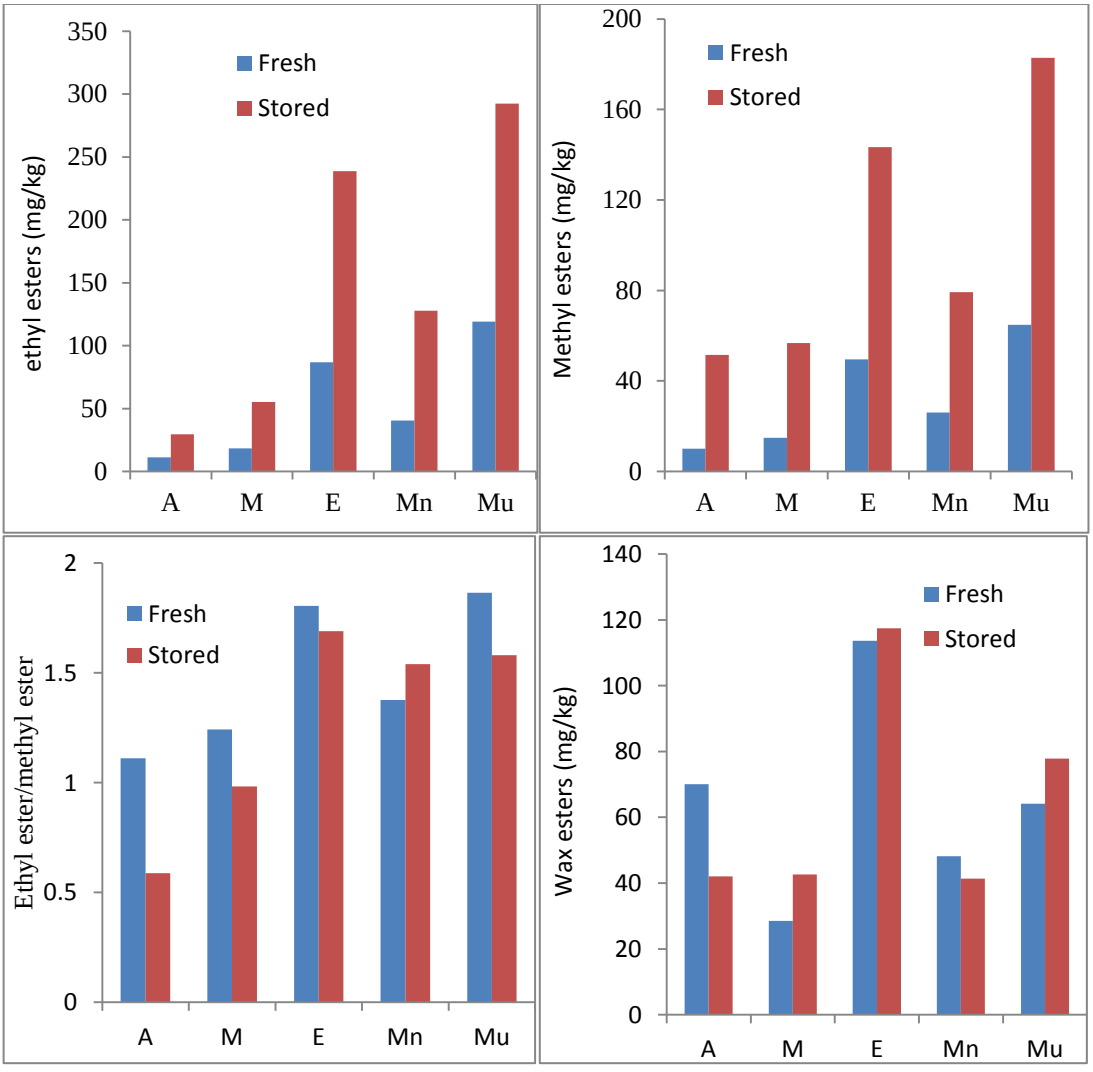

Figure 5.5.Changes in alkyl and wax esters (ethyl ester, methyl ester, ethyl:methylesters ratio and wax) of fresh and stored oils of different olive types (A: Ayvalik, M: Memecik, E: Erkence, Mn: Manisa region, Mu: Uzunkuyu region)

There was an upward trend in the content of methyl, ethyl and wax ester contents of the oil samples after storage. Table 5.7 and Fig .5.5 show the changes in these variables for different olive oil samples. The results revealed that Ayvalik and Memecik oils exhibited the highest percent increase in FAMEs and FAEEs and followed by Uzunkuyu. Ayvalik and Memecik oils are still within previous alkyl esters standard limits (75 -150 mg/kg). Erkence and Uzunkuyu oils had more than twice alkyl esters content of either of Ayvalik and Memecik oils. Free fatty acidity (FFA) is a factor which influences the rate of production of alkyl esters in olive oil. Erkence and Uzunkuyu oils were significantly high in FFA, which may have contributed to the overall concentration of these compounds in both. But it is very hard to reach a conclusion, since storage time did not influence FFA contents of the oil. Therefore, it is simply convenient to assume that alkyl esters are generated by other mechanisms that do not depend on FFA. Conte et al., (2014) suggested other probable mechanism that could lead to ester evolution when oils were stored. Ethanol or methanol esterification with fatty acids or transesterification (with triglycerides or partial glycerides) were the two probable reasons for the increase in alkyl esters content of olive oils with storage. Another plausible explanation could be a continuous esterification of FFA generated by available alcohols (not determined) almost at constant rate, thereby keeping the initial FFA contents constant.

There is no clear link between phenolic compounds and the rate of alkyl esters generation in olive oil. There is no study that predicts the interrupting effect of phenolic compound against methyl or ethyl esters production during olive oil storage. However, studies on fatty acid methyl esters have demonstrated the rate of autoxidation of linolenic esters is higher than linoleic esters, which in turn higher than esters of oleic acids (Pisoschi & Pop, 2015). Therefore, Ayvalik and Memecik oils having the lowest values of C18:2ME (linoleic methyl ester) when fresh (0.95 and 1.14 mg/kg) and stored (4.72 and 4.93 mg/kg) may be more resistant to oxidation as indicated so far. Surprisingly, the wax content decreased in Ayvalik, increased in Memecik and Manisa region oils, remained constant in Erkence and decreased slightly in Uzunkuyu oils. This random variation in wax content was also supported in literature (Pérez-Camino, et al., 2008).

5.5. Multivariate Analysis

The differences and similarities among the oil samples with respect to olive types and storage were investigated using models built separately with fatty acid profiles, quality parameters, phenolic profiles and ethyl-methyl and wax esters. Olive oils of Godence-Mix and Local-Mix from Urla were removed because of the uncertainty about their varieties. Each of the models was built with 54 observations. The principal components (PCA) were graphically displayed as a set of scores (score plot), and a set of loadings (loading plot). In order to improve separation among oils of different varieties and fresh/stored samples, OPLS-DA models were built for each data matrix. OPLS-DA discriminant analysis maximized covariance between the measured data and the response variable Y, which is the class information based on either variety or year. Finally, in order to unveil the influence (positive or negative) of fatty acids, ethyl-methyl esters, phenols and quality parameters on oxidative stability (OSI) of olive oils, an OPLS regression model was built.

5.5.1. Quality Parameters

A PCA model with 3 PCs, explaining 86% of total variance and R^2_{CV} of 0.69 showed the quality and storage differences between oils obtained from different olive types. The data matrix was made up of 54 observations and 11 variables. These variables include TPC, FFA, 5 color parameters, chlorophyll and carotenoids, and Kvalues. The score and loading plots are shown in Fig. 5.6A and 5.6B, respectively. The first half of the control ellipse was shared predominantly between fresh and stored Erkence oils with few intrusions from other varieties. The first quadrant on the lower left corner was mainly occupied by fresh Erkence oils and the upper part made up of stored ones. FFA, L* and H were the parameters defining fresh Erkence oils and stored Erkence oils are described by higher K values. Ayvalik, Memecik and Manisa oils are located at the second half of the ellipse and their clustering were influenced by TPC, OSI, pigments, and some color parameters. The most compelling pattern in this PCA model is the quality differences among the oil samples. Erkence oils and few others that shared the first half of the ellipse were significantly high in natural degradation products of triglycerides leading to increased FFAs and K values. On the other hand

potentially significant antioxidant chemical indices such as TPC, OSI and pigments helped in separating majority of Memecik, Ayvalik, and some Manisa oils. Olive oils, rich in TPC, pigments and visually appealing to consumers in terms of color, are likely to be of higher nutritional and economic values.

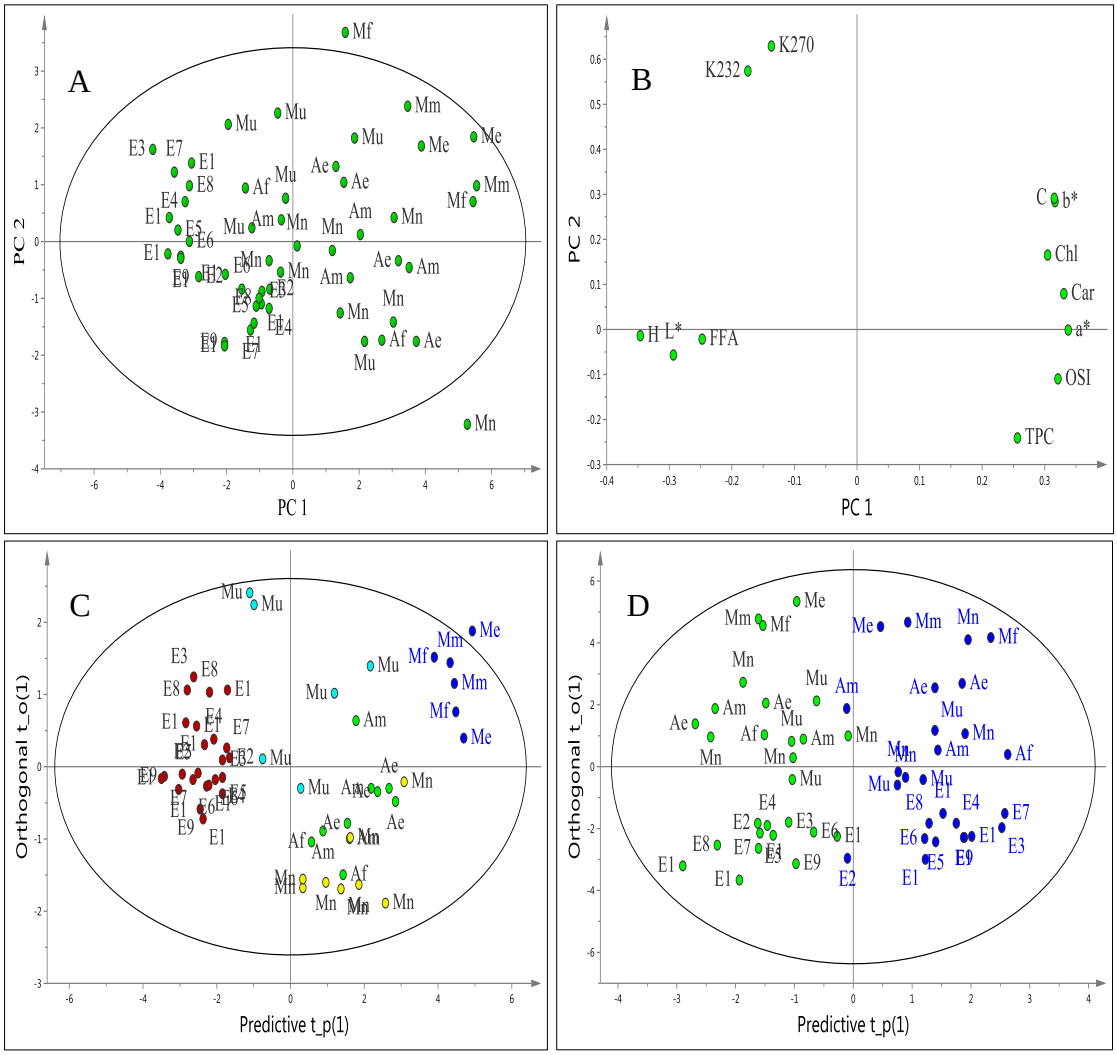

Figure 5.6 Multivariate model on influence of quality parameters: Score plot of PCA model (A), Loading plot of PCA model (B); Score plot of OPLS-DA model for o classification; **Erkence**, **Uzunkuyu**, **Manisa**, **Ayvalik** (e: early, m: mid, f: late harvest) and **Memecik** (e: early, m: mid, f: late harvest) oils (C), Score plot of OPLS-DA model of **fresh** and **stored** oil classification (D).

OPLS-DA model for varietal discrimination has 3+4 PCs, R^2 of 0.97 and R^2cv of 0.43. As shown in the score plot (Fig. 5.6C), there is a clear separation of Erkence olive oils. The precision in the classification of Erkence oils was significantly higher than other varieties. As stated earlier FFA, L* and H variables distinguised Erkence oils

from others. It is obvious that some of the Uzunkuyu oils have varying quality characteristics, preventing them from forming a distinct cluster. Unlike Memecik oils, there was no clear distinction between oils from Manisa and Ayvalik as both are localized at the lower right side of the ellipse. It is noteworthy to state that, Memecik oils had the highest values of the desirable quality properties (TPC, OSI and pigments) that characterize the right side of the ellipse. Ilyasoglu, et al., (2010) reported higher TPC and other oxidative stability indices in olive oils of Memecik variety. In order to determine the influence of storage on olive oils, observations were grouped into two categories: fresh oils and stored oils (Y variables). The score plot of OPLS-DA model with 1+2PCs, R^2 of 0.85 and R^2cv of 0.77 is divided into two (Fig.5.6D). There was a clear distinction between fresh and stored oils among all the varieties. Erkence oils formed two separate clusters at different planes, with fresh and stored oils located at lower left and lower right of the ellipse, respectively. The same applies to other varieties as well.

5.5.2. Fatty Acids Profile

Fatty acids profile was examined separately as important descriptors of olive oil in terms of variety and storage time differences. The PCA model with 3 PCs, R^2 of 0.80 (explained variance), and R^2cv of 0.53 was built with 54 observations (oil samples), and 16 variables. The variables include 11 individual fatty acids, SFA, MUFA, PUFA, MUFA/PUFA and C18:1/C18:2 ratios. This model was sufficient to separate olive oils based on their variety (Fig. 5.7A). There were at least three distinct homogenous clusters on the score plot, each belonging to different variety regardless of freshness. At the left hand side of the ellipse there were Erkence oils. PUFA, linoleic, linolenic and arachidonic acids are the main descriptors of Erkence oils (Fig 5.7B). On the other hand, upper right of the ellipse consists of Memecik oils and they are defined by MUFA, oleic acid, palmitoleic acid, MUFA/PUFA and oleic/linoleic acid ratios. These fatty acids characteristics are typical of highly stable olive oils. Recent studies confirmed the positive contributions of oleic acid, high MUFA/PUFA and oleic/linoleic acid ratios to oxidative stability of olive oil (Borges et al., 2017, Jolayemi, Tokatli & Ozen, 2016). The ease of enzyme-modulated, free radicals formation and subsequent

oxidative degradation of olive oil has been linked to its PUFA-richness (Clodoveo et al., 2014).

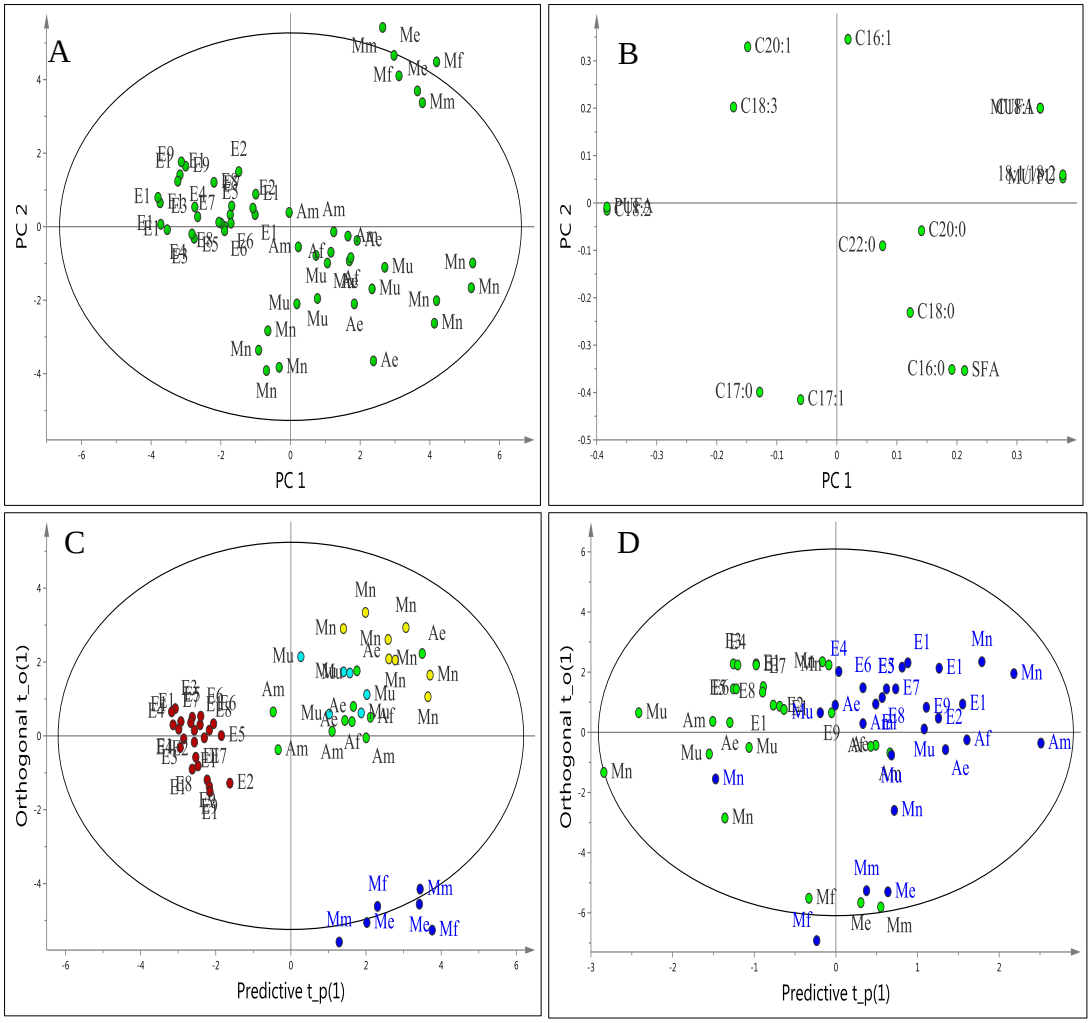

Figure 5.7 Multivariate models on influence of fatty acids profile: Score plot of PCA model (A), Loading plot of PCA model (B); Score plot of OPLS-DA model of olive types classification, **Erkence**, **Uzunkuyu**, **Manisa, Ayvalik** (e: early, m: mid, f: late harvest) and **Memecik** (e: early, m: mid, f: late harvest) oils (C) Score plot of OPLS-DA model of **Fresh** and **stored** oils classification (D)

This observation is highly significant in buttressing other quality properties of Erkence oils. Their high FFA, K_{270} and K_{232} values as well as low OSI, when taken all together, confirm that oxidative stability is not just a function of one single parameter, but rather by overall chemical properties (Borges et al., 2017). At the central part of right hand side of the ellipse, oils of Ayvalik overlapped with that of Uzunkuyu and some Manisa oils and were separated by their high values of SFA, C16:0, C20:0, and C22:0. Four of

Manisa oils (2 of each Mn 1 and 2) were slightly different from others, forming cluster of their own with C17:0 and C17:1 as the descriptors.

Varietal discrimination of the oils was done using OPLS-DA model with 3+1 PCs, R^2 of 0.64 and R^2cv of 0.53. As shown in the score plot (Fig. 5.7C), there were two homogenous clusters, Erkence, Memecik and a mixture of Ayvalik, Uzunkuyu and Manisa oils. As stated earlier, PUFA contents of Erkence oils were significantly higher than other varieties, which is responsible for their distinct projection. High content of linoleic acid has been linked to the continuous activities of oleate desaturase enzyme transforming oleic acid to linoleic acid (Reboredo-Rodríguez, et al., 2014). This enzyme may still be active in Erkence oil even after extraction. Memecik oils may likely have the lowest susceptibility to oxidation. This also explains the longer induction time observed in Memecik oils. The cluster of Ayvalik oils, with some of Uzunkuyu and Manisa regions oils simply implies that fatty acid composition is not an exclusive quality property. In order to evaluate the potential of fatty acid composition in differentiating fresh oils from stored ones, OPLS-DA of 1+1 PCs, R^2 of 0.47 and R^2cv of 0.38 model parameters was used (Fig. 5.7D). However, the weakness of the model is shown in the overlaps between oils of Ayvalik, Erkence, Manisa and Uzunkuyu. This is evidence that there is no significant impact of dark storage at room temperature on fatty acids composition of olive oil. This is supported by a previous study (Gomez-Alonso et al., 2007), where no significant changes were observed in fatty acid composition after 21-month storage period. This may be as a result of evolution of some phenolic compounds during storage, preventing lipid oxidation and compositional changes. Fresh and stored oils of Memecik variety clustered together showing no significant changes in their fatty acid compositions. On the other hand, Erkence oils were separated into two different clusters with the stored oils (right side ellipse) having high PUFA, C20:1, C20:0, C22:0, C17:0 and C17:1. These changes cannot be completely attributed to storage effect, owing to the low predictive capacity of the model and overbearing number of Erkence oil samples relative to others.

5.5.3. Phenolic Profile

In an attempt to explore probable intrinsic pattern of relationship between exploratory factors (olive types, storage) and olive oil phenolic composition, PCA

model containing 3 PCs, and R^2cv of 0.49 was built. The data matrix consists of 54 observations and 16 variables. The variables include 14 individual phenols, oleuropein derivatives (sum of hydroxytyrosol and tyrosol), and TPA (total phenolic acids). The PCA score plots obtained using these phenolic compounds as descriptors are displayed in a two-dimensional score plot considering first two principal components that covered 77% of the total variance (Fig. 5.8A). The most observable trend in the score plot is the changes in phenolic profile of the oil samples with storage. A hypothetical line can be drawn to separate the score plot into segments with the upper part preoccupied by mixed cluster of the fresh oil with little or no varietal separation pattern. Conversely, the lower segment of the ellipse is made up of stored oil samples. Within the stored oils, Erkence variety formed a cluster at the mid-center, while relatively higher quality-oils from Ayvalik, Memecik and Manisa are located at the far right. This is because of the weight differential between the concentration of individual phenol of fresh and stored olive oils. On the left-side of the score plot, there were two separate clusters; combination of Ayvalik, Memecik, Erkence oils and a mixed of Manisa and Uzunkuyu oils, which are all described by luteolin (Fig. 5.8B). On the other hand, all major phenolic compounds such as hydroxytyrosol, tyrosol, pinoresinol, phenolic acids, helped separate the stored oils.

This observation becomes clearer when modelled with OPLS-DA of 1+1 PCs, R^2 of 0.80 and R^2cv of 0.79 (Fig. 5.8C and 5.8D). The model was sufficient to separate fresh and stored oils and indicate the variables contributing to the separation. The loading plot showed the most influential variables discriminating the majority of stored oil of Memecik and Ayvalik are individual phenolic acids, apigenin, total phenolic acids and vanillin. In the same vein, hydroxytyrosol, tyrosol, pinoresinol and cinnamic acid are responsible for the classification of Erkence oils and few other overlaps of Memecik, Manisa, and Uzunkuyu oil samples. Model for varietal classification (not shown) with respect to phenolic compounds was not sufficient enough to classify the oil samples as the variance explained was less than 25%. Higher contents of phenols in the stored oil may be responsible for the same induction times (OSI) experienced between fresh and stored oil samples after 15 months of storage. It can be shown in the model that, storage impact of phenolic compounds outweighs that of varietal differences. The protective effect of phenolic compounds synergy especially o-diphenols family on oxidative changes in olive oil has been widely documented (Manai-Djebali et al., 2012). Hydroxytyrosol's antioxidant capacity has been attributed

to its efficiency in micelles system owing to it high lipophilicity (Fogliano et al., 1999). Ability of these phenolic compounds to form stable radicals in the lipophilic phase explained why FFA, PUFA, and K values remained unchanged after several months of storage of the oil samples. However, the antioxidant potency of tyrosol and other groups of phenols are relatively low compared to hydroxytyrosol and its derivatives (Baldioli et al., 1996). Similar changes in the phenolic compounds after a storage period were reported by other researchers (Gómez-Alonso et al., 2007, Kotsiou & Tasioula-Margari, 2016).

