EFFECTS OF STORAGE ON SOME CHEMICAL PARAMETERS OF OLIVE OILS

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ABSTRACT

EFFECTS OF STORAGE ON SOME CHEMICAL PARAMETERS OF OLIVE OIL

Mixing of fresh olive oil with olive oil from previous season is an emerging problem, and several chemical parameters have been proposed for the determination of this type of adulteration. The main objective of this study is to investigate the changes in several quality parameters (fatty acid alkyl esters, diacylglycerols and pigments) and spectral profiles of olive oils and olive oils adulterated with old olive oil as quality and authenticity indicators. For this purpose, fresh olive oil samples from North and South Aegean Regions and their blends with olive oil from previous year were stored for 15 months and changes in basic quality parameters (free fatty acidity, specific absorbances, fatty acids), fatty acid alkyl esters, diacylglycerol and pigment contents as well as ultraviolet-violet visible and mid-infrared spectral data were assessed to investigate effect of storage. Data were examined using multivariate analysis.

Free fatty acid limit of extra-virgin olive oils was exceeded in 6 months for the samples mixed with old oil at concentrations of 30% and higher. The most significant changes during storage were observed in free fatty acidity, fatty acid alkyl ester and pigment composition of the olive oils. Spectral data and pigment composition provided the best separation with respect to storage periods. None of the parameters investigated provided clear results in terms of detection of adulteration.

ÖZET

DEPOLAMANIN ZEYTİNYAĞLARIN BAZI KİMYASAL PARAMETRELERİ ÜZERİNDEKİ ETKİLERİ

Taze zeytinyağını önceki sezondan elde edilen zeytinyağı ile karıştırmak ortaya çıkan bir problemdir ve bu tip tağşişin tespiti için birkaç kimyasal parametreler önerilmiştir. Bu çalışmanın temel amacı, zeytinyağlarının ve eski sezon yağlarla tağşiş edilmiş zeytinyağlarının kimyasal parametrelerinin birkaçındaki değişikliklerini (yağ asidi alkil esterleri, diasilgliseroller ve pigmentler) ve spektrallerini kalite ve özgünlük indikatörü olarak incelemektir. Bu amaçla, Kuzey ve Güney Ege Bölgelerinden alınan zeytinyağı örnekleri ve onların önceki yıl zeytinyağıyla tağşiş edilen karışımları 15 ay boyunca depolanmış ve temel kalite özelliklerindeki (serbest yağ asitliği, özgül absorbans değerleri, yağ asit profili), yağ asidi alkil esterleri, diasilgliserol ve pigment içeriklerindeki değişiklikler ve aynı zamanda Ultraviyole ve görünür ışık (UV-Vis) absorpsiyon spektroskopisi ve orta kızılötesi spektroskopisi verileri depolamanın etkisini araştırmak için değerlendirilmiştir. Veriler çok değişkenli analiz kullanılarak incelenmiştir.

Natürel sızma zeytinyağları için serbest yağ asitliği limiti, %30 ve daha fazla konsantrasyonda eski yağla karıştırılan karışım örneklerinde 6.ayda aşılmıştır. Depolama boyunca en etkili değişimler zeytinyağlarının serbest yağ asitliği, yağ asidi alkil esterleri ve pigment kompozisyonlarında gözlemlenmiştir. Spektral veri ve pigment kompozisyonu depolama zamanına göre en iyi ayrımı sağlamıştır. Araştırılan parametrelerin hiçbiri tağşişi tespit etmede net bir sonuç sağlamamıştır.

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LIST OF ABBREVIATIONS

DAD	Diode-array detector
DAG	Diacylglycerols
EE	Ethyl esters
EU	European Union
EVOO	Extra virgin olive oil
FA	Fatty acids
FAAE	Fatty acid alkyl esters
FAME	Fatty acid methyl esters
FAEE	Fatty acid ethyl esters
FFA	Free fatty acid
FID	Flame Ionization Detector
FTIR	Fourier Transform Infrared Spectroscopy
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
IOC	International Olive Council
IS	Internal Standard
LVOO	Lampante virgin olive oil
MAGs	Monoacylglycerols
ME	Methyl esters
MSHFBA	N-Methyl-N-(trimethyl-silyl) heptafluorobutyramide
MUFA	Monounsaturated fatty acid
N	North Aegean Region olive oil
O	Old (previous harvest) olive oil
OPLS-DA	Orthogonal Partial Least Squares Discriminant Analysis
OVOO	Ordinary virgin olive oil
PCA	Principal Component Analysis
PDO	Protected Denomination of Origin
PLS	Partial Least Square
PLS-DA	Partial Least Squares Discriminant Analysis
PPP	Pyropheophytin
PTFE	Polytetrafluoroethylene