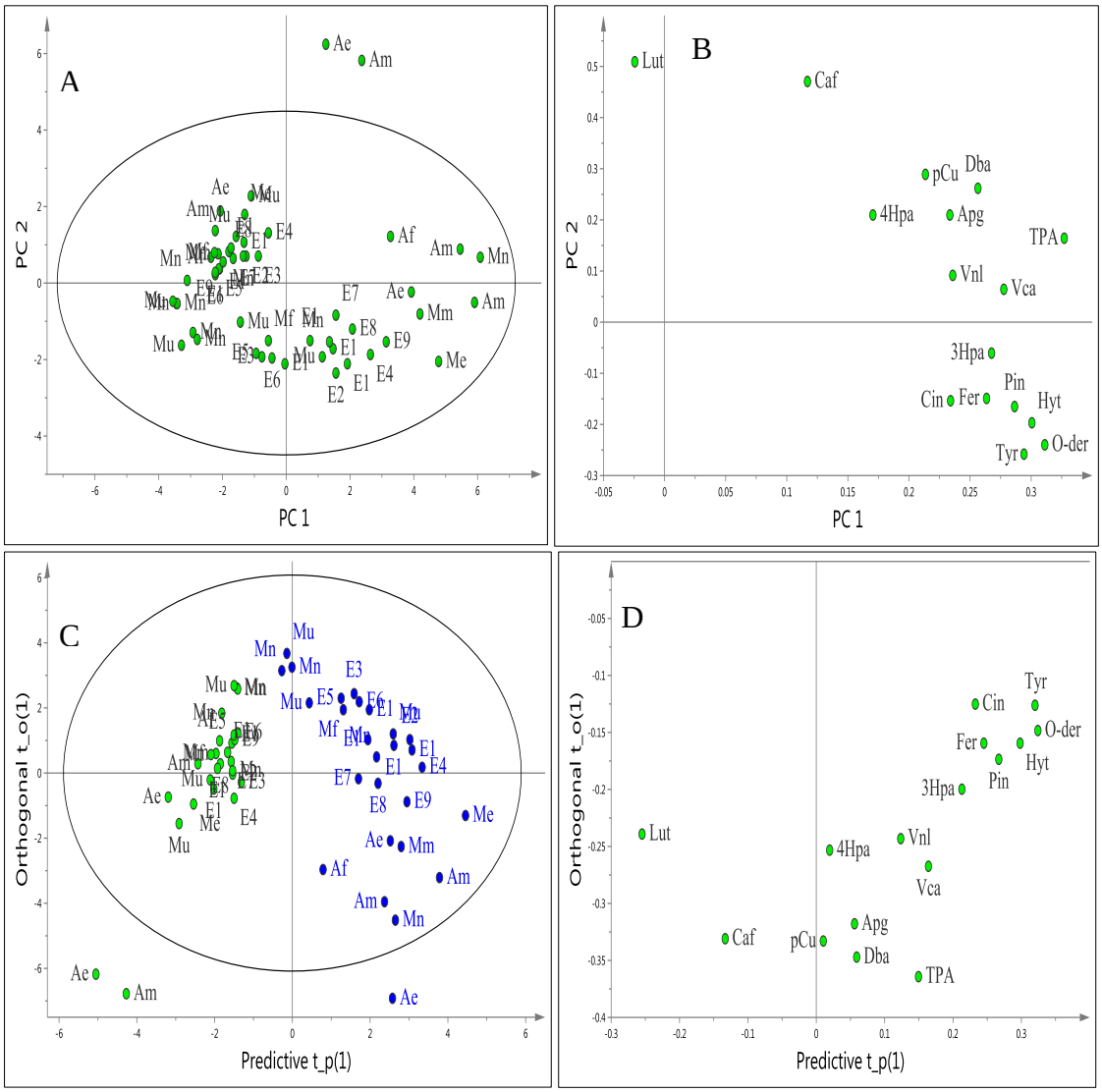

Figure 5.8 Multivariate model on influence of phenolic profile: PCA model Score (A), and Loading plots (B); OPLS-DA model Score (C) and Loading plot (D) of **Fresh** and **stored** oils

5.5.4. Alkyl Esters (FAAE) and Wax Contents

The determination of FAAE is a comprehensive indication of the extent of quality control of olive oil production steps, right from olive harvest to the end product. Fig. 5.9A and 5.9B show the score and loading plots of PCA model built with 99% of total variance represented, R^2cv of 0.94 and 4PCs. The model consists of 54 observations and 13 variables. These variables include 4 individual methyl esters, 4 individual ethyl esters, total wax content, sum of all methyl esters (FAMEs), sum of all ethyl esters (FAEEs), sum of ethyl and methyl esters (FAAEs) and FAEEs/FAMEs ratio. Observations on the score plot showed two distinct clusters; the upper and the lower parts of the ellipse. The upper left side of the ellipse, which is predominantly fresh Erkence oil samples, is well described the high wax concentration and ethyl: methyl ester ratio. Olive oils of high wax contents are usually discredited to be of low quality by International Olive Council and European Union Commision (IOC, 2015, EEC, 2013). This is because olive oil of high wax content is regarded as crude olive-pomace oil, lampante or adulterated virgin or extra virgin olive oil with refined oils based on their total aliphatic alcohol contents. In the same vein, high ratio of FAEEs/FAMEs is not encouraged for olive oil to be labelled as extra virgin. A ratio below 1.5 is the minimum requirement, provided the FAEE is within the threshold limits. However, the lower-left side of the ellipse contains a cluster of both fresh and stored Ayvalik, Memecik, and fresh Manisa oils located reasonably far from the two variables. This indicates a differential between wax and FAEE/FAME values of fresh Erkence oils and those of other varieties. The right-side of the ellipse contains majorly stored oils of Erkence, Manisa and Uzunkuyu. This section of the ellipse is completely devoid of Ayvalik and Memecik oils, indicating the stability of these varieties to storage with respect to alkyl esters profiles. The upper-right side was defined by ethyl esters and FAEE, while the lower part by methyl esters and FAMEs. The formation of these compounds is not static and it has been demonstrated to increase under suitable storage conditions (Gomez-Coca et al., 2016). This is evidenced in the progressive increase in ethyl and methyl esters content of oils from other varieties except Ayvalik and Memecik, which are relatively more stable. The stability of these varieties may be attributed to their consistency in other chemical characteristics such as phenolic compounds, OSI, FFA and the likes.

OPLS-DA models (4+1PCs, R^2 of 0.49, and R^2cv of 0.32) built to differentiate between oils of different varieties with respect to alkyl esters distribution was shown in Fig. 5.9C. Because of the discrepancy between contents of alkyl esters and wax content of Erkence oils relative to other varieties, none of the variables represents described oils of other varieties. However, the score plots showed some overlaps between oils of similar quality characteristics. Therefore, alkyl esters profile of olive oils is more of quality discriminant chemical parameters rather than varietal indicator, a view supported by a recent study (Beltran et al., 2015).

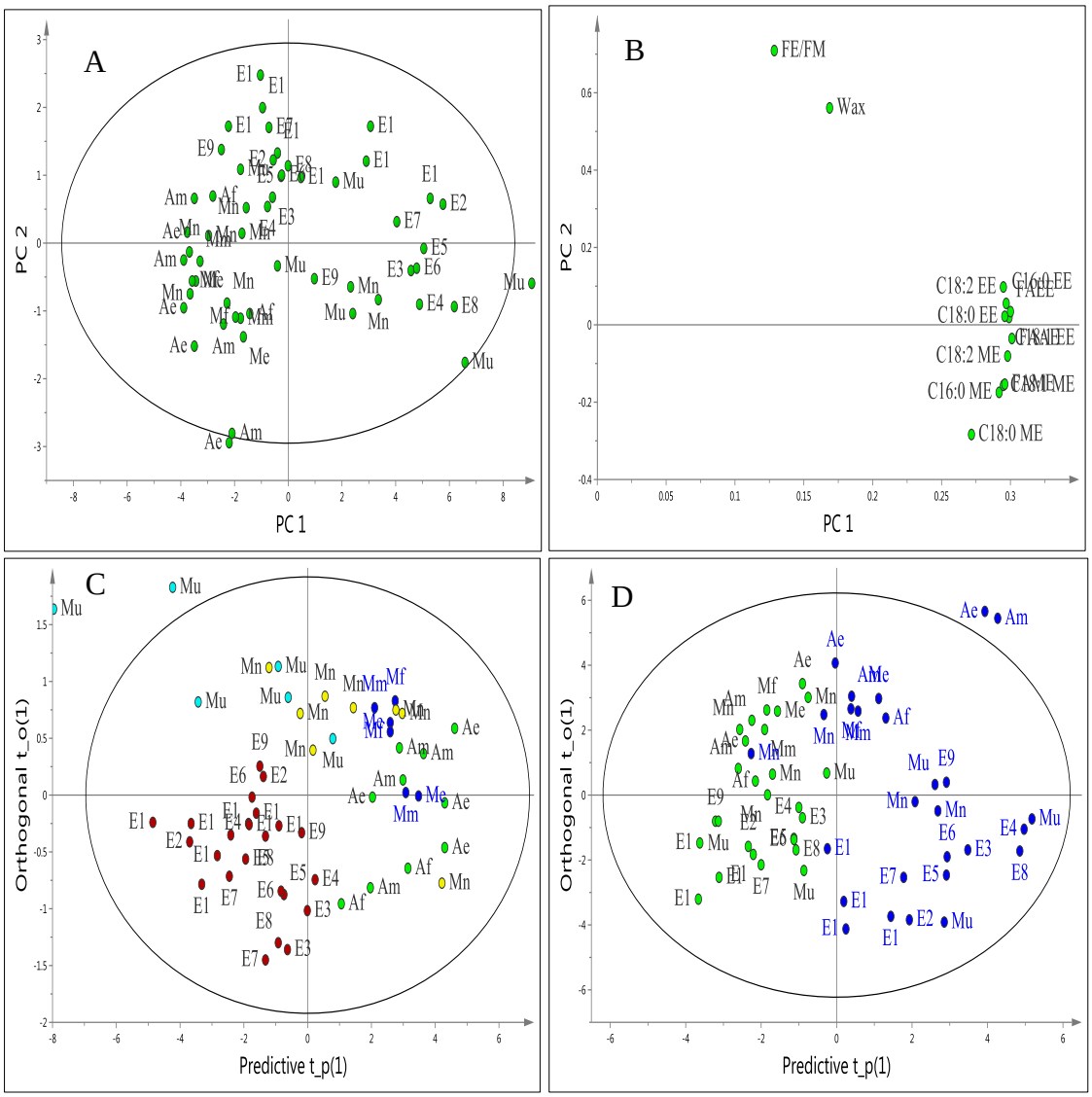

Figure 5.9. Multivariate model on influence of alkyl and wax esters: Score plot of PCA model (A), Loading plot of PCA model (B); Score plot of OPLS-DA model of olive types classification, **Erkence**, **Uzunkuyu**, **Manisa**, **Ayvalik** (e: early, m: mid, f: late harvest) and **Memecik** (e: early, m: mid, f: late harvest) oils (C) Score plot of OPLS-DA model of **Fresh** and **stored** oils classification (D)

Storage effect on the alkyl esters and wax content of the oil was evaluated using OPLS-DA model of 1+2PCs, R^2 of 0.64, and R^2cv of 0.60. The model simply amplifies the PCA with the score ellipse divided into, with upper and lower hemispheres consisting of two heterogeneous clusters (Fig 5.9D). Fresh and stored oils of Ayvalik, Memecik and some Manisa occupied the upper part (positive axis of 2^{nd} PC). The lower half is mainly Erkence, Uzunkuyu and some Manisa oils. High substrate concentrations such as short chain alcohols (ethanol or methanol), acidity and suitable temperature are keys to alkyl esters evolution during storage (Gómez-Coca, et al., 2016). Acidity contents of Erkence and Uzunkuyu oils are comparatively higher than other varieties. However, there was no significant change in acidity of the oil samples after storage, thereby weakening the direct proportionality of FFA to alkyl esters. Therefore, this significant increment in alkyl esters after 15 month of storage in these particular oils may be due to continuous consumption of substrate (FFA and ethanol) to form products (alkyl esters), thereby keeping the products of triglyceride hydrolysis relatively constant. Moreover, olive oils obtained under a well-controlled extraction conditions are likely to have lower content of short-chain alcohols because filtration prior to storage, reduces residual moisture as stated in literature (Gómez-Coca, et al., 2016).

5.5.5. Analysis with Combined Chemical Parameters

PCA and OPLS-DA models were built to evaluate the influence of all chemical variables in separation of olive oils with respect to their varieties and storage differences. Size of the data matrix was 54 x 54, with 54 olive oil samples (n observations) and 54 combined chemical characteristics (k variables). These variables include fatty acids, phenolic and alkyl ester profiles and quality parameters. The PCA preliminary data elaboration model explained 83% of the model variance, with R^2_{cv} of 0.65 and 6 PCs. There are four heterogeneous clusters, with each of them occupying separate quadrant of the score plane (Fig 5.10A). The first quadrant on the left hand side is predominantly occupied by fresh Erkence oils and few others from different varieties. This first quadrant is represented by high FFA, PUFA, Wax, FAEE/FAME, linoleic, linolenic acid, C17:1, C17:0 and L* (Fig 5.10B). The significance of these parameters indicates that fresh Erkence oils are not of best quality characteristics that can qualify them as virgin olive oil. High acidity and polyunsaturated fatty acids are

reflection of probable weak oxidative stability and susceptibleness to degradation, radical generation and subsequent off flavor. The L* is an index of low level or loss of pigments. High value of L* indicates lightness of the oil, which is the peculiar characteristics of oils from Erkence olive variety. The second quadrant on the left-hand side contained mainly stored oils of Erkence and few others. The variables describing this segment include all individual ethyl and methyl esters, FAEE, FAAE, FAMEs, K_{232}, K_{270}, tyrosol, pinoresinol, 3Hpa, and oleuropein derivative (sum of tyrosol and hydroxytyrosol). Except arachidonic acid (C20:1) there was no other fatty acid separating Erkence oils by the end of storage time. It can be observed that, some important phenolic compounds such as tyrosol, oleuropein derivatives and pinoresinol shared the same quadrant with alkyl esters and secondary oxidative indices (K values), with respect to stored Erkence oils. This probably shows that evolution of alkyl esters and phenolic compounds in olive oil during storage are independent of one another.

The second half of the ellipse contained two clusters with each occupying separate quadrant. The lower quadrant is localized predominantly with the mixed cluster of fresh Ayvalik, and Memecik oils and some other varieties, while the upper quadrant contained stored samples of the same varieties. Total phenol content, pigments, luteolin and saturated fatty acids are the variables discriminating fresh oils of Ayvalik and Memecik. TPC, pigments and phenolic compounds may have contributed to the stability of the oil and the saturated nature of their fatty acids made them relatively less prone to rapid oxidation. The upper quadrant of the right hemisphere contained mainly stored oils of Ayvalik and Memecik. Hydroxytyrosol, apigenin, phenolic acids, oleic acid, MUFA/PUFA, C18:1/C18:2 ratios, high molecular weight fatty acids and C, b* chromatic color parameters are the chemical characteristics responsible for this separation. The evolution of phenolic compounds such as hydroxytyrosol during the storage may be responsible for the oxidative stability of the oils. This is also evidenced in the comparative high values of oleic, MUFA/PUFA, and C18:1/C18:2 ratios, which are all positive contributors to the overall stability of olive oil. Invariably, it can be suggested that, the upper left half of control ellipse dominated with Erkence oil (fresh and stored) have lower quality and stability than the second hemisphere dominated by Ayvalik and Memecik oils.

Varietal classification using all variables with an OPLS-DA model (4+2 PCs, R^2 of 0.79, and R^2cv of 0.67) is presented in Fig 5.10C. Oils of Ayvalik and Memecik were almost inseparable owing to the various overlap between their chemical

properties, such as phenolic compounds, pigments, OSI, TPC, MUFA/PUFA and C18:1/C18:2. Manisa and some Uzunkuyu oils are mainly distinguished by their saturated fatty acids (SFA). Erkence oils with high FFA, ethyl, methyl and wax esters, PUFA and K values are in a discrete class.

OPLS-DA model of 1+2PCs, R^2 of 0.95 and R^2cv of 0.93 separated the samples into two broad categories; fresh and stored oils (Fig. 5.10D). Apart from revealing the class modelling results, varietal differences were also very apparent as shown in Fig. 5.11A. High values of FAEEs/FAMEs, FFA, wax and PUFA were the properties of fresh Erkence oils and few others (from Manisa and Uzunkuyu). A significant increase in alkyl esters, secondary oxidative dienes and trienes (K values) occurred in Erkence oils during storage. It is important to state that, there was no phenolic compound as a descriptor for the classification of fresh and stored Erkence oils (Fig. 5.11B). The upper part of ellipse divided into two segments (Fresh and stored) composed mainly of Ayvalik and Memecik oils. TPC, luteolin, OSI, pigments, SFA, C16:1, color chromatics (a* b* and C) were higher in fresh oils. After 15 months of storage, the oil's oxidative stability parameters and health-crucial variables such as oleuropein derivative (hydroxytyrosol + tyrosol), MUFA and MUFA/PUFA ratios changed. These changes were substantial enough to cause the clear separation of stored oils from fresh ones. Storage caused the failure of the majority of the oils from satisfying total ethyl esters (FAEEs) requirement (30 mg/kg) (IOC, 2015; EEC, 2013). Additional negative influence of storage includes: reduced color intensity of the oil (increased L*), reduced pigments, increased secondary oxidative products, and a slight reduction in oxidative stability index (OSI).

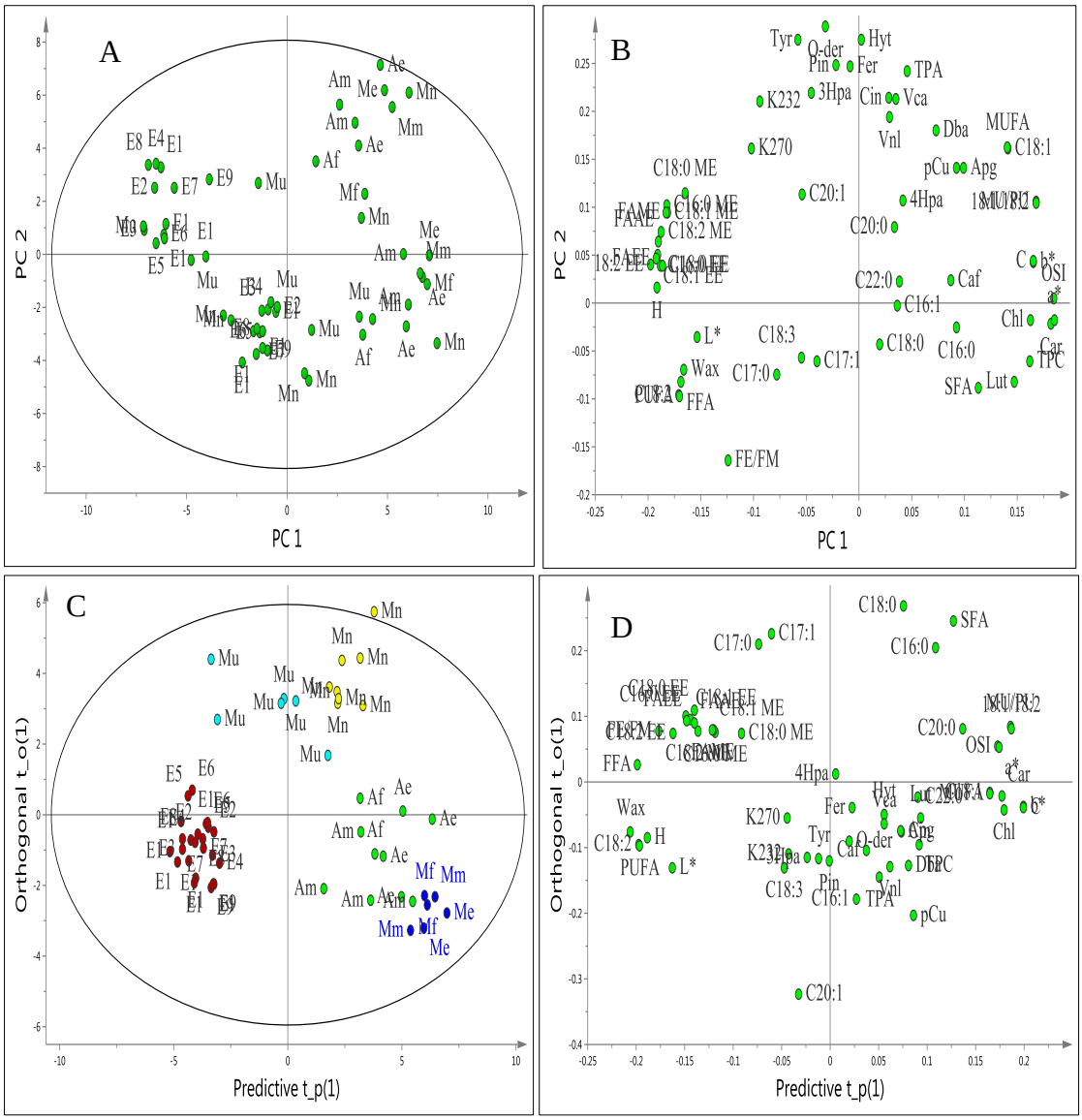

Figure 5.10. Classification with all variables with respect to variety: Score plot (A) and loading plot (B) of PCA model; Score plot of OPLS-DA model of olive types classification, **Erkence**, **Uzunkuyu**, **Manisa, Ayvalik** (e: early, m: mid, f: late harvest) and **Memecik** (C) and loading plot (D) of OPLS-DA model

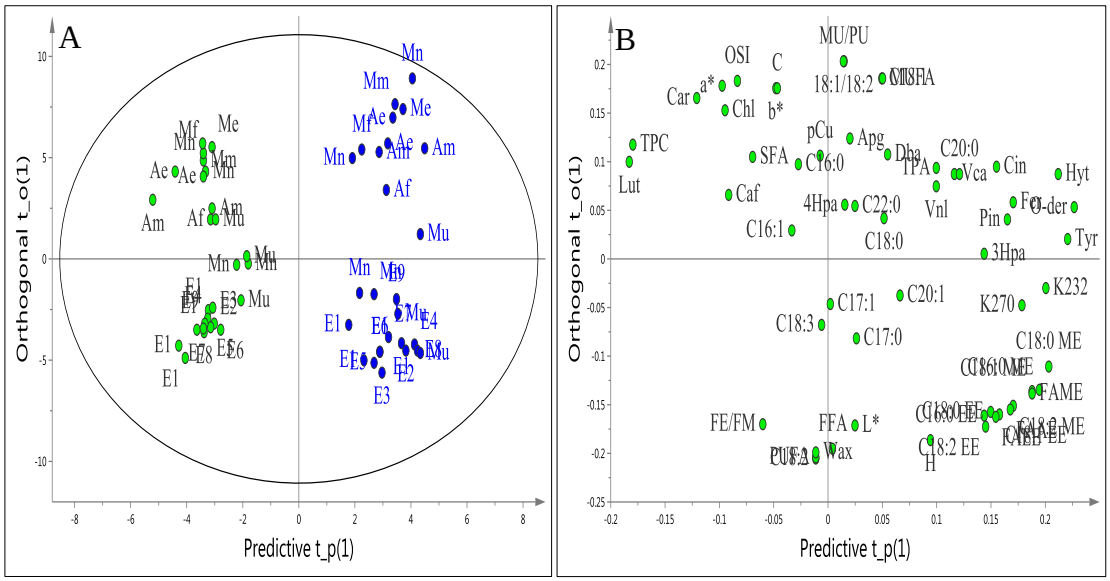

Figure 5.11. Classification with all variables with respect to storage effect: Score plot (A) and loading plot (B) of OPLS-DA model of **fresh** and **stored** oils

5.5.6. OPLS Regression of Oxidative Stability Index (OSI)

In this case, the variables (with VIP < 0.3) that were removed from the model are; hydroxytyrosol, oleuropein-derivatives, C16:1, C17:1, C22:0, vanillic, ferulic, cinnamic and total phenolic acid. This observation deviated slightly from our previous finding with respect to hydroxytyrosol and oleuropein derivative, both which were found positively contributive to OSI (Jolayemi, Tokatli & Ozen, 2016). This deviation was clearly due to the storage effect on these compounds, leading to significant increase in their values. However, increases in the values of these positive contributors were not parallel with those of negative contributors, thereby altering the model significantly. The effects of parameters were discussed in terms of weights of the OPLS regression model. The loadings weights and regression plots of the model with 1+4 PCs, R^2 of 0.90 and R^2cv of 0.79 are given in Fig. 5.12. Total phenol contents, oleic/linoleic acid ratio, oleic acid, saturated fatty acid, chlorophyll and carotenoid pigments were found as positive contributors for OSI. Variables of lesser positive impacts are; flavonoids (luteolin and apigenin), and some phenolic acids (Fig. 5.12A). On the other hand, polyunsaturated fatty acid, acidity (FFA), wax content, ethyl and methyl esters as well as K values were determined as the negative contributors, which were also reported for the previous olive oil analysis given in Chapter 4.