ROO	Refined olive oil
S	South Aegean olive oil
SIMCA	Soft Independent Modelling of Class Analogy
SPE	Solid-phase extraction
TAG/TG	Triacylglycerols or triglycerides
UV-Vis	Ultraviolet-visible
VOO	Virgin olive oil

CHAPTER 1

INTRODUCTION

Since olive oil is an important source of antioxidants and essential fatty acids it is one of the valuable food products in human diet. The importance of olive oil production is not only due to its nutritional benefits but also its annual economic value which has been exceeded €10 billion over the years (Wiesman, 2017). Moreover, consumers are demanding better quality olive oil because of the more consciousness they attained about their daily food consumption.

Adulteration is a commonly encountered major problem for olive oil and is also difficult to be detected by the consumers. Adulteration can be done by mixing with other vegetable oils as well as adding lower economical value olive oils to the higher quality olive oils, especially to extra virgin olive oil. EU regulation 29/2012 states that 'the optional marking of the harvest year should only appear on the label when 100% of the contents within the packaging comes from that harvest'. Therefore, a need has been also arisen to determine the adulteration of fresh olive oils with old olive oils from the previous harvest year to prevent the unjust profit (Borowicz and Petrovsky, 2006). Various analytical techniques have been used and also proposed for the detection of adulteration. These techniques could be classified as targeted (conventional wet methods; HPLC, GC, etc.) and non-targeted (mainly spectroscopic methods) approaches (Aparicio et al., 2013). While targeted approaches are based on the measurement of individual components of a matrix, general profiles of the samples are obtained in non-targeted methods. New adulterants are always introduced by the fraudsters as a response to improvements in detection techniques. Besides, variability in olive oil composition due to olive cultivation locations, olive growth practices and processing parameters makes the identification of adulteration even more difficult. In addition, changes in the chemical composition of the olive oil during its storage causes further challenge regarding the detection of adulteration. Therefore, there is always a need for the new perspectives in olive oil authenticity. Although there are various studies about the changes that take place in several parameters of olive oils during storage it has not been encountered any study

which monitors the alterations in the quality of olive oil mixed with old olive oil throughout the storage.

In this study, changes in various quality parameters of fresh olive oil and fresh olive oil adulterated with old olive oil at various concentrations were monitored by using traditional as well as innovative methods throughout the storage to evaluate their changes and reliability with respect to time. For this purpose, free fatty acid content, fatty acid profile, fatty acid methyl and ethyl esters, waxes, diacylglycerol and pigment contents and Ultraviolet-visible (UV-Vis) and infrared spectral profiles of all olive oil samples were followed up throughout 15 months of storage period and the results were analyzed with multivariate statistical analysis methods.

In the first part of this thesis (Chapter 2), olive oil and the importance of its chemical composition, especially its minor components are explained by summarizing information from the literature. Then, the effects of storage on olive oil quality and the detection of adulteration by using different methodologies were investigated and the methods used in this investigation are explained in Chapter 3. Extra virgin olive oil mixtures from two different geographical regions were monitored during 15 months of storage and the results are provided and discussed in Chapter 4. In the last part of this thesis (Chapter 5), conclusions regarding the possible critical chemical parameters to detect the olive oil quality and adulteration during storage and the necessity of additional studies are explained.

CHAPTER 2

LITERATURE REVIEW

2.1. Olive Oil

The olive, *Olea europaea,* is one of the oldest agricultural products which is known to be specific to Mediterranean climate. While consumption of healthier, cheaper, longer shelf life and easily accessible food products has been increasing, desire of consumers to understand the physical and chemical properties of these products also have risen.