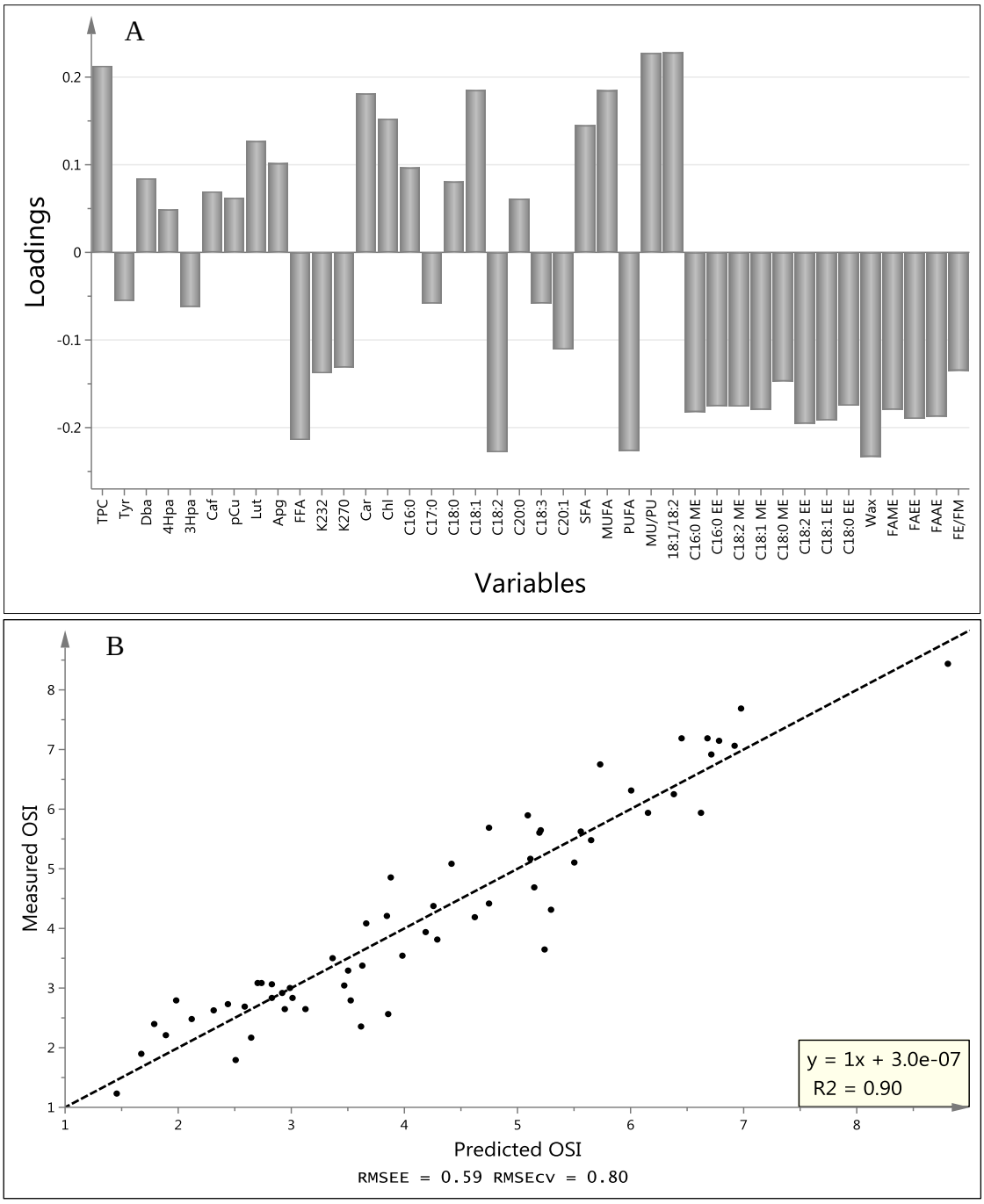

Figure 5.12. Results of OSI regression. The degree of influence (loading weights) of chemical variables (A) and scatter plot of predicted vs measured OSI values (B). RMSEE: root mean square error of estimation; RMSEcv: root mean square error of cross validation

The observations for OSI are in agreement with quite a number of studies. Rotondi et al., (2004) reported a positive correlation between OSI and TPC. The positive effects of chlorophyll and carotenoid to oxidative stability are ultimately due to the dark storage, as the pigments act as antioxidants in the dark and prooxidants in the presence of light (Manai-Djebali et al., 2012). Therefore, the influence of MUFA and

PUFA to the models are expected to be opposite as shown in Fig. 5.12B. FFA and alkyl esters (ethyl and methyl esters) have a close relationship as the formal is required to facilitate the generation of the later. The most prominent fatty acids in olive oil are the C18 and C16 at a suitable condition. A good correlation between FFA and alkyl esters should be expected because FFA is measured as percentage unesterified free oleic acid. It depends on the initial condition of olives, the time lag between harvesting the fruits and their processing, as well as the storage temperature. This relationship forms the bases for the negative influence of FFA, ethyl and methyl esters on oxidative stability. Waxes are esters of long chain alcohol with probable tendency to follow the same trend as alkyl esters as observed.

CHAPTER 6

MULTIVARIATE ANALYSIS OF DATA: CLASSIFICATION OF OLIVE OILS AND PREDICTION OF CHEMICAL PARAMETERS

UV-vis, mid-infrared, near-infrared spectra and electronic-nose data in conjunction with multivariate statistical techniques are elucidated in this section. Olive oil classification with respect to variety and harvest year were verified and the chemical parameters were predicted spectroscopically. Whole UV-vis spectra and selected regions of MIR and NIR spectra were used in building the models. All spectra were pretreated with second derivative and/ or in conjunction with standard normal variate (SNV) transformation.

6.1. Spectra Interpretation

The spectroscopic analyses given in this section were performed in the Department of Food Engineering at IYTE. Descriptive examples of the UV-vis and MIR spectra are presented (Fig. 6.1). In the UV-vis spectra, there are useful informations embedded in the band of the spectrum that can create both qualitative and quantitative differences in olive oil purity. The range of 200-350 nm is the ultraviolent region. The intensities among oil samples differ particularly between 300-350 nm. The most informative parts lie between 350-530 nm and 600-700 nm. There are three weaker peaks along the spectrum: 540 nm, 610 nm and 670 nm compared to the broad range in the ultraviolent region. These weaker peaks are only observed in high quality olive oils as in the case of Ayvalik and Memecik oils. In the low quality oils (most of the Erkence oils), peaks of smaller absorption intensities tend to disappear and those of larger absorption band shift towards shorter wavelengths. This observation is supported by other researchers (Tarakowski, Malanowski, Kościesza, & Siegoczyński, 2014). All the samples have absorption in the near ultraviolet and blue bands representing carotenoids and chlorophylls. The broad absorption range of 430-460 nm corresponds to carotenoid pigment (Aroca-Santos et al., 2015). The peaks at 670 nm are caused by electronic transitions between grounds and first excited state of chlorophylls. It can be

stated that transitions to higher energy state are responsible for the absorptions in shorter wavelengths (Giannakopoulos, Isari, Bourikas, Karapanagioti, Psarras, Oron, & Kalavrouziotis, 2016). The shape and width of peaks indicate broadening of those states. Strong absorption bands at near UV and blue region (400-500 nm) were observed in both high and low quality olive oils, but the shape of the spectra differ from that of those containing significant amount of chlorophyll (Memecik and Ayvalik). This view is equally shared by Tarakowski, Malanowski, Kościesza, & Siegoczyński, (2014).

Each significant MIR spectra band represents vibrational response of the chemical composition of the oil at molecular level. The two preceding bands (3006-2854 cm^{-1} and 1746-1654 cm^{-1}) as well as the fingerprint region (1464-983 cm^{-1}), represent a number of vibrational modes depending on the predominant and most responsive functional groups of that region. For example, a small shoulder-like band at 3006 cm^{-1} is due to the stretching vibration of cis double-bond, as expected in any natural edible oil. Asymmetric and symmetric stretching vibration of CH_2 aliphatic functional group is responsible for the bands at 2924 and 2854 cm^{-1}. Oils are esters of unsaturated fatty acids and the bands representing ester carbonyl functional group are found at 1746 cm^{-1}, while the weak band at 1654 cm^{-1} is associated with C=C vibration. Bending and rocking vibrations are the common modes of vibration within the fingerprint regions (1464-983 cm^{-1}) (Guillén & Cabo, 2002).

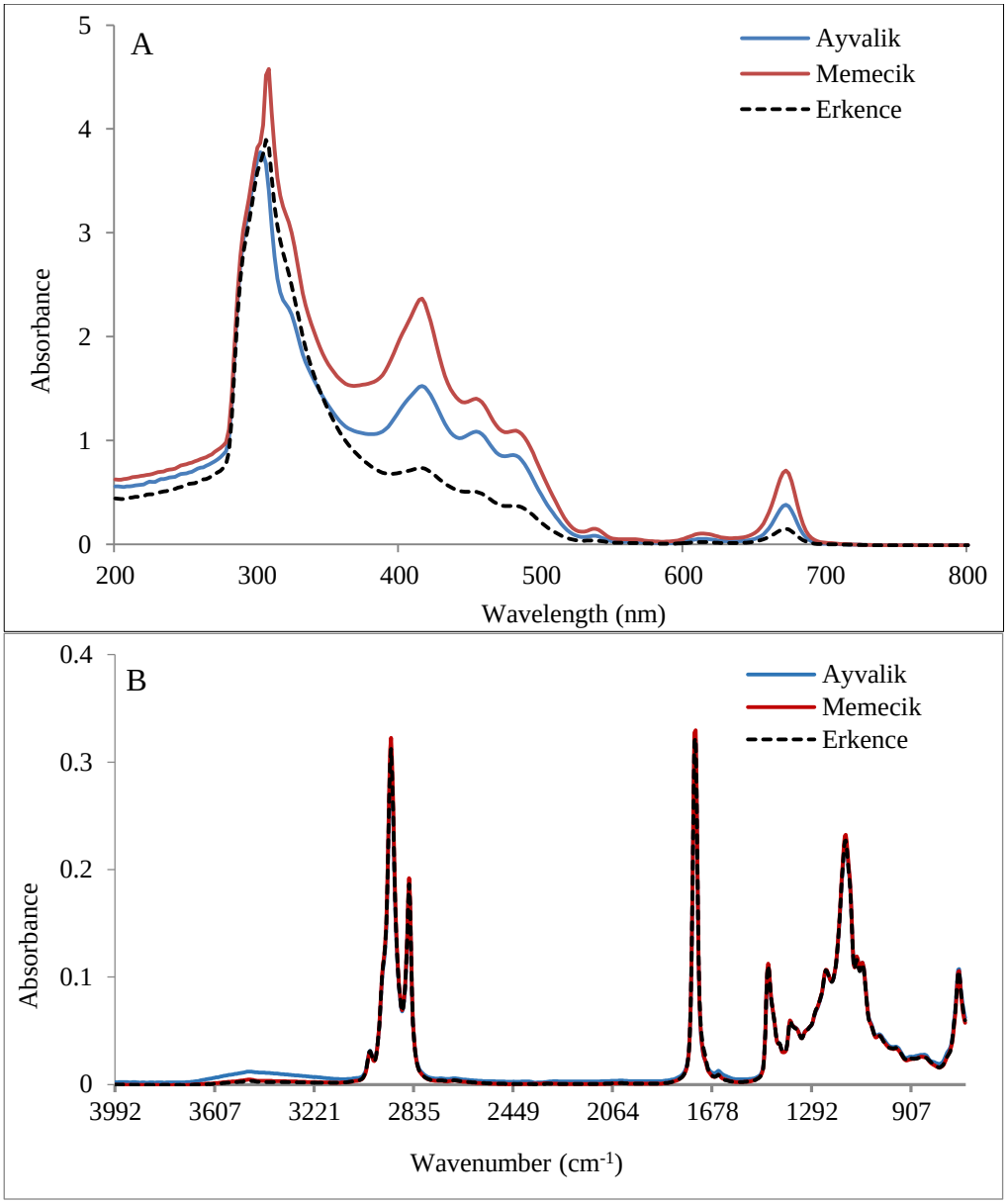

Figure 6.1. Raw UV-vis (A) and MIR (B) spectra olive oils of Ayvalik, Memecik and Erkence varieties

6.2. PCA Data Visualization

Prior to the multivariate analysis, all spectra matrices were subjected to standard normal variate (SNV) transformation in conjunction with second order derivative (2der) filtering techniques. The selection of these methods was largely due to the nature of the data and prior experience. SNV is a simple row-oriented spectra transforming algorithm effective for scattering correction by using centering and scaling of

individual spectra. This helps to minimize spectra matrix dimensionality effects by normalizing the variables to the same scale (Zeng, Huang, Xu, Ma, & Wu, 2016)..

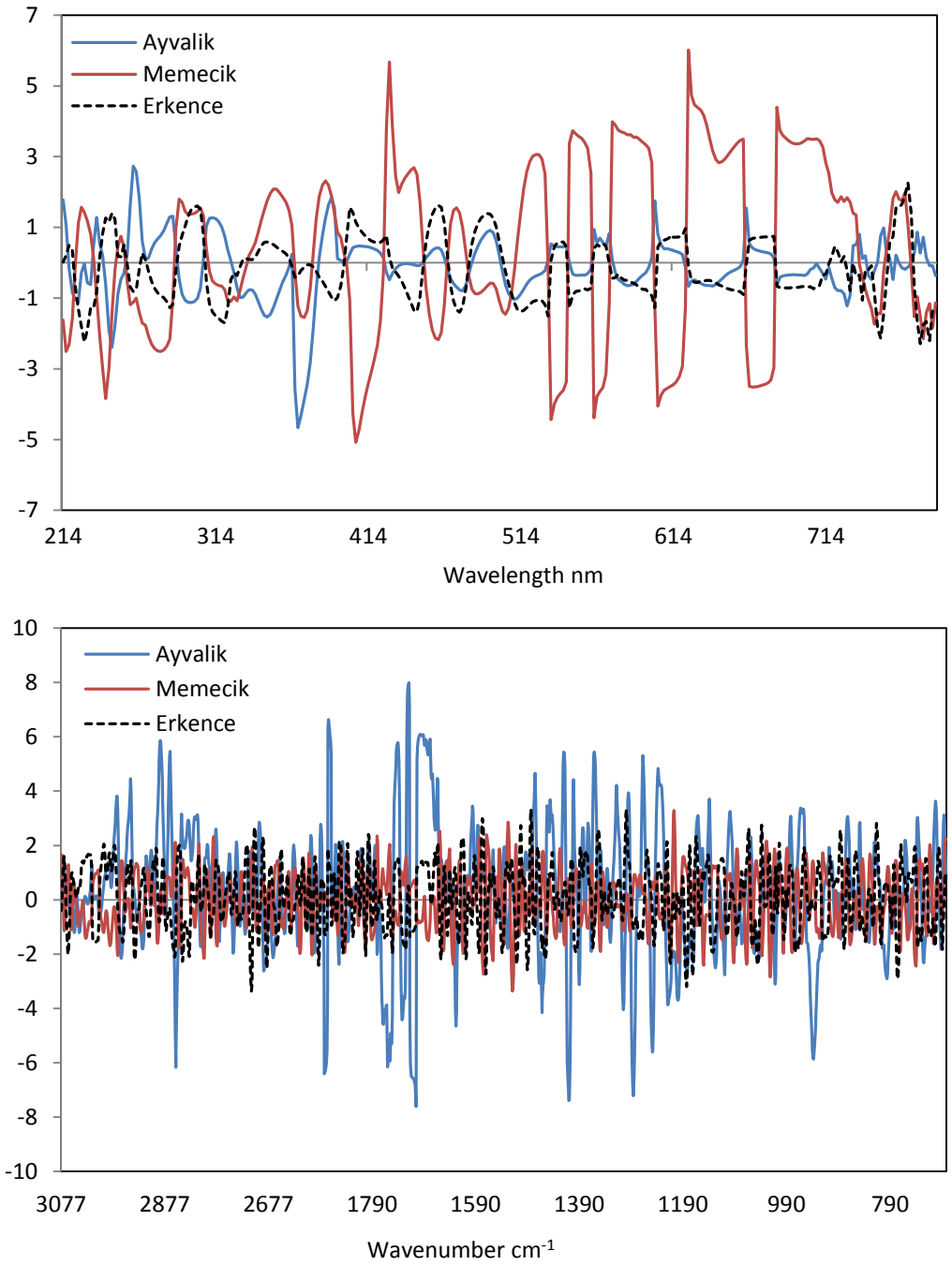

Figure 6.2. SNV+2der pretreated UV-vis (A) and MIR (B) spectra of olive oils of Ayvalik, Memecik and Erkence varieties.

Second order derivative on the other hand is calculated by obtaining the differences between two consecutive points, by smoothing, specified gap distance or Savitzky-Golay polynomial fitting. This improved signal-to-noise ratio by the removal of

random noise (Xu et al., 2008). Fig. 6.2 shows the transformed spectra output after application of these noise-counseling techniques

Being the first step in data exploratory process, PCA is expected to give brief information on the natural clustering pattern of each matrix to visualize observable trends among observations and determine the total amount of variance represented in the model. PCA score plots of three data matrices, revealing patterns of projection of observations are shown in Fig 6.3. The common observation to all the models is the sufficiency of PCA to clearly distinguish oils of Erkence variety from others. There are varying degrees of overlap between Ayvalik and Memecik olive oil samples. PCA model of the mid-infrared (MIR) data with 9 PCs, R^2_{cal} of 0.56 and R^2cv of 0.37 and that of fused-spectra (UV-IR) with 8PCs, R^2_{cal} of 0.71 and R^2cv of 0.64 created visually similar score plots with clear separation of Erkence olive oils (Fig. 6.3B and 6.3C). UV-vis sparingly showed some discrepancy between oils of Memecik and Ayvalik. Mid-infrared could not provide a clear observable difference within oils of Ayvalik variety. On the other hand, UV-vis PCA score plot suggests some intrinsic sub-classes within oils of Ayvalik variety. This is evidenced in the cluster of oils labelled as "Ae" (Ayvalik-early harvest) at the upper part of the ellipse and some Memecik oils at the left hand corner.

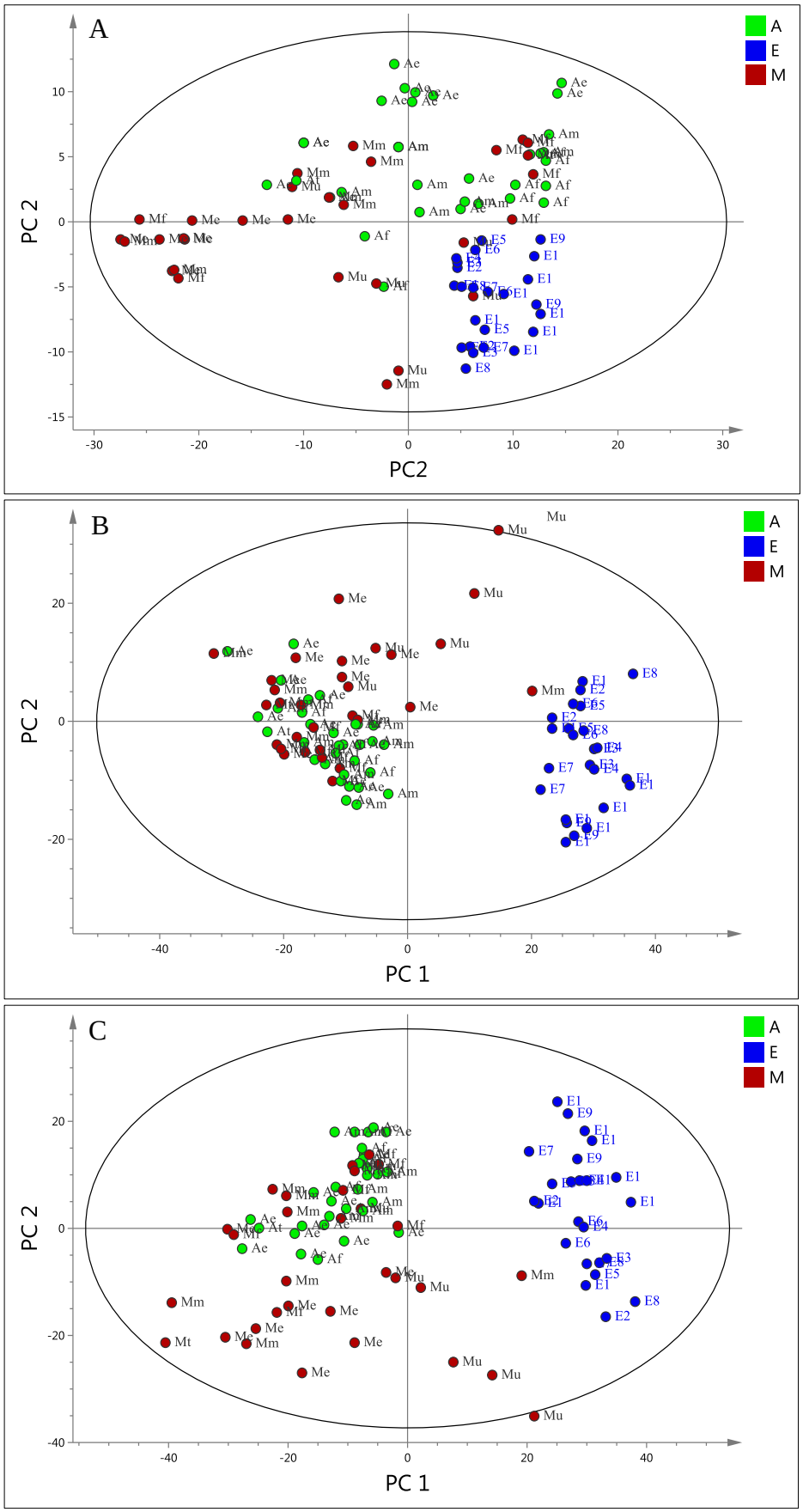

Figure 6.3. PCA score plots of SNV+2der treated UV-vis (A), MIR (B) and combined-spectra (UV-IR) of oils of Ayvalik, Memecik and Erkence varieties

6.3. Varietal Discrimination

OPLS-DA models were built with classes 1, 2 and 3 ascribed to oils of Ayvalik, Erkence and Memecik varieties, respectively, where 88 olive oil samples of known varieties were considered (Fig. 6.4). 60 samples were used in the calibration model (training) and 28 randomly selected samples as external validation set (test). Numerical representation of the output of each model is given as percentage correct classification and coefficient of calibration (R^2_{cal}) and cross-validation (R^2cv) for UV-vis, MIR and combined UV-IR spectra in Table 6.1 and 6.2. The varietal discrimination (A, M and E) with UV-vis, MIR and UV-IR provided over 95% correct classifications in each class for both calibration and validation. There was no misclassified observation between the three classes of olive oil in UV-vis and combined UV-IR models, with each having 100% correct classification in calibration and validation. In comparing models performances, UV-vis gave higher coefficients of calibration and cross-validation values (0.94 and 0.74 respectively) than MIR models (R^2 of 0.86 and R^2cv of 0.51) whereas combined spectra produced R^2 of 0.97 and R^2cv of 0.74, showing the influence of UV-vis in enhancing the model performance. Oliveri et al. (2011) showed the high predictability of class models by UV-vis spectra. This synergistic benefit of concatenated spectra is apparent because it involves fusion of the most informative part of UV-vis and MIR (Borràs et al., 2015). Visual distinctions between the score plots show significant improvements in UV-IR models. Observation projections in score plot of combined spectra shows a more distinct cluster between Ayvalik and Memecik oils compared to UV-vis and MIR alone. These discriminant analysis results align with the previous studies. Sinelli et al., (2010) observed sensitivity above 90% for LDA three-class modelling of olive oils. Similarly, correct classification of up to 100% was observed by LDA class models using UV-vis spectra and different spectra by others (Casale, Armanino, Casolino, & Forina, 2007).

Table 6.1. OPLS-DA model parameters for varietal discrimination

OPLS-DA models	PC	R^2_{cal}	R^2cv
UV-vis_SNV+2der	2+8	0.94	0.74
FT-IR_SNV+2der	2+2	0.86	0.51
UV-IR_SNV+2der	2+4	0.97	0.74

Table 6.2. Correct classification rates of OPLS-DA models with respect to variety for calibration and validation sets

Model	Member	A	M	E	%CC[a]
Calibration					
UV-vis_SNV+2der					
A	22	22	0	0	100
M	21	0	21	0	100
E	17	0	0	17	100
Average	60	22	21	17	100
FT-IR_SNV+2der					
A	22	22	0	0	100
M	21	1	20	0	95.2
E	17	0	0	17	100
Average	60	23	20	17	98.4
UV-IR_SNV+2der					
A	22	22	0	0	100
M	21	0	21	0	100
E	17	0	0	17	100
Average	60	22	21	17	100
Validation					
UV-vis_SNV+2der					
A	11	11	0	0	100
M	10	0	10	0	100
E	7	0	0	7	100
Average	28	11	10	7	100
FT-IR_SNV+2der					
A	11	11	0	0	100
M	10	0	9	1	90
E	7	0	0	7	100
Average	28	11	9	8	96.7
UV-IR_SNV+2der					
A	11	11	0	0	100
M	10	0	10	0	100
E	7	0	0	7	100
Average	28	11	10	7	100

[a] %CC: percentage of correct classification

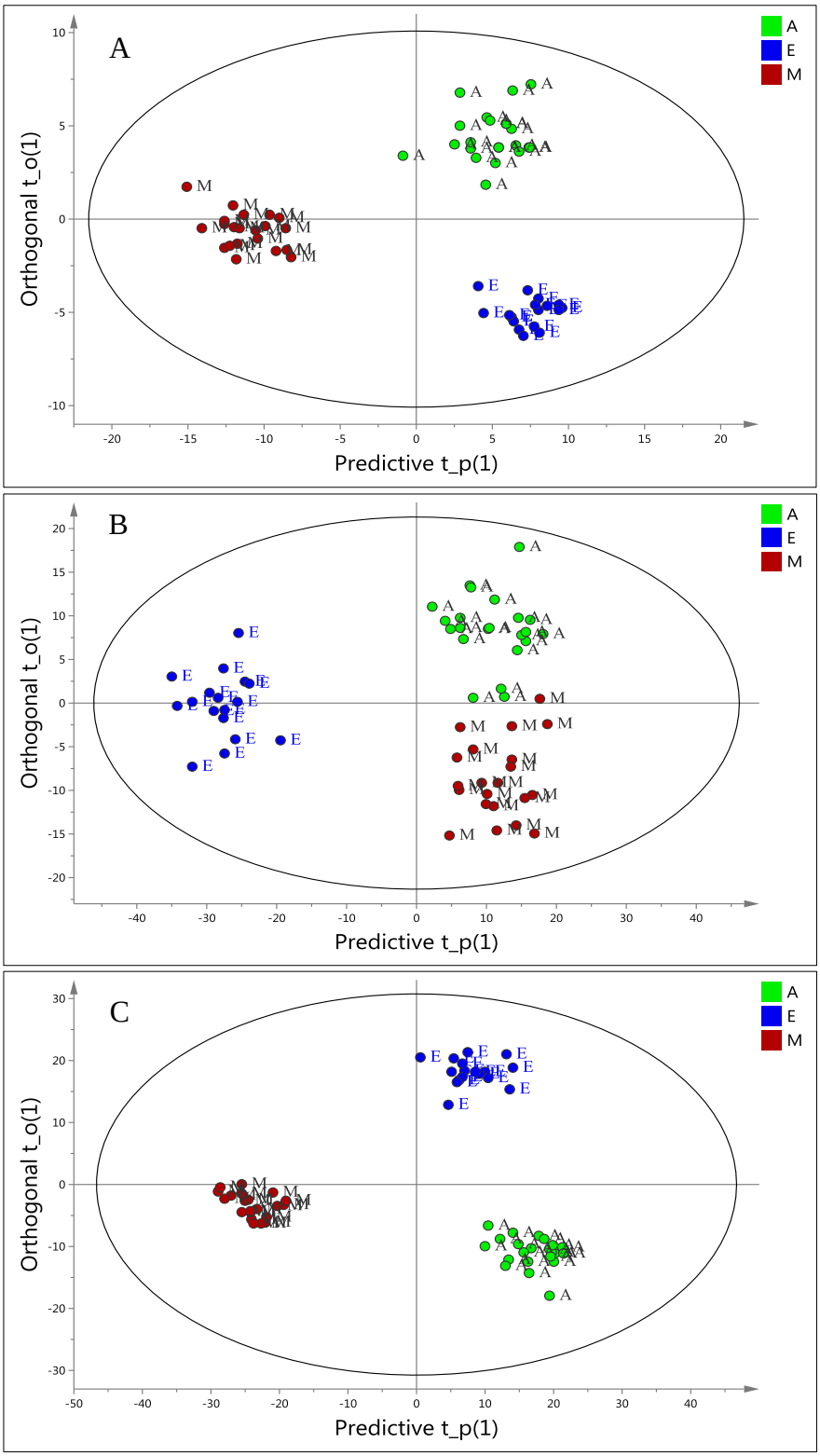

Figure 6.4. Score plots of OPLS-DA calibration models with UV-vis (A), MIR (B) and combined spectra UV-IR (C) with respect to variety (Ayvalik, Memecik and Erkence)

6.4. Harvest Year Discrimination

UV-vis and MIR spectra of 103 olive oil samples obtained from four harvest years (2012, 2014, 2015 and 2016) were evaluated using OPLS-DA. User-defined Y variables are coded as 2, 4, 5 and 6 representing samples obtained in 2012, 2014, 2015 and 2016, respectively. 30 observations were used to validate the predictability of the models. These observations were selected from classes with reasonably high number of samples (2012, 2015 and 2016). Due to the fewer number of samples in 2014, it was not included in the validation set. The performances of OPLS-DA model for each spectroscopic technique (UV-vis and MIR) and their combination (UV-IR) are presented in model output and correct classification tables (Table 6.3 and 6.4). The model of UV-vis spectra that explained about 76% of the total variance with R^2cv of 0.36 is sufficient to classify olive oils with almost absolute precision (Fig 6.5). It is known that, when the number of class categories considered in discriminant analysis is high, a proper balance in the number of observations per class must be ensured (Forina et al., 2015). Despite this short-coming, UV-vis was able to correctly classified oils of 2014 class containing just four observation as compared to MIR. MIR gave a 100% correct classification of 2012 oils compared to 96% in UV-vis. Sensitivity is the percentage of samples of the modelled class that are correctly accepted by the class model and specificity measures the percentage amount of members of other class rejected by the model of the class under consideration (Javidnia, Parish, Karimi, & Hemmateenejad, 2013). Sensitivity of 100% was achieved by MIR with respect to oils of 2012 compared to 91% for UV-vis.

OPLS-DA model of the unified matrix (UV-IR) created visually better class projection as shown in the score plots (Fig. 6.5) and higher discriminant capacity (Table 6.4). There was no misclassification in all the observation categories in classification and validation models with each class perfectly separated with 100% correct classification. The model of UV-IR have slightly better total variance represented (0.87) and higher coefficient of cross validation (0.63). Theoretically, combined spectra are expected to improve the precision of individual models constituting it, because of its all-inclusive nature. Pizarro et al., (2013) recorded better model outputs when visible spectra and quality parameters are combined.

Table 6.3. OPLS-DA model parameters for harvest year discrimination

OPLS-DA models	PC	R^2_{cal}	R^2cv
UV-vis_SNV+2der	3+3	0.76	0.36
FT-IR_SNV+2der	2+3	0.68	0.46
UV-IR_SNV+2der	3+7	0.87	0.63

Table 6.4. Correct classification rates of OPLS-DA models with respect to harvest year for calibration and validation sets

Model	Member	2012	2014	2015	2016	%CC[a]
Calibration						
UV-vis_SNV+2der						
2012	25	24	0	0	1	96
2014	4	0	4	0	0	100
2015	21	0	0	21	0	100
2016	23	0	0	0	23	100
Average	73	24	4	21	24	99
FT-IR_SNV+2der						
2012	25	25	0	0	0	100
2014	4	3	0	0	1	0
2015	21	0	0	21	0	100
2016	23	0	0	0	23	100
Average	73	28	0	21	24	75
UV-IR_SNV+2der						
2012	25	25	0	0	0	100
2014	4	0	4	0	0	100
2015	21	0	0	21	0	100
2016	23	0	0	0	23	100
Average	73	25	4	21	23	100
Model	Member	2012	2015	2016	%CC [a]	
Validation						
UV-vis_SNV+2der						
2012	11	10	1	0	91	
2015	9	0	9	0	100	
2016	10	0	1	9	90	
Average	30	10	11	9	94	
FT-IR_SNV+2der						
2012	11	11	0	0	100	
2015	9	0	9	0	100	
2016	10	0	1	9	90	
Average	30	11	10	9	97	
UV-IR_SNV+2der						
2012	11	11	0	0	100	
2015	9	0	9	0	100	
2016	10	0	0	10	100	
Average	30	11	9	10	100	

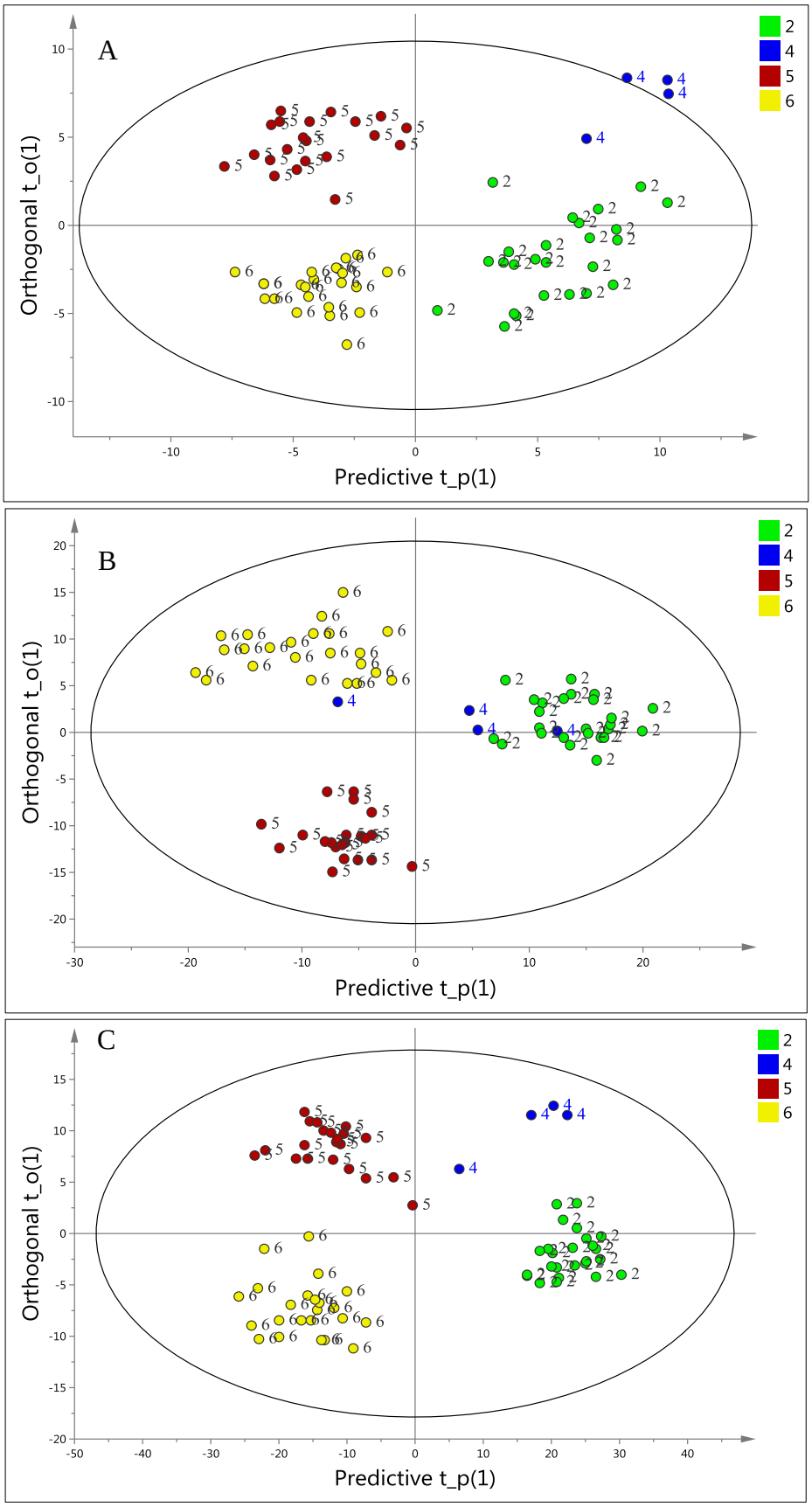

Figure 6.5. Score plots of OPLS-DA calibration models with UV-vis (A), MIR (B) and combined spectra UV-IR (C) with respect to harvest year: **2 (2012), 4 (2014), 5 (2015)** and **6 (2016)** harvest years

6.5. Prediction of some Chemical Parameters Using UV-vis and MIR Spectroscopy.

OPLS regression technique was applied to evaluate and compare the predictive capacities of MIR and UV-vis spectra. Total of sixty three olive oil samples were used in the analysis. The samples were separated into training (40 observations) and validation (23 observations) sets. Models were built separately for different variable categories. Variables considered were selected based on their significance in determining overall quality characteristics of olive oils. Table 6.5 shows the minimum, maximum and median values of each of the chemical parameters evaluated. Total phenol content (TPC) and individual phenols belonging to the group of phenolic alcohols (hydroxytyrosol and tyrosol), phenolic acids (total phenolic acids), flavonoids (luteolin and apigenin) and lignin (pinoresinol) were considered together. Fatty acids profiles includes MUFA, PUFA, SFA, ratios of MUFA/PUFA and oleic/linoleic acids were combined with alkyl esters and wax as lipid-based variables Alkyl esters includes total ethyl esters (FAEE), total methyl esters (FAME), total ethyl and methyl esters (FAAE) and total wax content. The last category consists of oxidative stability index (OSI), quality variables (FFA and K values) and color properties (chlorophylls and carotenoids pigments).

The most informative mid-infrared vibrational bands regions that are responsive to the functional groups in olive oil (3084-2563 cm^{-1} and 1874-675 cm^{-1}), with high signal to noise ratio were selected for MIR variables prediction (Gurdeniz & Ozen, 2009). The whole UV-vis range of 200-800 nm was used because of its slightly higher performance than the reduced form. The chemical information embedded in an absorption spectrum is revealed in the intensity and position of the absorption band. The second derivatives in conjunction with Standard Normal Variate spectra filterings were more efficient for spectroscopic variables prediction and it was applied in MIR, UV-vis and their combined spectra. The performances of statistical models are presented in Tables 6.6-6.8.

Table 6.5. Chemical variables predicted using spectroscopic data

Variables	Min.	Max.	Median
Phenols (mg/kg)			
TPC	233.25	504.75	325.84
Hyt	0.65	32.48	5.85
Tyr	0.58	39.26	6.62
O-der	1.23	62.32	12.04
Lut	0.29	14.99	3.20
Apg	0.58	7.59	2.76
Pin	3.34	45.39	12.56
TPA	0.52	13.48	2.83
Fatty acids (%)			
MUFA	66.27	74.12	69.32
PUFA	8.95	18.20	13.71
MU/PU	3.63	8.19	5.10
18:1/18:2	3.76	8.69	5.32
SFA	14.6	19.66	16.14
Alkyl & wax esters (mg/kg)			
FAEE	5.16	420.15	22.22
FAME	4.16	271.67	54.86
FAAE	9.32	691.82	118.94
Wax	17.39	145.36	64.15
Quality parameters			
OSI (hr)	1.24	9.00	3.93
FFA (%)	0.28	5.40	1.98
Chl (mg/kg)	0.36	7.72	2.46
Car (mg/kg)	0.67	3.12	1.33
L*	74.41	96.07	91.70
b*	43.25	114.2	66.87
K_{232}	2.04	2.77	2.51
K_{270}	0.08	0.34	0.22

6.5.1. Prediction of Phenols

Hydroxytyrosol and tyrosol were reasonably well predicted by MIR and combined spectra. MIR spectra produced a model slightly better than the combined spectra in predicting hydroxytyrosol with R^2 calibration of 0.94 and R^2cv of 0.47. Tyrosol on the other hand is predicted by combined spectra (UV-IR) with 98% total variance and R^2cv of 0.33 (Table 6.4). Luteolin is one of the major phenolic compounds belonging to flavonoid group that was characterized in the oil samples with

an average value of 3.9 mg/kg. OPLS model of MIR and combined spectra gave satisfactory prediction of luteolin with R^2_{cal} of 0.95, R^2cv of 0.45 and R^2_{cal} of 0.99, R^2cv of 0.40 respectively and relatively close values of $RMSE_{cal}$ and $RMSE_{Val}$ in both cases as shown in Fig. 6.6 and Table 6.6. A similar result was obtained in the case of apigenin when modeled with combined spectra. However the prediction of pinoresinol and total phenolic acid (TPA) could not be sufficiently validated as the coefficient of cross-validation in both variables were very low in all the three spectra models. UV-vis spectra produced the most acceptable prediction of total phenol content; slightly different from MIR (Tables 6.6). The total variance in the model (R^2_{cal} of 0.85) and the R^2cv of 0.30 were comparatively higher than others. The difference in RMSEcal and RMSEval observed in TPC was almost the same with that of Mailer, (2004), who achieved marginal success using NIR. Intrinsic variations that are common to the reaction kinetic during total phenol determination have been attributed to its low predictability spectroscopically (Cerretani et al., 2010).

If the coefficient of cross-validation (R^2cv) as a regression model parameter is below 0.50 (as it is in the case of some phenolic compounds) it is considered low when dealing with variable prediction and validation. However, in some frequently encountered circumstances, the value of this parameter depends strongly on the individual samples composed in the SIMCA internal and external validation subsets (Triba, Le Moyec, Amathieu, Goossens, Bouchemal, Nahon, Rutledge, & Savarin, 2015). This was probably the case in the evaluation of phenolics. Another plausible reason is the low number of observations in both training and validation sets, because the performance of poorly a predictive model is usually enhanced by large datasets.

Table 6.6. OPLS prediction of phenolic compounds with MIR, UV-vis and UV-IR combined spectra

Variable	Mean	PC	R^2_{cal}	R^2cv	RMSEcal	RMSEval	Equation
MIR spectra							
TPC	324.13	1+2	0.85	0.27	26.94	47.55	$y = x - 4.56*10^{-6}$
Hyt	9.19	1+2	0.94	0.47	2.34	6.68	$y = x + 5.56*10^{-7}$
Tyr	12.68	1+2	0.91	0.33	3.06	8.22	$y = x - 6.00*10^{-7}$
O-der	21.87	1+2	0.95	0.43	4.49	13.64	$y = x + 6.89*10^{-7}$
Lut	3.9	1+2	0.95	0.45	0.75	2.05	$y = x + 2.24*10^{-7}$
Apg	3.05	1+3	0.95	0.05	0.37	1.21	$y = x - 4.56*10^{-8}$
Pin	14.67	1+5	0.99	0.03	0.53	9.73	$y = x + 5.81*10^{-7}$
TPA	3.65	1+5	0.99	0.04	0.12	1.98	$y = x - 1.03*10^{-7}$
UV-vis spectra							
TPC	324.13	1+5	0.85	0.30	28.72	60.96	$y = x + 1.16*10^{-6}$
Hyt	9.19	1+1	0.51	0.33	6.78	6.68	$y = x - 3.19*10^{-7}$
Tyr	12.68	1+2	0.6	0.25	6.52	11.59	$y = x - 1.33*10^{-7}$
O-der	21.87	1+2	0.5	0.35	13.54	18.14	$y = x - 1.11*10^{-6}$
Lut	3.9	1+2	0.68	0.35	1.94	2.40	$y = x - 2.00*10^{-7}$
Apg	3.05	1+2	0.60	0.18	1.02	1.83	$y = x - 1.14*10^{-7}$
Pin	14.67	1+2	0.53	0.05	6.24	9.11	$y = x - 6.31*10^{-7}$
TPA	3.65	1+1	0.43	0.18	2.09	2.15	$y = x +1.64*10^{-7}$
UV-IR spectra							
TPC	324.13	1+3	0.91	0.12	20.98	44.5	$y = x - 1.51*10^{-5}$
Hyt	9.19	1+3	0.96	0.39	2.08	6.68	$y = x + 5.62*10^{-7}$
Tyr	12.68	1+4	0.98	0.33	1.63	10.56	$y = x + 5.62*10^{-7}$
O-der	21.87	1+7	0.99	0.44	0.56	16.95	$y = x - 3.46*10^{-7}$
Lut	3.9	1+6	0.99	0.40	0.18	2.31	$y = x - 2.35*10^{-7}$
Apg	3.05	1+5	0.99	0.39	0.11	1.50	$y = x + 2.09*10^{-7}$
Pin	14.67	1+2	0.90	0.10	2.85	8.67	$y = x - 1.90*10^{-6}$
TPA	3.65	1+6	0.99	0.04	0.08	1.61	$y = x + 1.40*10^{-7}$

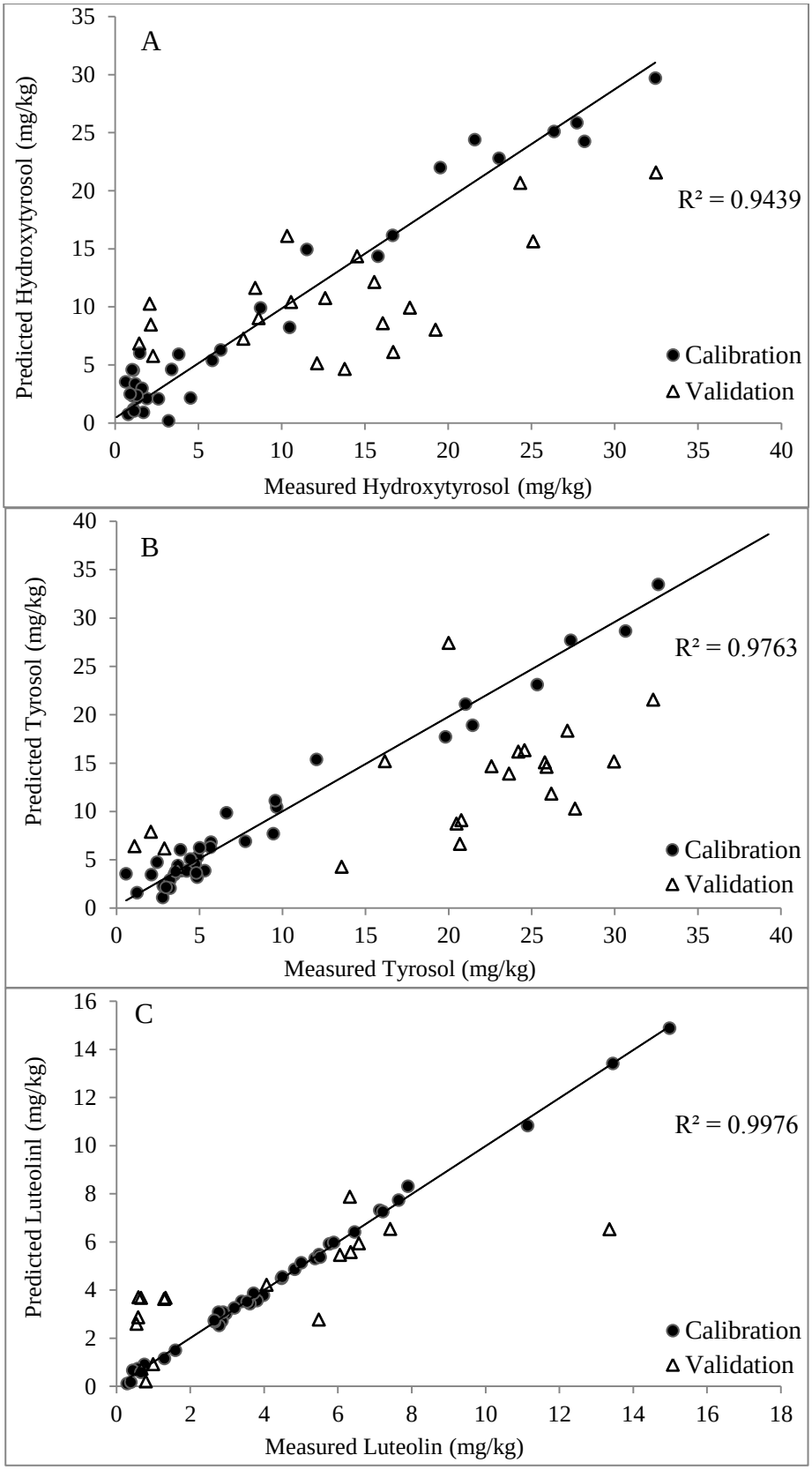

Figure 6.6. Results of OPLS regression for hydroxytyrosol with MIR (A), tyrosol (B) and luteolin with combined UV-IR (C) spectra

6.5.2. Prediction of Fatty Acids

Saturated fatty acids such as palmitic, heptadecanoic, stearic, arachidic and behenic acids were summed up as total saturated fatty acid (SFA), palmitoleic, Cis-10-heptadecanoic, Cis-11-eicosenoic and oleic acids as total monounsaturated fatty acid (MUFA) and linoleic and linolenic as polyunsaturated (PUFA). Therefore, the table invariably contained all the fatty acids quantified in the oil samples (Tables 6.5). Oleic acid constitutes more than 98% of monounsaturated fatty acids in olive oil. A preliminary assessment of the predictive abilities of these spectroscopic techniques showed no difference between oleic acid and MUFA. The same was also true for PUFA and linoleic acid MUFA and PUFA contribute positively and negatively to the oxidative stress of olive oil, respectively (Jolayemi, et al., 2016). How vulnerable olive oil is to oxidation is in part determined by the ratio of MUFA to PUFA (MU/PU) (Goncalves, et al., 2014).