The olive was originated from South Asia and the olive tree, which is from Oleaceae family, is extremely resistant to severe climate conditions and well adapted to the similar types of environments (Quiles et al., 2002). The tree was spread firstly straight to Middle East, Europe, Australia and Latin America by birds and migrants. In ancient civilizations, wine and wheat along with olive oil were generally consumed as basic food products in the east of Mediterranean region (Valavanis, 2004). It is still not clear when and how the first olive oil was produced but the Phoenicians were most probably the first civilization attempting to produce olive oil (Boskou, 2008). Since ancient times olive oil has been valuable owing to its unique nutritional and chemical properties (Boskou, 2008). Unique properties of this product caused increase in its consumption and production all over the world.

Olive oil is quite stable when it is stored under appropriate conditions and also keeps its quality if it is consumed before expiration date which is specified on the label. There are basic regulations and standards set by different regulatory agencies in different countries to determine the chemical freshness of this oil. A good quality extra virgin olive oil (EVOO) should be stored in dark glass bottle, which would minimize the risk of oxidation, and also air and light contact must be avoided as much as possible (Muzzalipo, 2012). However, olive oil quality decreases over time even if it is kept at ideal conditions. There are various quality parameters such as free fatty acid value, K indices and fatty acid profile to follow the state of olive oil during storage.

Pre-harvest, harvest and post-harvest factors not only influence the basic quality and chemical characteristics of olive oil, but also its annual production. The production amount of olive oil, which is the result of many factors such as climate, olive variety, storage time and conditions, varies from year to year (Figure 2.1). Each year the same yield could not be obtained or all of the products could not be sold. As a result, fresh olive oil could be blended with oil from the earlier/older harvests or other vegetable oils.