Considerably, good predictive models of fatty acid profiles were obtained with each spectroscopic technique and their combination. However, the best results were obtained from MIR and combined UV-IR models with respect to MUFA, PUFA, MU/PU, and oleic/linoleic acid ratio (Fig 6.7). In MIR with respect to MUFA, R^2cal of 0.97, R^2cv of 0.83 was achieved. UV-vis was fairly satisfactory with comparatively low predictive capacity (R^2cal of 0.68, R^2cv of 0.25 and 1+2PCs). This is understandable owing to the fact that, the first and second overtones (C-H stretching vibrations) common to infrared regions are function of triglycerides; a mixture from where esters of saturated or monounsaturated fatty acids are formed. This explained why good predictions are obtained from high percentage fatty acids compared to lower ones. This is also in agreement with earlier observations (Inarejos-Garcia et al., 2013; Gurdeniz, Ozen & Tokatli, 2010; Galtier et al., 2008). The dominance effect of MIR in the fused-spectra was apparent, considering the closeness in the model output parameters obtained in MIR and UV-IR (Table 6.7). The responsiveness of UV-vis to intrinsic functional groups in olive oil is somewhat lower than MIR. Apart from the vibrational to electronic absorption differences in the energy level between MIR and UV-vis, the non-chromophore nature of fatty acids make their differences less pronounced in UV-vis spectroscopy. MUFA/PUFA and Oleic/linoleic acids ratio prediction outputs were very similar in both MIR and combined UV-IR spectra and the performance of MIR

model is better than UV-vis and the combination of the spectra did not significantly improve the model output In summary, higher percentage fatty acids composition of olive oils have better predictability compared to lower ones. MUFA, PUFA, MUFA/PUFA, C18:1/C18:2 and SFA are well predicted especially with MIR and combined spectra (Fig .6.7 and 6.8). The observations are in agreement with earlier studies (Galtier et al., 2008, Mailer, 2004).

Table 6.7. OPLS prediction of fatty acids, alkyl and wax esters with MIR, UV-vis and UV-IR combined spectra

Variable	Mean	PC	R^2_{cal}	R^2cv	RMSEcal	RMSEval	Equation
MIR spectra							
MUFA	69.8	1+2	0.97	0.83	0.46	0.63	$y = x - 1.68*10^{-5}$
PUFA	13.78	1+2	0.99	0.96	0.20	0.23	$y = x + 5.16*10^{-7}$
MU/PU	5.30	1+2	0.99	0.92	0.15	0.21	$y = x - 1.18*10^{-7}$
18:1/18:2	5.57	1+2	0.99	0.92	0.17	0.24	$y = x - 1.76*10^{-8}$
SFA	16.42	1+5	0.99	0.30	0.09	0.72	$y = x + 3.81*10^{-7}$
FAEE	112.91	1+1	0.86	0.73	41.45	43.62	$y = x - 5.71*10^{-6}$
FAME	74.04	1+1	0.83	0.66	27.26	26.02	$y = x - 3.59*10^{-6}$
FAAE	186.95	1+1	0.86	0.71	67.16	67.02	$y = x + 1.32*10^{-5}$
Wax	75.26	1+2	0.99	0.68	4.73	20.53	$y = x - 7.00*10^{-7}$
UV-vis spectra							
MUFA	69.8	1+2	0.68	0.25	1.40	1.07	$y = x + 5.10*10^{-6}$
PUFA	13.78	1+2	0.78	0.49	1.34	0.77	$y = x - 7.45*10^{-7}$
MU/PU	5.3	1+2	0.80	0.50	0.61	0.42	$y = x + 9.99*10^{-8}$
18:1/18:2	5.57	1+2	0.80	0.51	0.66	0.47	$y = 1.07x - 0.42$
SFA	16.42	1+1	0.45	0.18	0.97	0.97	$y = x + 3.46*10^{-7}$
FAEE	112.91	1+3	0.83	0.48	47.4	55.07	$y = x - 6.69*10^{-6}$
FAME	74.04	1+3	0.84	0.49	27.5	32.14	$y = x + 2.95*10^{-6}$
FAAE	186.95	1+3	0.84	0.49	73.84	84.78	$y = x - 1.11*10^{-5}$
Wax	75.26	1+6	0.97	0.55	7.45	19.47	$y = x + 5.97*10^{-6}$
UV-IR spectra							
MUFA	69.8	1+5	0.99	0.82	0.09	0.60	$y = x - 1.01*10^{-5}$
PUFA	13.78	1+2	0.99	0.94	0.34	0.49	$y = x - 4.84*10^{-7}$
MU/PU	5.3	1+3	0.99	0.91	0.24	0.38	$y = x + 9.73*10^{-8}$
18:1/18:2	5.57	1+3	0.99	0.91	0.13	0.27	$y = x - 5.06*10^{-7}$
SFA	16.42	1+3	0.95	0.24	0.29	0.55	$y = 1.00x - 0.16$
FAEE	112.91	1+2	0.89	0.63	37.82	39.46	$y = x - 3.43*10^{-7}$
FAME	74.04	1+2	0.88	0.59	23.57	23.74	$y = x + 6.01*10^{-6}$
FAAE	186.95	1+2	0.89	0.62	60.37	60.33	$y = x + 8.80*10^{-6}$
Wax	75.26	1+3	0.98	0.63	5.52	19.47	$y = x + 3.38*10^{-6}$

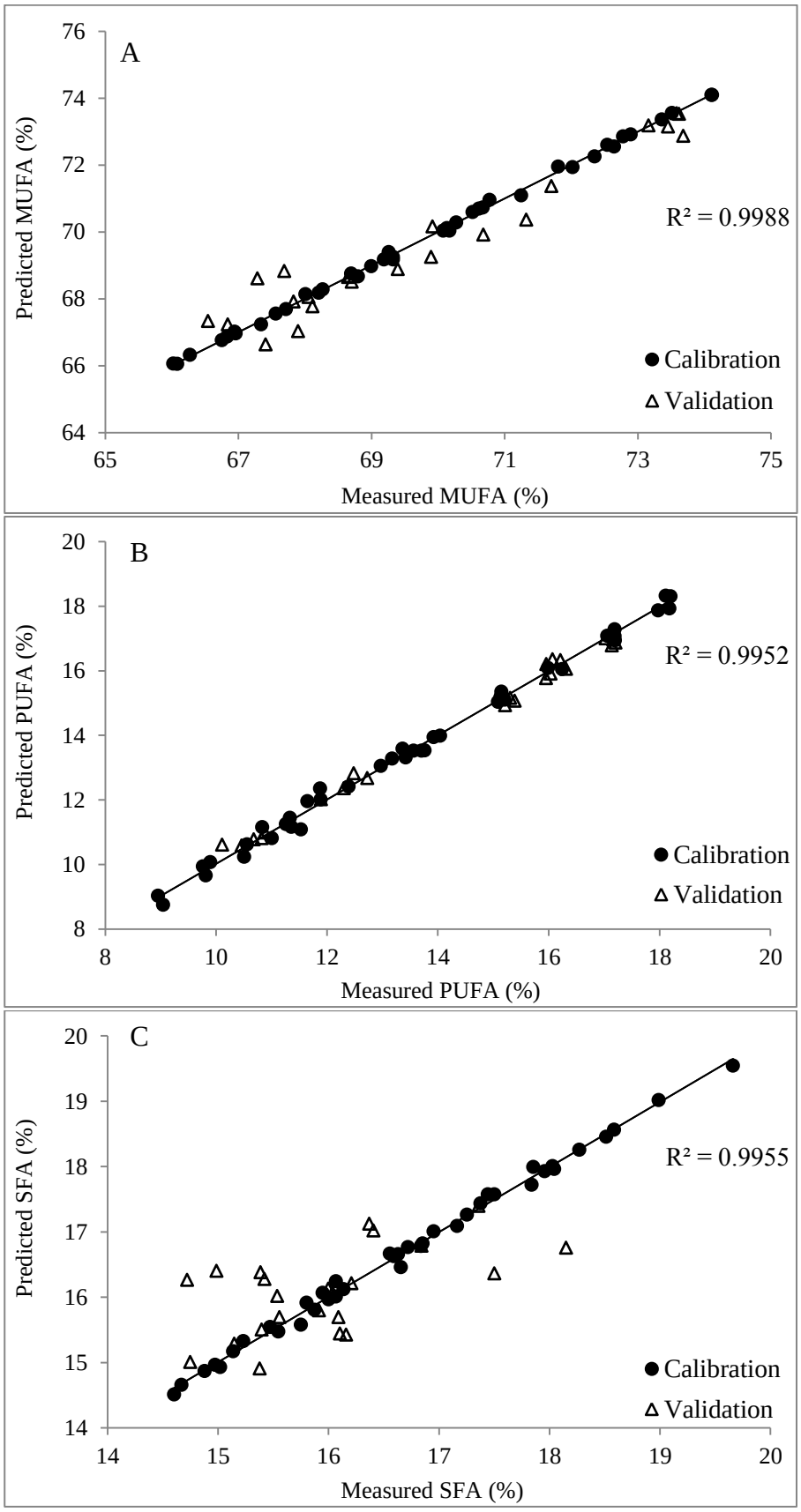

Figure 6.7. Results of OPLS regression for MUFA with combined UV-IR (A), PUFA (B) and SFA (C) with MIR spectra

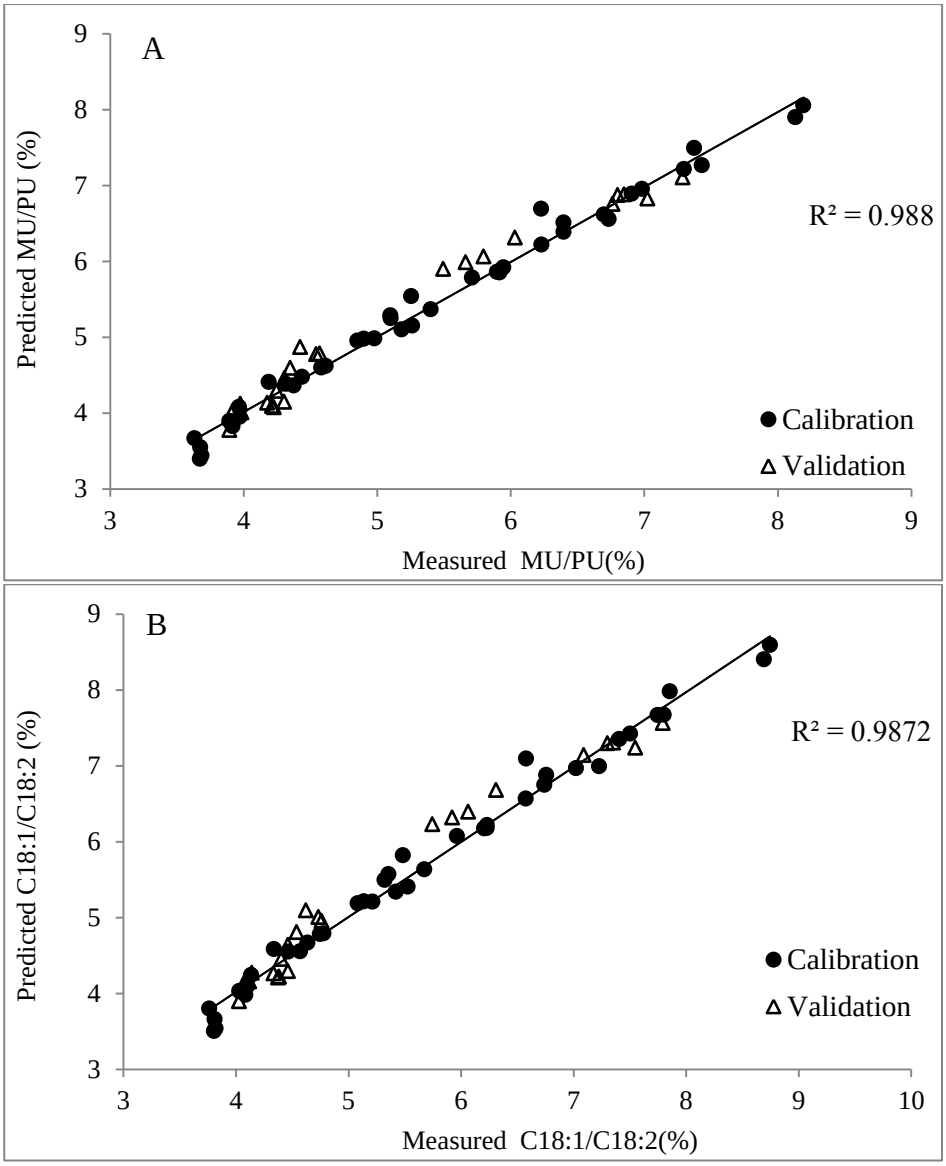

Figure 6.8. Results of OPLS regression for MUFA/PUFA (A) and oleic/linoleic acid (B) ratio with MIR spectra

6.5.3. Prediction of Alkyl Esters and Wax

Establishing the genuineness of olive oil with absolute certainty is an ever growing area of study. Apart from protecting the market share of compliant producers, quality authentication can be a significant parameter for consumers (Aroca-Santos et al., 2015). Alkyl esters and wax concentrations of olive oil are quality authentication parameters that reflect the initial state and wholesomeness of olive fruits at the moment of extraction (Perez-Camino et al., 2008). Fatty acid ethyl esters (FAEE) are the major concerns in determining the purity and quality of olive oil. Its high predictability using

this rapid spectroscopic method can be a time-saving adulteration detection procedure that can replace the tedious elution process. As shown in the Table 6.7, MIR successfully predicted total ethyl and methyl esters (FAEE and FAME). OPLS regression model for the prediction of total ethyl esters (FAEE) using MIR spectra contained 1+1PCs, 86% variance represented and R^2cv of 0.73. As shown in Fig 6.9, the prediction of FAEE content of the oils was very satisfactory. Model built on MIR spectra proved more suitable in predicting FAEE compared to UV-vis. The combined spectra did not improve the model significantly in terms of validation, due to the dominance of MIR. The same observation was true for FAAE, which is the summation of FAEE and FAME, with MIR showing better predictive capacity (86% of the total variance and R^2cv of 0.71, with 67.16 and 67.02 RMSEcal and RMSEval respectively). As in the case of fatty acid profile, MIR compatibility with lipid-based functional groups in olive oil is basically due to highly distinctive and specific vibrational fingerprint patterns that can qualitatively elucidate them. OPLS models of combined spectra gave slightly higher coefficients of calibration and lower cross validation of FAAE (0.89 and 0.62) due to the inclusion of UV-vis data. The results are in agreement with earlier observations (Valli et al., 2013), where slightly higher coefficients were found for calibration and cross validation when MIR was used in the prediction of FAAE. Despite the comparatively low number of observations and the use of external validation, the predictions were satisfactory.This shows how amenable these quality properties of olive oil are to spectroscopic qualitative evaluation.

The wax fraction of olive is composed of esters of varying degree of saturation and chain lengths, and is classified as short-chain esters, saturated wax ester, unsaturated wax ester, benzyl esters, and diterpenic esters (Reiter & Lorbeer, 2001). In this study only the long-chain esters were quantified (C40, C42, C44 and C46 wax ester) and their summation is reported as wax. As a result of considerable high content of wax in solvent-extracted olive oils, wax determination became a test of extra virginity of olive oil. Table 6.7 indicated that OPLS model of MIR has the most predictive capability for wax content with R^2cal of 0.99, and R^2cv of 0.68 (Fig. 6.9C). As observed in some other variables, synergistic effect of concatenated spectra on the performance of the model was not substantially evidenced. OPLS models of UV-vis performances were very encouraging as well.

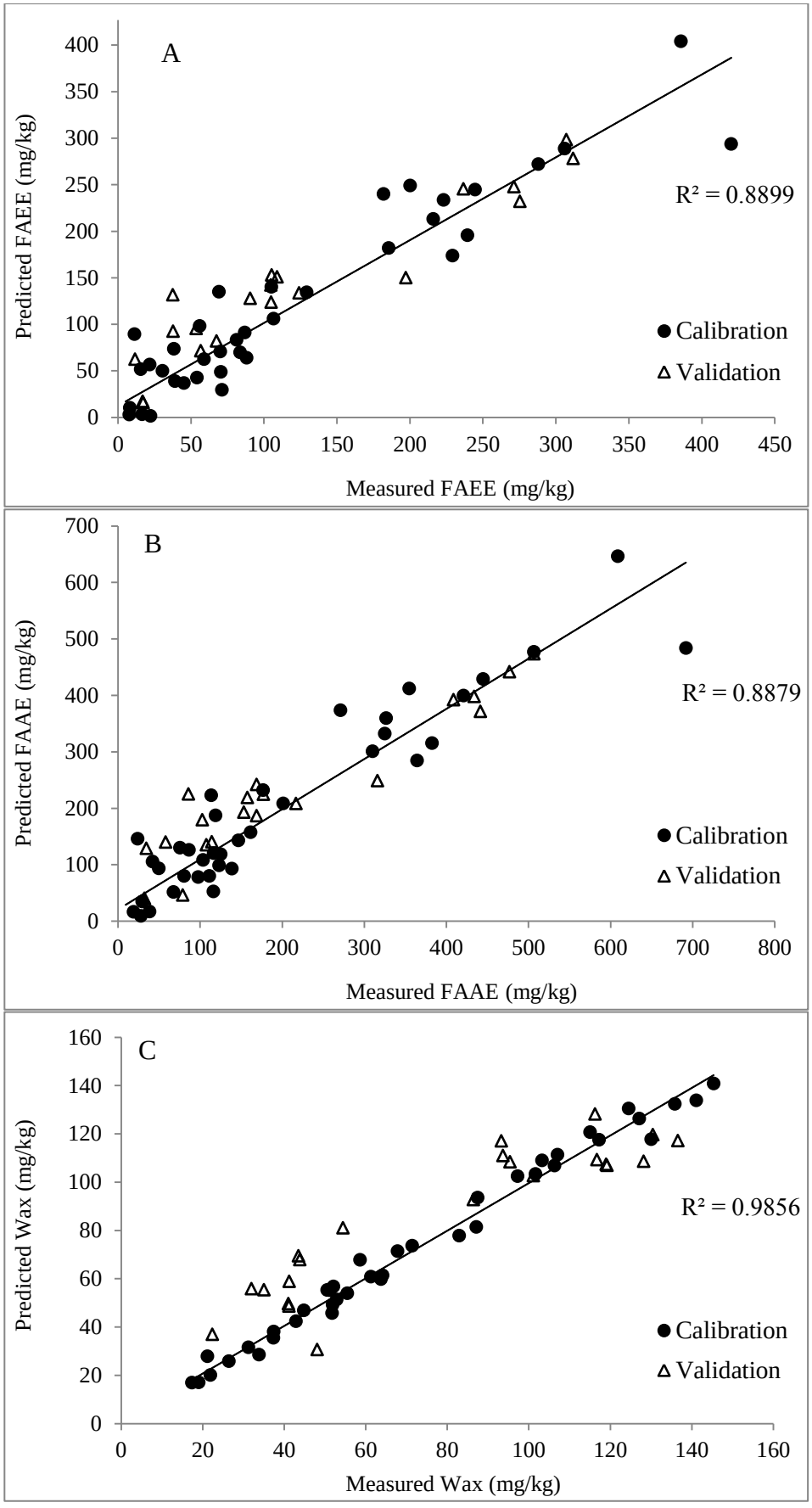

Figure 6.9. Results of OPLS regression for FAEE (A), FAAE with combined UV-IR (B) and wax with MIR (C) spectra

There are quite a number of quantitative determinations of ethyl, methyl and wax esters contents of olive oil in literature (Gómez-Coca et al., 2012; Jabeur et al., 2015; Pérez-Camino et al., 2008). However, these results can be an indication of practical and quick determination of ester and wax components and worth to be investigated further.

6.5.4. Prediction of Quality Parameters

6.5.4.1. Acidity (FFA), Oxidative Stability (OSI) and *K* values

Some of the most crucial quality differentiation parameters of olive oil that enables its classification, based on designation (grade) are FFA and ultraviolet light absorption K_{232} and K_{270} values. In addition to these standardized methods, accelerated oxidative stability test (OSI) is used to predict the shelf life of olive oil. Free fatty acids do not accumulate in healthy plant tissues. Hydrolysis of triacylglycerol by endogenous lipase enzyme is fundamentally responsible for the evolution of free fatty acids in olive oil (Clodoveo et al., 2014). The relationship between alkyl esters formation and FFA is due to this enzymatic hydrolysis. K values measure the amount of primary and secondary oxidative products in the form conjugated dienes and trienes, respectively. Linoleic and linolenic acids are 1-4 pentadiene compounds, which are suitable substrates for lipoxygenase enzyme, that form hydroperoxides and subsequent secondary degradative products (Gómez-Alonso et al., 2007). As shown in Table 6.8 OPLS models of FFA with MIR and UV-IR spectra were successful with each representing more than 90% of total variance. MIR is slightly better with R^2cv of 0.84 as against 0.61 in UV-vis model. The two models have RMSEcal and RMSEval that are very close to each other (0.38 and 0.62, 0.45 and 0.64 for MIR and UV-vis, respectively) and insignificantly small compared to the mean values of FFA in both cases. The same is true for the combined spectra with higher calibration and intermediate cross-validation coefficients (0.99 and 0.81). The regression plot of FFA is shown in Fig. 6.11A. The predictive capacity of MIR on FFA is understandable owing to the fact that, FFA measures the percentage of free oleic acid – the major fatty acid in olive oil. However, UV-vis spectroscopy is highly responsive to chromophore containing matrix and fatty acids do not possess strong chromophore in their structures (Monasterio, Fernández, & Silva, 2013). Therefore, in order to find a suitable

explanation for UV-vis prediction of oil acidity, regions of the UV spectrum were examined. These regions where major differences between olive oils (Extra virgin EVOO, virgin VOO, and lampante LOO) due to this parameter, are shown in Fig. 6.10. The most defining bands with respect to FFA occurred at visible region (400-540 nm and 600-700 nm). These two regions correspond to the absorption bands of chlorophylls and carotenoids (Aroca-Santos, Cancilla, Matute, & Torrecilla, 2015). Therefore, there is an indirect relationship between the intensity of these pigments in oil and FFA contents as shown in the Fig 6.10. The intensity of the bands decreased from EVOO to LOO. This relationship is plausible because chlorophyll and carotenoids possess antioxidant properties in the absence of light, which may delay the oxidative rancidity and subsequent FFA generation (Criado et al., 2008).

Oxidative stability index (OSI) was sufficiently predicted by OPLS model of MIR, with R^2cal of 0.99 and R^2cv of 0.60. The regression plot of OSI is shown in Fig 6.11B. Olive oil freshness has been evaluated in literature under rapid oxidative stress using MIR (Sinelli, Cosio, Gigliotti, & Casiraghi, 2007). In the same vein, a promising result was obtained when NIR spectroscopy was used in quantitative evaluation of rancimat induction time (Mailer, 2004). However, the performance of this model is fairly comparable with a recent study (Uncu & Ozen, 2015) where 0.99 and 0.81 as coefficients of calibration and cross-validation were reported. Cayuela Sanchez, Moreda & Garcia, (2013) observed larger coefficient of validation in the prediction of OSI using Vis/NIR transmittance and higher sample size. It is very most likely that the transmittance mode is more informative, because it involves the radiation going through the optical path which is the location of the sample, thereby providing more quantitative substance. Coefficients of calibration and cross-validation of 0.69 and 0.33 were obtained from OPLS model of OSI using UV-vis spectra while 0.97 and 0.49 when modelled with combined spectra, both with very low RMSEcal and RMSEval (Table 6.8).

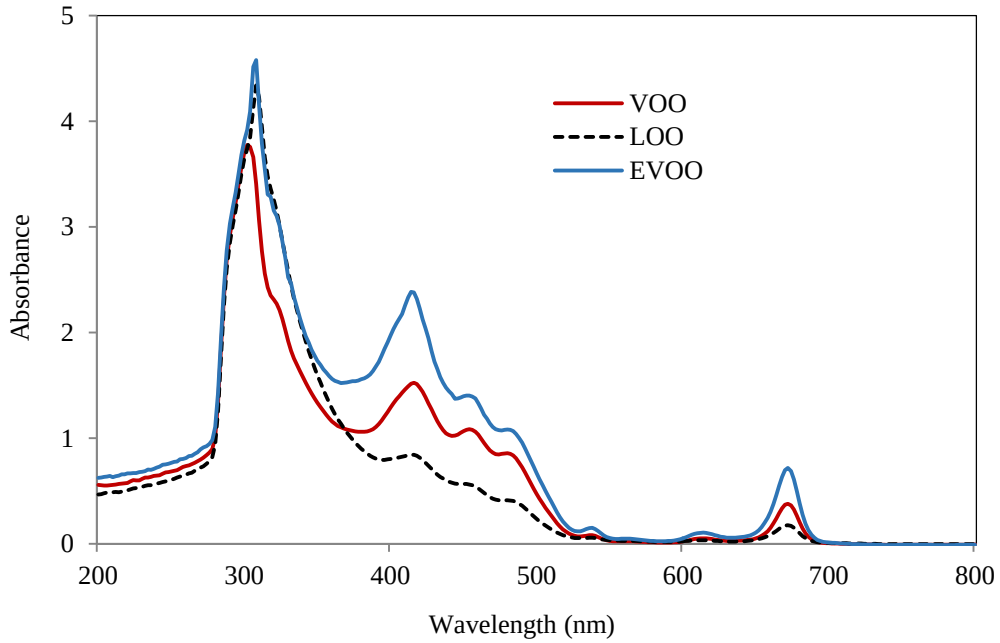

Figure 6.10. UV-vis spectra of extravirgin (EVOO), virgin (VOO) and lampante (LOO) olive oils

Prediction of K values were done using OPLS regression model of the two spectra separately and conjointly. Models built on UV-vis, MIR and combined spectra partially predicted K_{232} and K_{270} extinction values. Coefficients of calibration and cross validation obtained in fused-spectra with respect to K_{232} and K_{270} are 0.99 and 0.32 and 0.99 and 0.48 respectively. The R^2cv of the models are low in all the spectra matrices; however, a good prediction of these extinction coefficients would have helped in rapid extrapolation of possible shelf life of olive oil. This assertion is supported by others, who observed a direct correlation between K_{232} evolution and some other olive oil oxidative parameters (Gomez-Alonso et al., 2007). A significant correlation between K_{232} and oleic acid was found recently (Ruiz-Domínguez, Raigón, & Prohens, 2013). The prediction results of K_{232} in all the three cases (MIR, UV-vis and UV-IR) were slightly better than that of K_{270} (Table 6.8). The reason is probably due to the fact that, K_{232} indicates accumulation of primary oxidative products, which are usually common at early stage of oil defects. On the other hand, K_{270} is an index of secondary oxidative products that accompany advanced stage of oil deterioration.

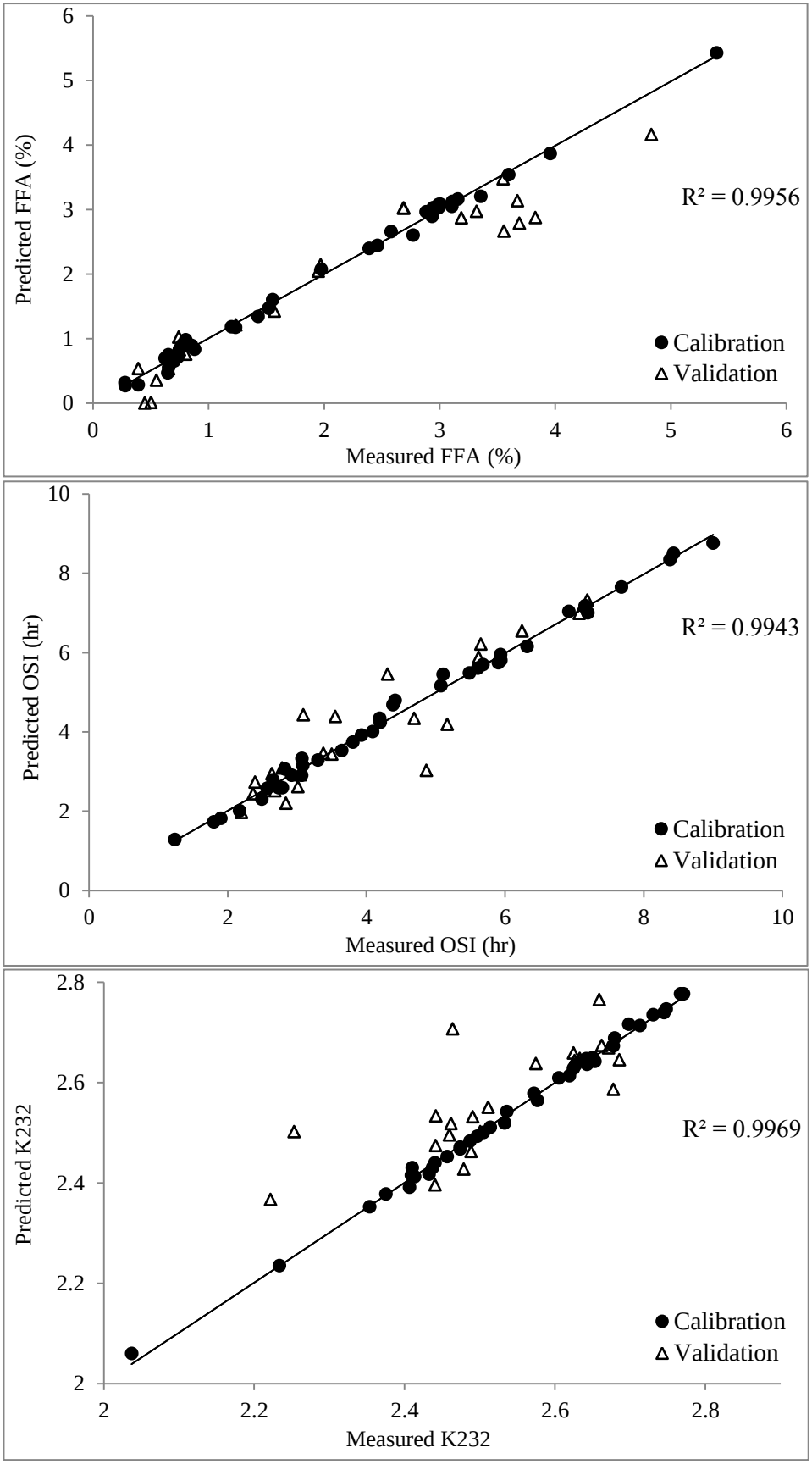

Figure 6.11. Results of OPLS regression for FFA with combined UV-IR (A); OSI with MIR (B) and K_{232} with combined UV-IR (C) spectra

Table 6.8. OPLS prediction of quality parameters with MIR, UV-vis and UV-IR combined spectra

Variable	Mean	PC	R^2_{cal}	R^2cv	RMSEcal	RMSEval	Equation
MIR spectra							
OSI	4.36	1+4	0.99	0.6	0.16	0.86	$y = x - 3.42*10^{-7}$
FFA	2.06	1+1	0.92	0.82	0.38	0.62	$y = x - 1.49*10^{-7}$
Chl	2.75	1+2	0.9	0.24	0.49	0.83	$y = x + 3.43*10^{-7}$
Car	1.45	1+2	0.92	0.33	0.18	0.24	$y = x + 7.22*10^{-9}$
L*	90.77	1+3	0.98	0.39	0.72	2.42	$y = x + 1.70*10^{-5}$
b*	69.76	1+2	0.93	0.51	4.63	9.95	$y = x + 7.84*10^{-7}$
K_{232}	2.53	1+2	0.92	0.36	0.04	0.10	$y = x - 2.66*10^{-7}$
K_{270}	0.22	1+2	0.92	0.29	0.02	0.05	$y = x + 9.94*10^{-9}$
UV-vis spectra							
OSI	4.36	1+2	0.69	0.33	1.18	0.89	$y = x + 1.49*10^{-7}$
FFA	2.06	1+3	0.89	0.61	0.45	0.64	$y = x - 7.20*10^{-8}$
Chl	2.75	1+8	0.99	0.70	0.18	0.83	$y = x - 1.11*10^{-7}$
Car	1.45	1+8	0.99	0.73	0.07	0.25	$y = x - 3.29*10^{-8}$
L*	90.77	1+7	0.98	0.65	0.82	2.9	$y = x + 6.58*10^{-6}$
b*	69.76	1+2	0.94	0.87	4.35	5.1	$y = x + 2.01*10^{-6}$
K_{232}	2.53	1+4	0.86	0.41	0.06	0.12	$y = x - 5.13*10^{-8}$
K_{270}	0.22	1+3	0.81	0.32	0.03	0.05	$y = x + 1.75*10^{-8}$
UV-IR spectra							
OSI	4.36	1+3	0.97	0.49	0.36	0.92	$y = x - 6.87*10^{-8}$
FFA	2.06	1+4	0.99	0.81	0.09	0.61	$y = x - 1.59*10^{-7}$
Chl	2.75	1+4	0.99	0.68	0.15	0.67	$y = x + 1.04*10^{-8}$
Car	1.45	1+4	0.99	0.72	0.06	0.22	$y = x + 3.86*10^{-8}$
L*	90.77	1+5	0.99	0.82	0.17	1.82	$y = x + 1.38*10^{-5}$
b*	69.76	1+2	0.97	0.92	3.07	5.15	$y = x - 6.73*10^{-6}$
K_{232}	2.53	1+6	0.99	0.48	0.01	0.09	$y = x + 3.27*10^{-8}$
K_{270}	0.22	1+6	0.99	0.32	0.00	0.05	$y = x + 1.41*10^{-8}$

6.5.4.2. Pigments Prediction

The olive oil color is directly linked to the chlorophyll and carotenoid contents. It has been proposed as a characterizing factor and as a quality index related to the oil extraction method and olive variety (Malheiro, et al., 2013). Carotenoids, besides their contribution in yellow pigmentation of fruits, vegetables and oil, are bioactive compounds linked with provitamin properties (Aparicio-Ruiz & Gandul-Rojas, 2014). Some of the valuable functional properties of carotenoid derivatives (beta carotene and beta-cryptoxanthin) as precursor of vitamin-A include the ability to prevent age-related degeneration and eyes defect formation in the case of lutein (Lopez et al., 2014). The

degree of acceptability of olive oil to consumer, mostly depend on superficial characteristics of the oil; color inclusive. Chlorophyll and carotenoid contribute to this positive visual appeal of olive oil.

High chromophore-containing chemical compounds like these pigments, are highly compatible with spectroscopic analysis; especially at ultraviolet and visible regions. OPLS model of UV-vis gave better prediction of chlorophyll and carotenoids content of the oil samples than MIR and their combination. According to the earlier evaluation of Aroca-Santos, Cancilla, Matute, & Torrecilla (2015) chlorophyll (420-670 nm) and carotenoid (470 and 495 nm) contents of olive oils produced the absorption bands observable in the visible spectra. As shown in Fig 6.12, UV-vis and MIR spectra of three olive oil samples corresponding to extra virgin, virgin and lampante oils are plotted with 0.66, 1.20 and 3.16 FFA, respectively. The intensity of the band between 370-520 nm and 600-700 nm in UV-vis spectra helped in the separation of the oils into their quality categories. However, these visual differences in the spectra are not obvious in MIR. This explained the better performance of UV-vis models (Table 6.8). A similar calibration coefficient was obtained in case of combined spectra (0.99) showing the dominance of UV-vis in the model. OPLS calibration curve of chlorophyll and carotenoids with respect to UV-vis spectra showed a reliable predictability of these parameters (Fig 6.13).

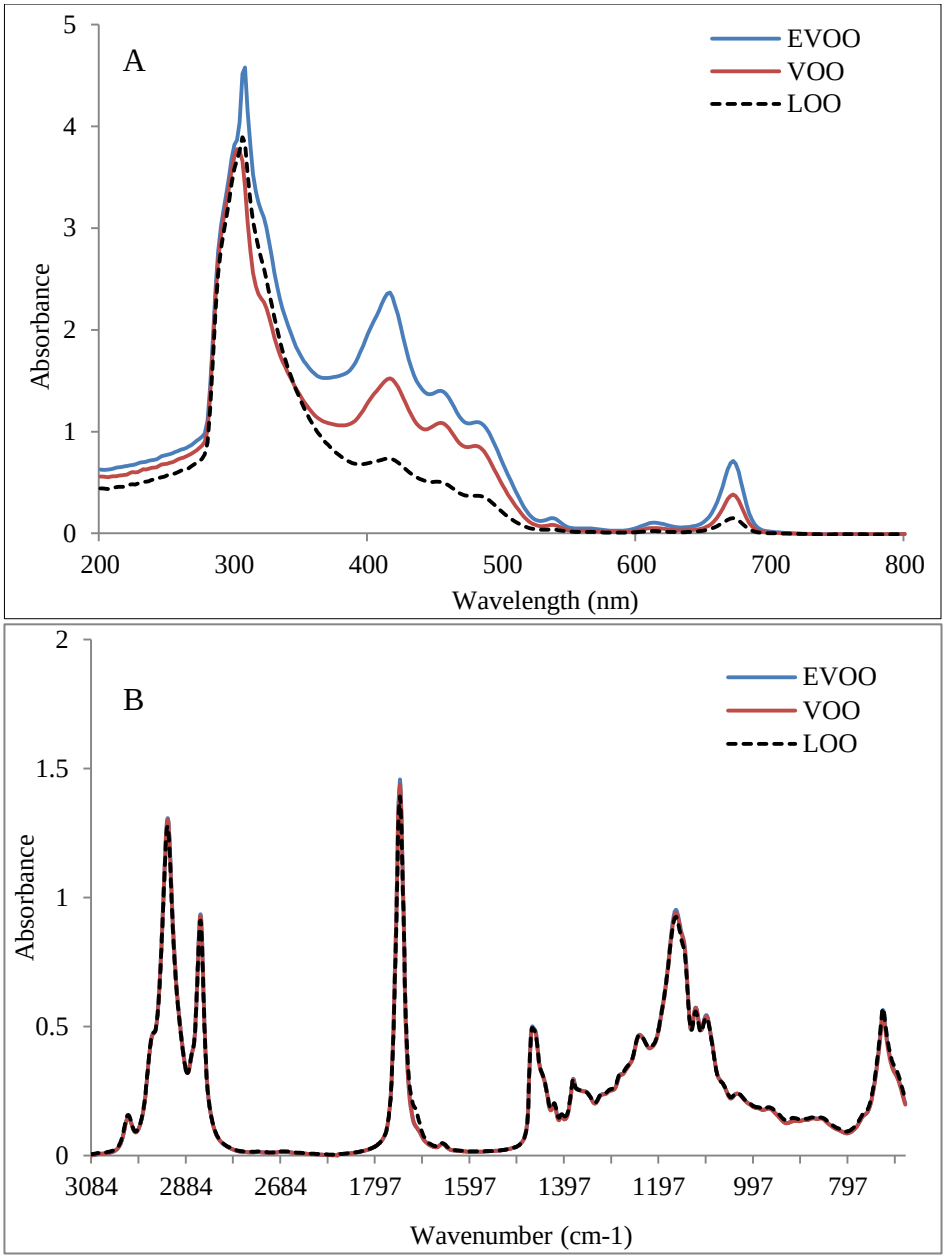

Figure 6.12. UV-vis (A) and MIR (B) Absorption spectra of three olive oil samples corresponding to EVOO (blue), VOO (red) and LOO (black)

Application of these defining regions on visible spectroscopy (650-700 nm) has been applied for quantitative determination of adulteration in EVOO, where the concentration of adulterant in EVOO inversely proportional to the absorption intensity (Aroca-Santos, et al., 2015). With the use of advanced statistical tools, these UV-vis spectra regions can turn into some reliable methods, such as detecting less than 10% adulteration level in olive oil (Torrecilla, Rojo, Dominguez, & Rodriguez, 2010). A more recent application of MIR in the prediction of chlorophyll and carotenoid proved plausible, with high coefficient of calibration (Uncu & Ozen, 2015). Practically,

differences in these pigments depend on the olive cultivar, geographical location, degree of ripeness, extraction technique, and storage conditions (Karabagias et al., 2013). UV-vis absorption spectrum can be considered as a putative signature and a univocal fingerprint of olive oil. Chemometric elaboration of the spectra can be an all-inclusive method of predicting agronomical and technological differences in olive oils.

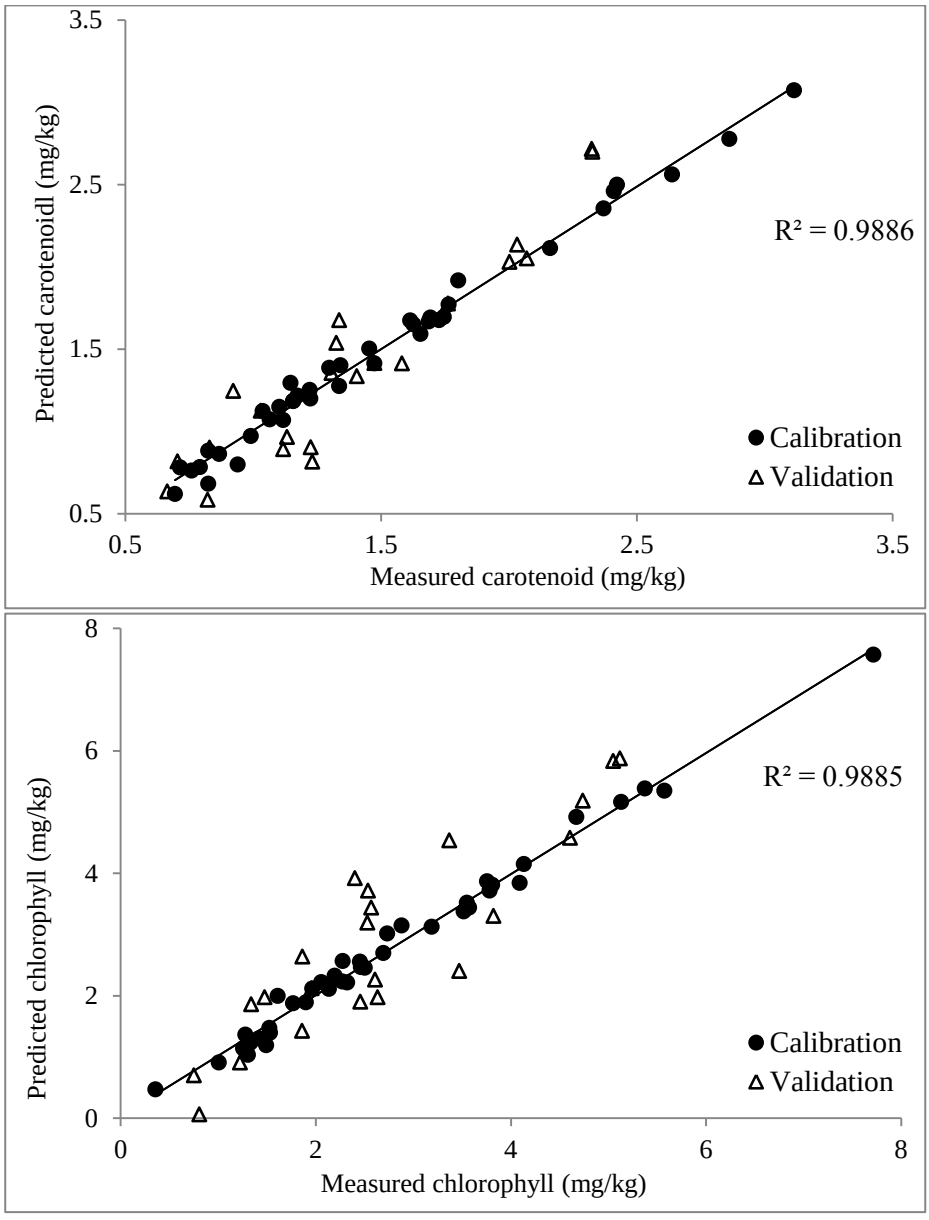

Figure 6.13. Results of regression plots for carotenoid (A) and chlorophyll (B) contents with UV-vis spectra.

6.6. Near Infrared Discrimination of Olive Oils

6.6.1. Spectra Interpretation

Sixty three olive oil samples of Ayvalik, Memecik and Erkence varieties were analyzed using near infrared spectroscopy. NIR spectra of olive oil as displayed showed peaks/bands that are much weaker and broader compared to that of MIR spectra. NIR spectrum contains more overlapped bands (Yang, Irudayaraj, & Paradkar, 2005). Considering the NIR spectra (12,500-400 cm^{-1}) as shown in Fig. 6.14, there are four major informative regions. As stated by others, most of the shoulders and peaks of NIR of olive oil are ascribable to functional groups (Bendini et al., 2007). The 4800-4500 cm^{-1} range indicates a combined CH stretching vibration in conjunction with other vibrational modes. The band of 6000-5300 cm^{-1} consisting of two conjoined peaks, represent methyl, methylene and ethylene first overtone stretching vibration. Variation in the frequency of this region is an index of oxidative quality of olive oil. The 7400-6250 cm^{-1} range consists of combined CH stretching vibrations. Finally, 8700-8100 cm^{-1} band is the second overtone of CH stretching vibration of methyl; methylene and ethylene interrupted or conjugated groups (Laroussi-Mezghani et al., 2015).

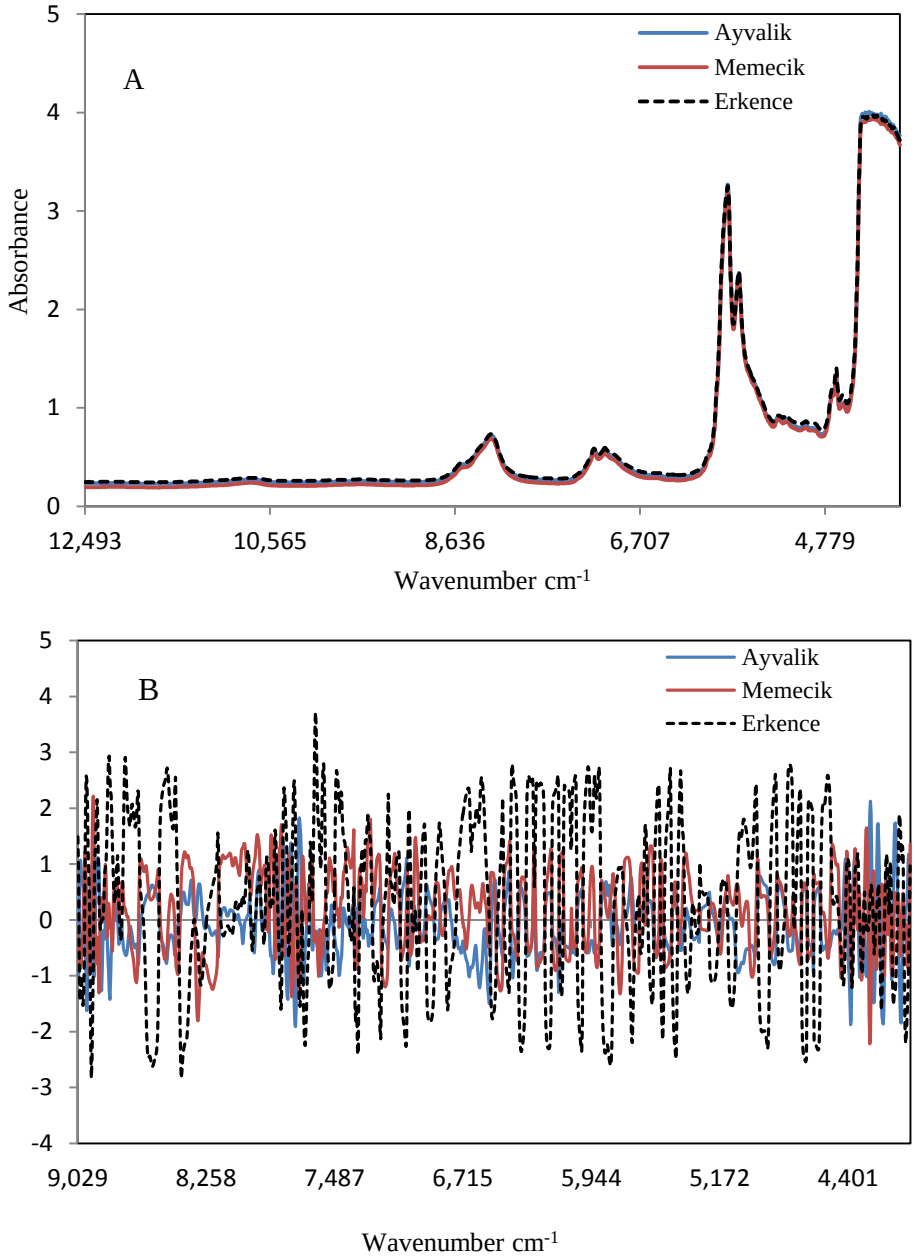

Figure 6.14. NIR spectra (A) and SNV+2der pretreated spectra (B) of Ayvalik, Memecik and Erkence oils.

6.6.2. PCA and OPLS-DA Results

The most informative parts of the NIR spectra with low signal-to-noise ratio (9056-4000 cm^{-1}) were pretreated using the Standard Normal variate (SNV) and second order derivative (2der) filtering techniques prior to chemometric elaboration. Experiences have shown that, application of one or more of these transformations can

improve overall performance of the model. PCA and OPLS-DA score plots of results are presented in Fig. 6.15. In PCA the model explained 83% total variance with R^2cv of 0.73 using the first 7PCs. This model was sufficient to separate oil samples of Erkence varieties at the right hand side of ellipse (Fig. 6.15A). In addition, the model sparingly showed some discrepancy between oils of Memecik and Ayvalik varieties.

OPLS-DA discriminant model of NIR spectra built using 40 observations as calibration and 23 observations as validation sets produced R^2 of 0.94 and R^2cv of 0.55 was able to distinguish oils of different varieties using 2+5 PCs (Fig. 6.15B). Memecik, Ayvalik and Erkence oils Erkence and Ayvalik varietal classes were distinctively classified with 100% accuracy using OPLS-DA of NIR as shown in Table 6.9. Misclassification of only one observation was experienced with respect to Ayvalik varietal class in the validation set. The results of these analyses were comparable with previous observations by other authors (Laroussi-Mezghani et al., 2015, Dupuy, et al., 2010; Sinelli et al., 2010; Yang, Irudayaraj, & Paradkar, 2005) with slight differences in the degree of success achieved. Casale & Simonetti, (2014) reported 98% precision in classification of pure and adulterated olive oils with NIR analysis. Accurate monitoring of olive oil routine quality characteristics was successfully determined using NIR reflectance spectroscopy (Mailer, 2004). Besides the oil-food-based evaluation, NIR in conjunction with other rapid analytical techniques was successful in other food system endeavors (Alamprese, Casale, Sinelli, Lanteri, & Casiraghi, 2013).

Table 6.9. OPLS-DA calibration and validation model results of NIR: correct classification rates of the oil samples

FT-NIR_SNV+2der	Member	A	M	E	% CC [a]
Calibration					
A	17	17	0	0	100
M	15	0	15	0	100
E	8	0	0	8	100
Average	40	17	15	8	100
Validation					
A	10	9	1	0	90
M	9	0	9	0	100
E	4	0	0	4	100
Average	23	9	10	4	97

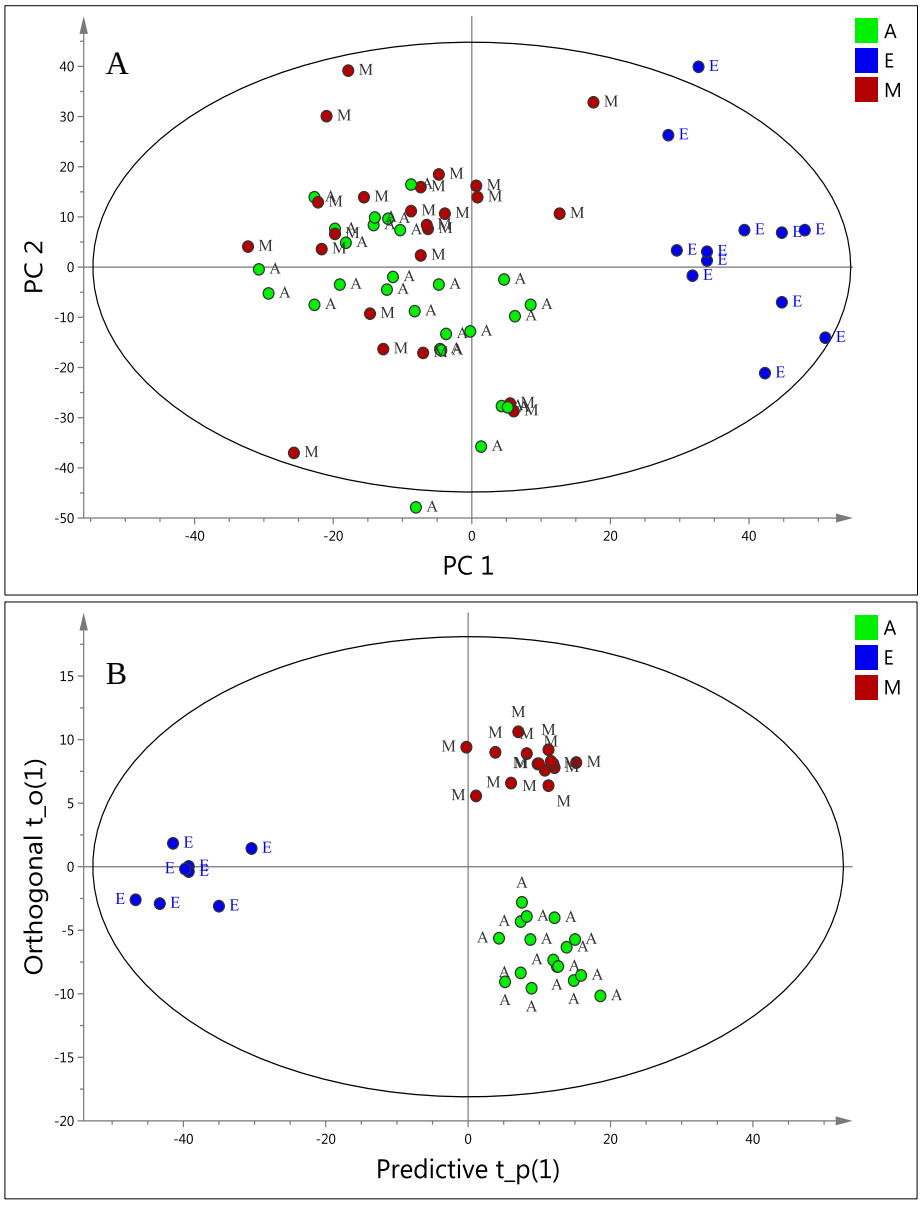

Figure 6.15. PCA (A) and OPLS-DA calibration model (B) Score plots of SNV+2der treated NIR spectra

6.7. Electronic Nose Classification of Olive Oils

6.7.1. E-nose Profiles of Olive Oils

E-nose profiles elucidating the resistivity (ohms) of each sensor corresponding to aromatic characteristics of the oil samples are shown in Fig. 6.16. The most visual difference between the oil samples are observed in sensor W5S, W1S and W2S. These sensors are responsive to broad-range, broad-methane and broad-alcohol flavor

compounds, respectively. Other important groups of sensors that enhance olive oil varietal discriminant ability of e-nose data matrix are: W1C, W3C and W5C (aromatic-based compounds) and W1W and W2W (Sulphur-organic compounds) (Buratti et al., 2006) as shown in Table 6.10. Olive oils volatile and aromatic characteristics have been attributed to various compounds which include: carbonyl compounds, alcohols, esters and hydrocarbons (Haddi et al., 2013). PEN2 electronic nose is a very handy and rapid method of determining these volatile profiles in olive oil with the aid of highly responsive MOS sensors.

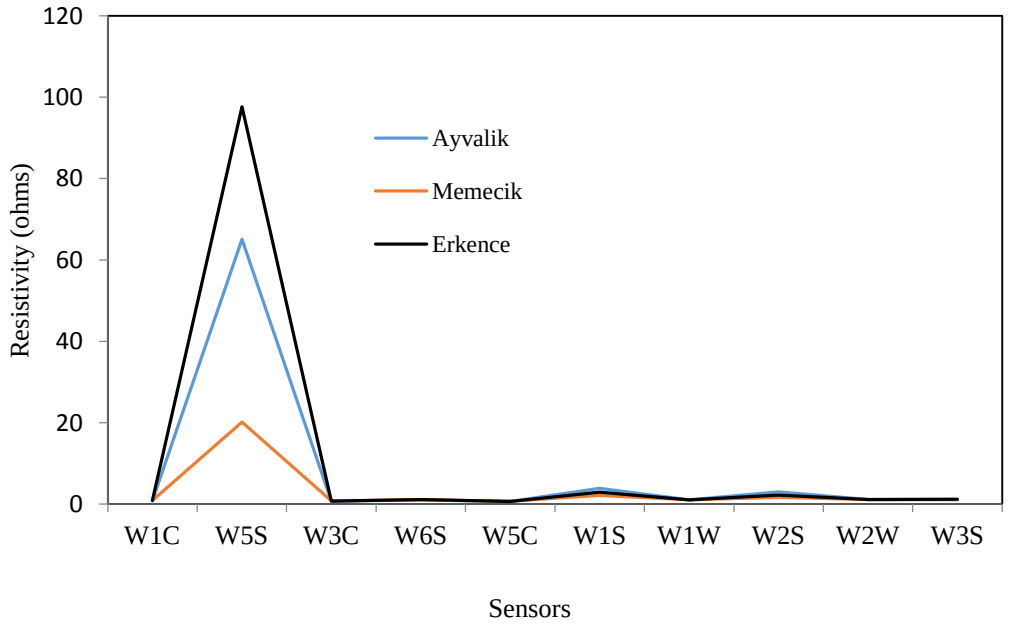

Figure 6.16. E-nose profile of olive oil of Ayvalik, Memecik and Erkence varieties

Table 6.10. Description of metal oxide semi-conductor sensors (MOS) Electronic Nose (Source: Buratti et al., 2006)

Sensor-name	General Description	Reference, mLm^3
W1C	Aromatic compounds	Toluene, 10
W5S	Very sensitive broad-range reactive to nitrogen oxides, sensitive with negative signal	NO_2, 1
W3C	Ammonia, used as sensor for aromatic compounds	Benzene, 10
W6S	Selective hydrogen	H_2, 0.1
W5C	Alkenes, Aromatic compounds, less Polar compounds, Aromatic-aliphatic	Propane, 1
W1S	Sensitive to methane broad-range & Sulphur-organic	CH_3, 100
W1W	Reactive to sulphur-containing compounds e.g. smell of limonene pyridine, terpenes	H_2S, 1
W2S	Detects alcohols, partially aromatic compounds, Broad-range alcohols	CO, 100
W2W	Sulphur-organic, aromatic compounds Sulphur-chloride	H_2S, 1
W3S	reacts on high concentration of methane Very sensitive	CH_3, 100

6.7.2. PCA and OPLS-DA of E-nose Data

E-nose data showed somewhat different projection with only oils of Erkence having a distinct cluster. PCA model with R^2 of 98% and R^2cv of 0.80 did not provide a distinct pattern among the samples unlike the spectra models. The indefinite cluster of oil among different varieties may be due to the close threshold response in their volatiles. The visual and numerical results of OPLS-DA calibration and validation model of e-nose data with respect to varietal classification is shown in Fig. 6.17 and Table 6.11 respectively. Ayvalik olive oils are localized at the lower right corner of the control ellipse with slight overlap with Memecik and Erkence varieties near the origin. The variables corresponding to the observation projection on the score plane are shown in the loading plot (Fig. 6.17B). W1S, W2S, W3S sensors, which are for partially aromatic, broad-range alcoholic, methane and sulphur-organic volatile compounds, are largely responsible for the discrimination of Ayvalik olive oils. The main drawback about any conclusion that could be made is that, the concentration of alcohol-based flavor compounds is usually much in the vapor phase than in liquid (Pierpauli et al., 2008). A study conducted by Buratti et al., (2006) correlated these sensors (W1S, W2S) to ethyl acetate and acetaldehyde, when e-nose was combined with volatile concentrations.

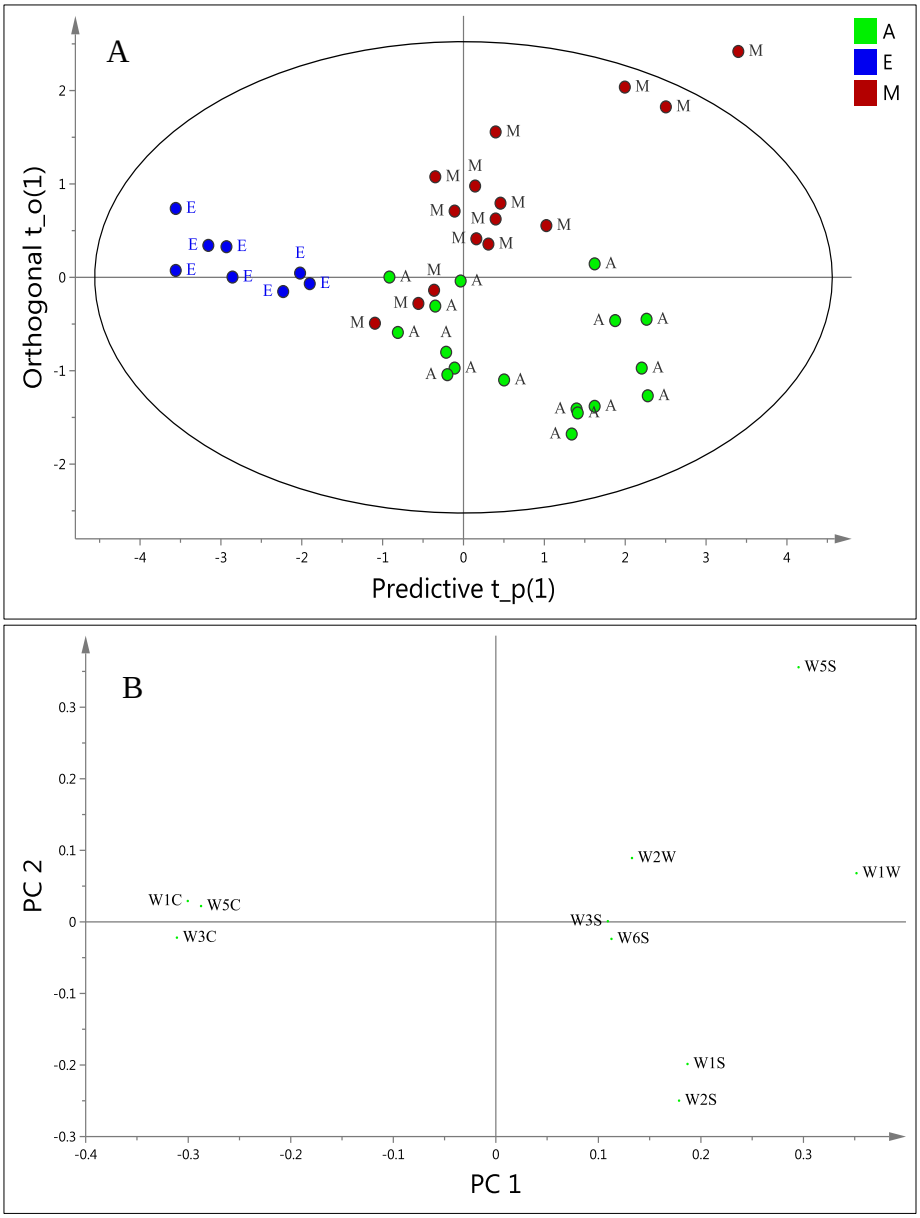

Figure 6.17. Result of varietal classification with e-nose data: OPLS-DA calibration model score (A) and Loading (B) plots

Similarly, W6S, W2W and W5S sensors, which are sensitive to selective hydrogen, sulphur-organic aromatic, and NO_2 sensitive broad-range volatiles respectively, seem significant for the characterization of Memecik oils. Erkence oil samples are precisely separated by very low response at aromatic compounds sensors (W1C, W3C and W5C). The sensitivities of OPLS-DA model of e-nose were comparatively lower than that of spectra as shown in Table 6.11.

Table 6.11. OPLS-DA calibration and validation model results of e-nose data: correct classification rates of the oil samples

E-nose data	Member	A	M	E	% CC
Calibration					
A	17	16	0	1	94
M	15	3	12	0	80
E	8	0	0	8	100
Average	40	19	12	9	91
Validation					
A	10	9	0	1	90
M	9	0	5	4	56
E	4	0	0	4	100
Average	23	9	5	9	82

However, it should be added that e-nose results are important in the consideration of sensory attributes of olive oil, which is equally relevant as chemical characteristics. Electronic nose responses; in terms of the nature of flavor compounds represented can be further validated if the tool is integrated with real-time sensory evaluation by panels (Lerma-Garcia et al., 2010

CHAPTER 7

CONCLUSION

Total of 103 olive oil samples composed mainly of Ayvalik, Memecik and Erkence varieties obtained between 2012 and 2016 were characterized under three separate studies. The first study involved qualitative evaluation of the effects of malaxation temperature (27, 37 and 47^{o}C), olive variety (Ayvalik and Memecik) and harvest time/maturation (early, mid and late) on the chemical characteristics of olive oils. The second study determined the effect of storage in the dark at room temperature on the chemical properties of olive oils. The third study included the multivariate analysis of UV-visible, mid and near-infrared spectra and e-nose data for the varietal, harvest year discrimination and for the prediction of important chemical parameters. PCA unsupervised multivariate statistical model was applied to visualize observation projections and natural clustering of samples. OPLS-DA supervised discriminant analysis was employed for classification. OPLS was used for the prediction of variables from spectroscopic data and for the prediction of oxidative stability from chemical variables.

The degree of maturation as early, mid and late harvest olives was the most influential factor on the chemical and quality characteristics of olive oils from Ayvalik and Memecik varieties. The impacts of malaxation temperatures between 27 and 47^{o}C were highly substantial on the distribution of important phenolic compounds such as hydroxytyrosol, tyrosol, pinoresinol, p-coumaric acid contents and peroxide values. The interaction effect of olive variety and harvest time was found significant with respect to some chemical variables, which means, different types of oils possessed different characteristics depending on the harvest time. Based on the result of multivariate classification models, early and mid-harvest Memecik oils had similar properties, and Ayvalik oils of early harvest had distinctive chemical attributes compared to its mid and late harvest. This study further revealed the possibility of producing olive oils of high oxidative stability and nutritional values from early harvest olive even at high temperatures up to 47^{o}C.

The influence of storage was significant in all the quality parameters except acidity and OSI. The color intensity and pigment contents of the oils decreased with time, where oils of Erkence variety and oils from Uzunkuyu and Manisa regions were found to have the greatest decline. However, fatty acid profiles of the oils remained unchanged, making it an unsuitable tool for monitoring storage effects. Evolution of phenolic alcohols (hydroxytyrosol and tyrosol) was significant due to the increase in their concentrations compared to other groups of phenols. There were over 50% decreases in luteolin of oils of all varieties, while apigenin and pinoresinol were relatively stable. The initial alkyl ester contents of Ayvalik and Memecik and some of the oils of Manisa region were within the EEC and IOC statutory requirements. At the end of 15-month storage time, alkyl esters contents of these samples have exceeded the threshold limits. In the multivariate analysis results, oils of Erkence before storage formed a distinctive cluster by their high FFA, PUFA, wax, linoleic, linolenic and L* values, which are all reflection of weak oxidative stability. Stored oils of Erkence variety were described by alkyl esters, as well as tyrosol, oleuropein derivatives and pinoresinol, which probably indicate that evolution of phenols and alkyl esters are independent of one another. Fresh and stored oils of Ayvalik and Memecik reserved most of the positive contributors to oxidative stability of olive oils: TPC, OSI, hydroxytyrosol, oleic acid, luteolin, apigenin, phenolic acids, MUFA/PUFA and oleic acid/linoleic acid ratios.

Multivariate regression analysis indicated that lipid-based variables are the most consistent major contributors (positive or negative) to olive oil oxidative stability. Oleic acid, oleic/linoleic and MUFA/PUFA ratio and saturated fatty acid positively influenced the stability of olive oils. On the other hand, FFA, PUFA, ethyl, methyl and wax esters, PV and K values negatively influenced olive oil stability. However, phenolic alcohols (hydroxytyrosol and tyrosol), total phenol content, pigments, some flavonoids and phenolic acids tend to impact positive effect on oxidative stability of olive oil.

UV-vis, MIR, NIR spectroscopies and e-nose data were shown effective as varietal and harvest season discriminating tools. Combined spectra of UV-IR produced 100% classification of samples to different harvest year, in spite of the disproportionate number of observations among different classes. There was no misclassification in all the observation and the model have coefficient of calibration and cross validation. Visual parameters such as color pigments (chlorophyll and carotenoid) were best

predicted by UV-vis spectra, while MIR performed better in the prediction of fatty acids, alkyl esters, oxidative stability, free fatty acid and some of the phenolic compounds.

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APPENDIX A

ANOVA P-VALUES OF CHEMICAL PARAMETERS AND HPLC-DAD INSTRUMENTAL CALIBRATION MODELS

Table A.1. ANOVA p-values showing the effect of factors and their interactions (O: olive type, H: harvest time, T: malaxation temperature (^{0}C) on the phenolic profiles

Responses	O	H	T	O+H	O+T	H+T	O+H+T
Hyt	ns	**	**	ns	ns	**	ns
Tyr	**	**	**	**	*	**	*
4Hpa	**	**	ns	**	ns	ns	ns
3Hpa	*	ns	ns	*	ns	ns	ns
Caf	*	**	ns	*	ns	ns	ns
Pin	**	**	*	**	**	*	**
Dba	*	**	ns	ns	ns	ns	*
Vnl	ns	*	ns	ns	ns	ns	ns
pCu	**	**	*	**	**	ns	*
Fer	ns	**	ns	ns	ns	ns	ns
Lut	ns	**	ns	**	**	ns	ns
O-der	**	**	**	**	**	**	*
TPA	**	**	ns	**	ns	ns	ns

Hyt: Hydroxytyrosol, Tyr: Tyrosol, 4Hpa: 4-hydroxyphenyl acetic acid, 3Hpa: 3-hydroxyphenyl acetic acid, Caf: caffeic acid, Pin: Pinoresinol, Dba: 2,3dihydroxylbenzoic acid, Vnl; vanillin, pCu: p-Coumaric acid, Fer: Ferulic acid, Lut; Luteolin, O-der: sum of tyrosol and hydroxytyrosol, TPA: Total phenolic acids. ns: not significant; *: p-value < 0.05; **: p-value < 0.01.

Table A.2. ANOVA p-values showing the effect of factors and their interactions (O: olive type, H: harvest time, T: malaxation temperature (^{0}C) on the quality parameters

Responses	O	H	T	O+H	O+T	H+T	O+H+T
TPC	ns	ns	ns	ns	ns	ns	ns
FFA	**	**	ns	*	*	ns	ns
PV	**	ns	*	ns	ns	ns	ns
OSI	ns	ns	ns	ns	ns	ns	ns
Chl	**	**	ns	*	ns	ns	ns
Car	**	**	ns	**	ns	ns	ns
L*	**	**	ns	**	ns	ns	ns
a*	**	**	ns	**	ns	ns	ns
b*	**	**	ns	*	ns	ns	ns
C	**	**	ns	*	ns	ns	ns
H	**	**	ns	**	ns	ns	ns

TPC: total phenol content (mg/kg), FFA: Free fatty acid (% Oleic acid), PV: Peroxide value (meq O_2/kg), OSI: Oxidative stability index (hr), Chl: Chlorophylls (mg/kg), Car: Carotenoids (mg/kg), CIELAB color parameters: L* (lightness-darkness), a* (greenness-redness), b*(blueness – yellowness), C (Chroma), H (Hue angle ^{o}). ns: not significant; *: p-value < 0.05; **: p-value < 0.01.

Table A.3. ANOVA p-values showing the effect of factors and their interactions (O: olive type, H: harvest time, T: malaxation temperature (^{0}C)) on the fatty acids profile

Responses	O	H	T	O+H	O+T	H+T	O+H+T
C16:0	**	**	ns	ns	ns	*	ns
C16:1	*	**	ns	*	ns	ns	ns
C17:0	**	ns	ns	**	ns	ns	ns
C17:1	**	ns	ns	*	ns	ns	ns
C18:0	ns	**	ns	**	ns	ns	ns
C18:1	ns	*	ns	*	ns	ns	ns
C18:2	ns	ns	ns	*	ns	ns	ns
C18:3	**	**	ns	ns	ns	ns	ns
C20:0	ns	ns	ns	ns	ns	ns	ns
C20:1	ns	ns	ns	ns	ns	ns	ns
C22:0	ns	ns	ns	ns	ns	ns	ns
SFA	**	**	ns	ns	ns	ns	ns
MUFA	ns	ns	ns	*	ns	ns	ns
PUFA	ns	ns	ns	*	ns	ns	ns
MUFA/PUFA	ns	ns	ns	**	ns	ns	ns
C18:1/C18:2	ns	ns	ns	**	ns	ns	ns

C16:0: Palmitic acid, C16:1: Palmitoleic acid, C17:0: Margaric acid, C17:1: Cis-10-heptadecanoic acid, C18:0: Stearic acid, C18:1: Oleic acid, C18:2: Linoleic acid, C18:3: Linolenic acid, C20:0: Arachidic acid, C20:1: Cis-11-Eicosenoic acid, C22:0: Behenic acid, SFA: Saturated fatty acids, MUFA: Monounsaturated fatty acids, PUFA: Polyunsaturated fatty acids. ns: not significant; *: p-value < 0.05; **: p-value < 0.01.

Table A.4. HPLC-DAD instrument analytical parameters of phenolic profile analysis of olives and olive oil of 2012-2014 harvest year

Compounds	Max λ	Rt*olive oil	R^2olive oil	Calibration Curve OO	Max λ	Rt* olive fruit	R^2olive fruit	Calibration Curve OF
Gallic acid	280	6.544	ISTD	-	280	8.083	0.999	y = 4.4394x
Hydroxytyrosol	280	9.608	0.998	y = 0.5094x - 0.0057	280	12.033	0.999	y = 0.7969x
Tyrosol	280	13.17	0.999	y = 0.3072x - 0.0023	280	16.357	0.999	y = 1.2122x
Oleuropein	-	-	-	-	280	34.260	0.999	y = 0.5899x
3-hydroxyphenyl acetic acid	280	15.621	0.999	y = 0.3162x - 0.0009	-	-	-	-
4-hydroxyphenyl acetic acid	280	14.17	0.996	y = 0.0471x - 0.0005	-	-	-	-
2,3-dihydroxylbenzoic acid	320	16.202	0.999	y = 6.8915x - 0.0212	-	-	-	-
Vanillic acid	280	16.00	0.999	y = 1.2508x	240	19.949	0.999	y = 3.4830x
Vanillin	280	18.652	0.999	y = 25.692x + 0.0761	240	23.924	0.999	y = 4.0215x
p-Coumaric acid	280	23.719	0.999	y = 53.511x - 0.1047	240	26.085	0.999	y = 10.228x
m- Coumaric acid	280	31.459	0.999	y = 4.1269x + 0.0017	-	-	-	-
o-Coumaric acid	280	40.736	0.999	y = 3.4831x - 0.0011	240	32.307	0.999	y = 12.607x
Syringic acid	280	17.028	0.999	y = 1.7819x + 0.0069	240	20.896	0.999	y = 11.011x
Chlorogenic acid	280	14.01	0.999	y = 16.5556x	-	-	-	-
Caffeic acid	280	16.298	0.999	y = 1.5391x - 0.0037	240	20.633	0.999	y = 6.1063x
Ferulic acid	280	27.21	0.997	y = 26.164x + 0.1151	240	27.805	0.999	y = 6.1080x
Cinnamic acid	320	31.792	0.999	y = 4.0963x	-	-	-	-
Apigenin-7-glucoside	320	46.388	0.999	y = 23.259x - 0.1338	-	-	-	-
Rutin	-	-	-	-	240	29.165	0.999	y = 1.823x
Quercetin	-	-	-	-	240	40.595	0.999	y = 3.059x
Luteolin	320	59.724	0.999	y = 20.507x - 0.0065	240	41.210	0.999	y = 4.2169x
Pinoresinol	280	53.533	0.994	y = 0.5133x - 0.0091	-	-	-	-

*Rt : Retention time, OO: olive oil, OF: Olive fruit

Table A.5. HPLC-DAD instrument analytical parameters of phenolic profile analysis of olive oils 2015 -2016 samples

Compounds	Max λ	Rt*2015	R^2 2015	Calibration Curve 2015	Max λ	Rt*2016	$R^2$2016	Calibration Curve 2016
Gallic acid	280	5.985	ISTD		280	5.906	ISTD	
Hydroxytyrosol	280	9.608	0.998	y = 0.415x - 0.0302	280	7.687	0.996	y = 0.2641x - 0.0508
Tyrosol	280	13.17	0.998	y = 0.9171x - 0.0763	280	10.624	0.998	y = 0.5289x - 0.0162
3-hydroxyphenyl acetic acid	280	15.621	0.993	y = 0.5253x - 0.0251	280	12.004	0.999	y = 0.3788x + 0.0071
4-hydroxyphenyl acetic acid	280	14.17	0.996	y = 0.3810x - 0.008	280	11.133	0.998	y = 0.6033x - 0.0134
2,3-dihydroxylbenzoic acid	320	16.202	0.999	y = 0.1413x + 0.0212	320	12.248	0.998	y = 9.4797x - 1.1555
3,4-dihydroxylbenzoic acid	320		0.994	y = 2.8972x + 0.1106	-	-	-	-
Vanillic acid	280	16	0.999	y = 1.0908x - 0.0225	280	13.791	0.999	y = 1.5193x - 0.0014
Vanillin	320	18.652	0.998	y = 37.649x - 0.6658	280	16.158	0.999	y = 3.7737x - 0.2964
p-Coumaric acid	280	23.719	0.999	y = 2.2577x - 0.0369	320	18.964	0.999	y = 9.9864x - 5.6601
m- Coumaric acid	280	31.459	0.999	y = 4.3508x + 0.0209	280	-	-	-
o-Coumaric acid	280	40.736	0.997	y = 3.1404x - 0.0168	280	-	-	-
Syringic acid	280	17.028	0.999	y = 2.1856x - 0.0279	280	-	-	-
Chlorogenic acid	320	14.01	0.999	y = 6.4379x + 0.035	320	-	-	-
Caffeic acid	280	16.298	0.999	y = 0.467x - 0.0137	280	14.229	0.998	y = 1.6762x - 0.0435
Ferulic acid	280	27.21	0.992	y = 31.907x - 0.0932	280	22.522	0.999	y = 2.0999x - 0.0402
Cinnamic acid	320	31.792	0.999	y = 18.785x - 0.1818	280	42.138	0.999	y = 6.4667x - 0.266
Apigenin	320		0.998	y = 16.555x + 7.137	320	65.033	0.999	y = 45.045x - 3.8153
Luteolin	320	59.724	0.999	y = 5.7446x + 0.0975	320	61.442	0.999	y = 22.199x - 3.550
Pinoresinol	280	53.533	0.994	y = 0.8929x - 0.0568	280	46.205	0.998	y = 0.8414x - 0.0223

*Rt : Retention time

APPENDIX B

THE HPLC CHROMATOGRAMS OF OLIVE OIL SAMPLES

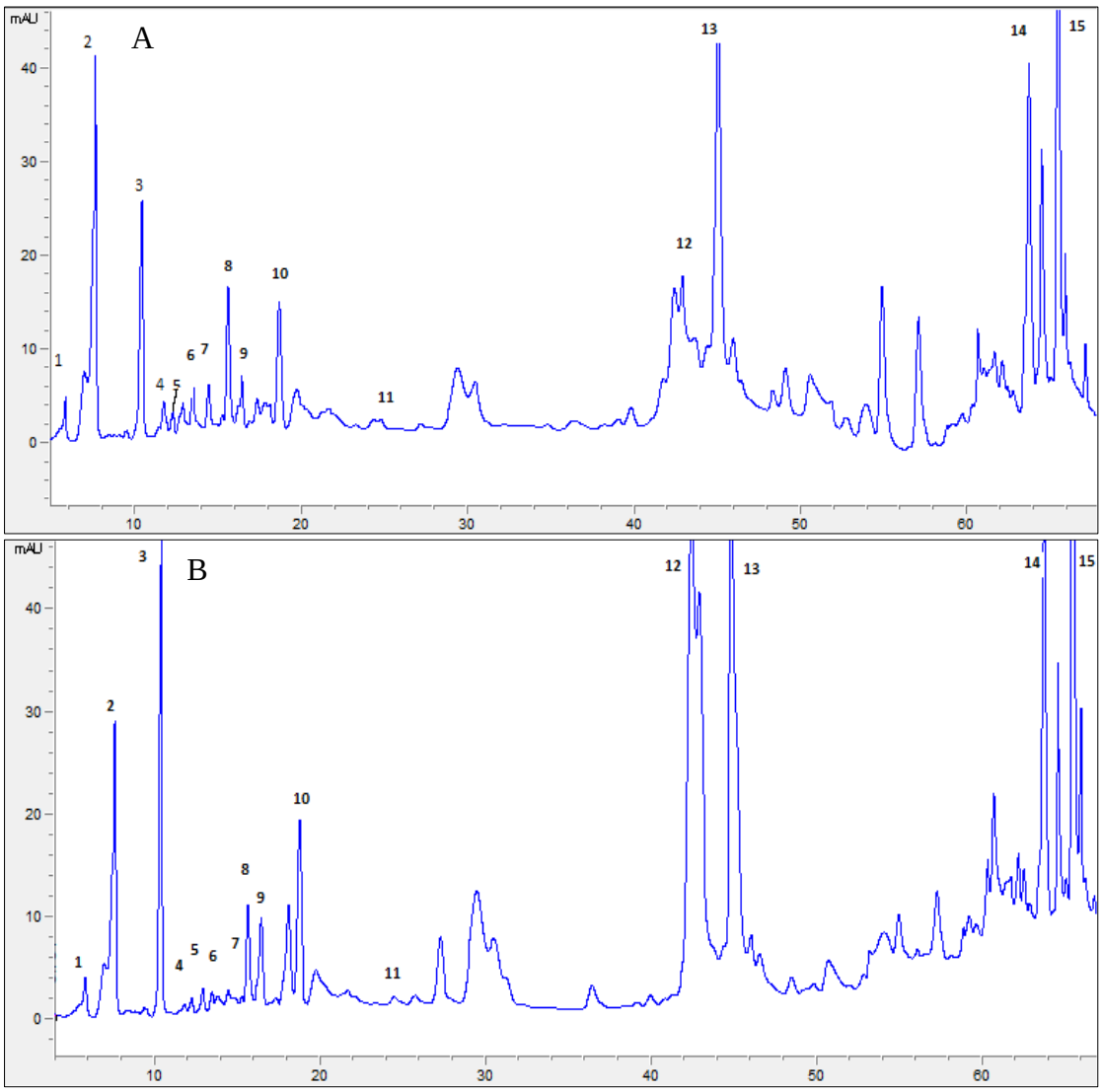

Figure B.1. HPLC phenolic chromatograms of Ayvalik (A), Memecik (B), and Erkence (C) olive oil samples. Peak assignments: 1: gallic acid, 2: hydroxytyrosol, 3: Tyrosol, 4: 4-hydroxyphenylacetic acid, 5: 3-hydroxylphenylacetic acid, 6: 2,3-dihydroxylbenzoic acid, 7: vanillic acid, 8: caffeic acid, 9: vanillin, 10: pcoumaric acid, 11:ferulic acid, 12:cinnamic acid, 13: pinoresinol, 14: luteolin, 15: apigenin.**(Cont. on the next page)**

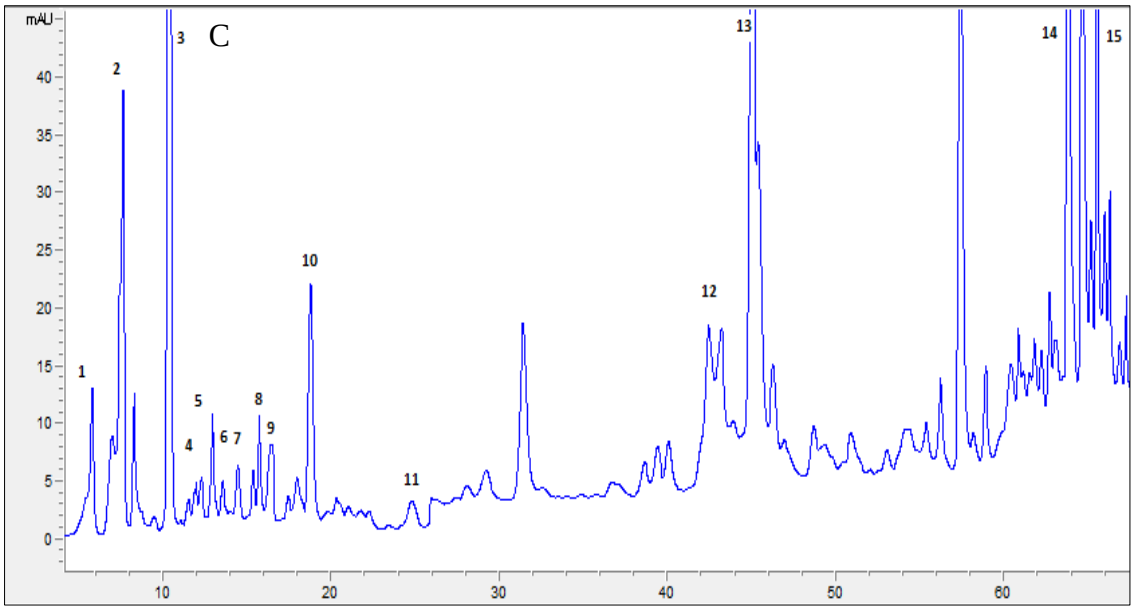

Figure B.1. (Cont.)

APPENDIX C

THE CALIBRATION CURVES OF TOTAL PHENOL CONTENT ANALYSIS

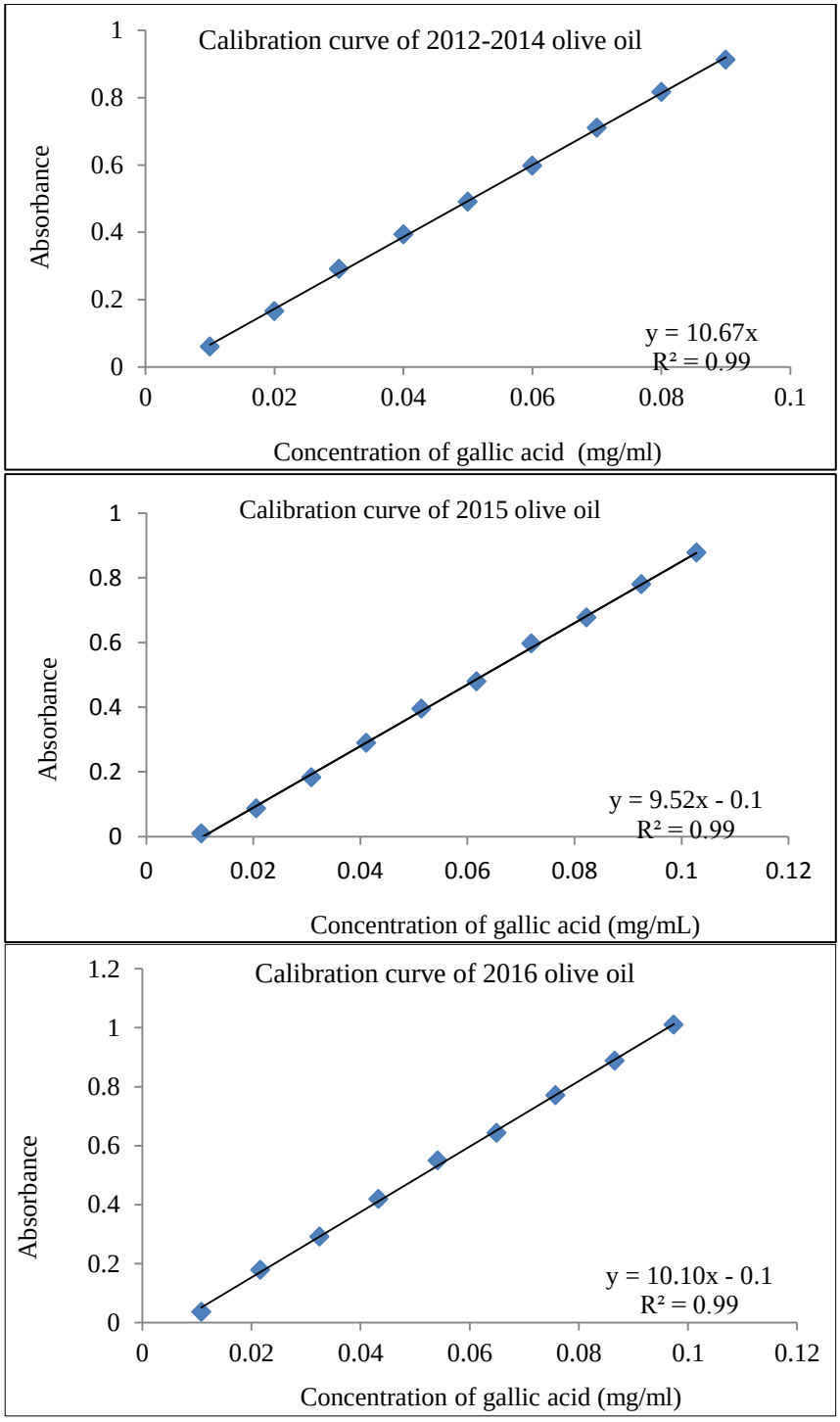

Figure C.1. The calibration curve of total phenol content analysis

APPENDIX D

GC CHROMATOGRAMS OF ALKYL AND WAX ESTER OF OLIVE OIL

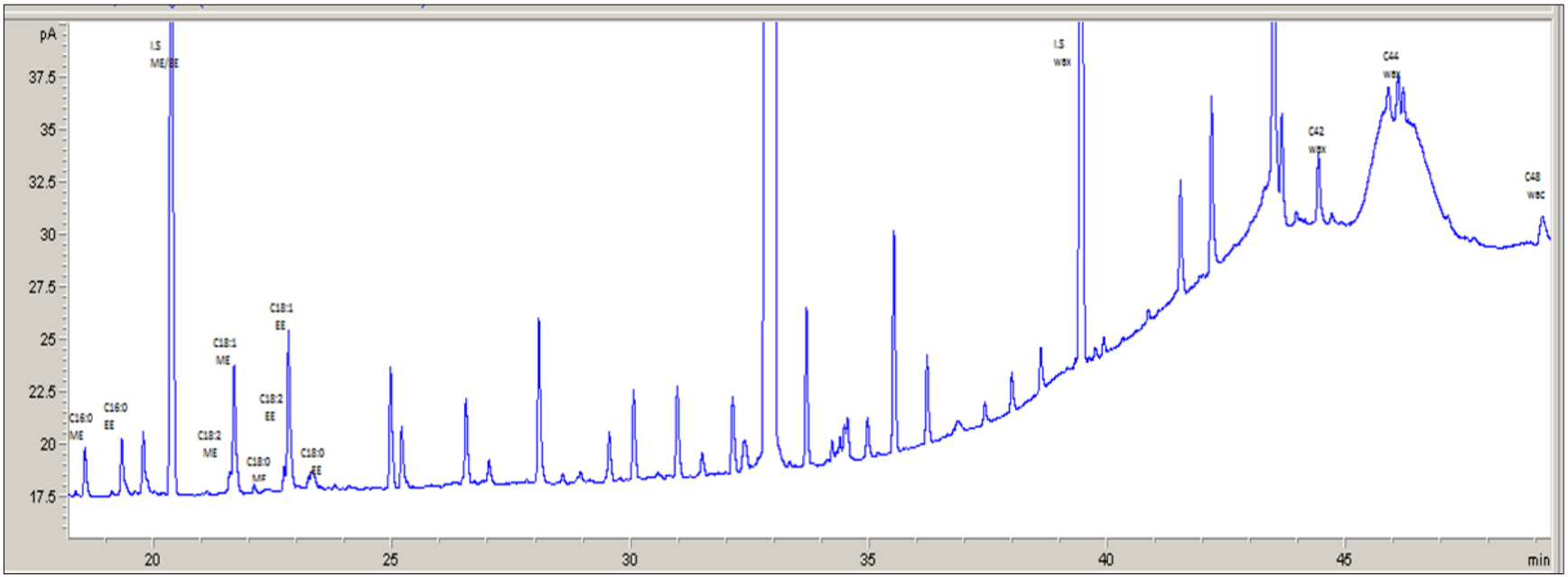

Figure D.1 Chromatogram of an olive oil showing good resolution of important ethyl and methyl esters with the following peak ID: C16:0 ME: methyl palmitate, C16:0 EE: ethyl palmitate, I.S ME/EE: alkyl ester Internal standard (methyl heptadecanoate), C18:2ME: methyl linoleate, C18:1ME: methyl oleate, C18:0ME: methyl stearate, C18:2EE: ethyl linoleate, C18:1EE: ethyl oleate, C18:0EE: ethyl stearate, I.Swax: wax Internal standard (lauryl arachidate), C42, C44 and C48: identified waxes.

VITA

Date and Place of Birth: 04.12.1985, Akure Nigeria

EDUCATION

2012-2016: Doctor of Philosophy (PhD): Izmir Institute of Technology, Department of Food Engineering (*Effect of malaxation temperature, olive variety and harvest time on the chemical characteristics of olive oils*)

2010-2011: Master of Technology (M.Tech): The Federal University of Technology, Department of Food Science and Technology, Nigeria (*Course–works*)

2005-2009: Bachelor of Technology (B.Tech – First Class): The Federal University of Technology, Department of Food Science and Technology, Nigeria (*Physico-thermal properties of soy-melon enriched garri selmolina*)

2002-2005: National Diploma (OND – Upper Credit): The Federal Polytechnic Ado, Department of Food Technology, Ekiti State Nigeria (*Food Safety and Regulation: A Review*)

PROFESSIONAL EXPERIENCE

2011-Cont., Research Assistant: The Federal University of Technology, Akure Nigeria

PUBLICATIONS

Jolayemi, O.S, Tokatli, F., & Ozen, B (2016). Effect of malaxation temperature and harvest time on the chemical characteristics of olive oils. *Food Chemistry, 211,* 776-783.

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AWARDS: 2nd Best Poster Award, 2016 (Trend in Chemometric conference)
FST and SAAT Best Graduating Student (2007-2009)
BEA Scholarship Award (YTB), Turkey (2011 – 2016)