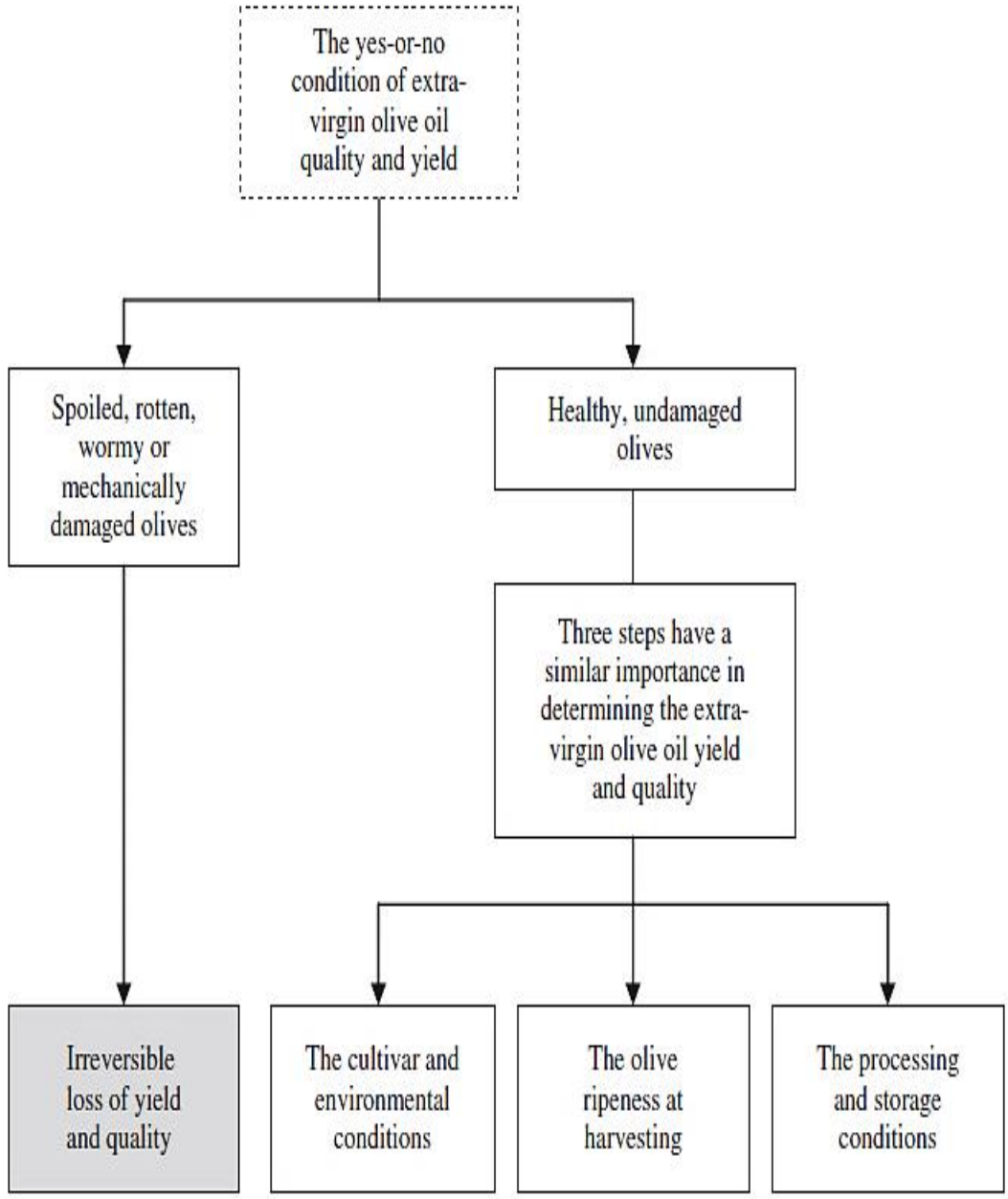


Figure 2.1. Important steps and conditions for yield and quality of extra-virgin olive oil (Source: Peri, 2014)

2.1.1. Olive Oil Production and Consumption

There are more than 12 million hectares of olives grown in 48 countries. The yield of olives is determined by factors such as tree age, size, variety and growing conditions; however, each olive tree yields between 15 and 40 kg of olives per year. (IOC, 2010).

Olive oil production in 2017 was about 3.3 million tons which were obtained from approximately 750 million olive trees, mostly cultivated in the Mediterranean region (IOC, 2018).

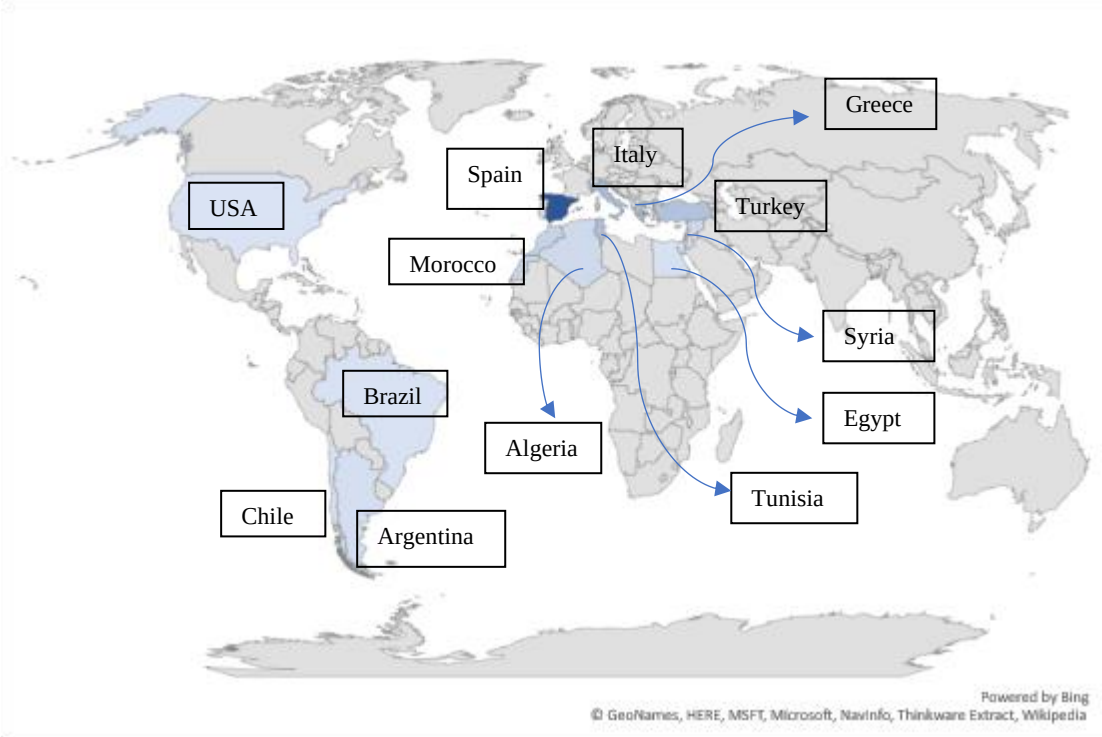


Figure 2.2. Major olive oil producing locations
(Source: IOC, 2018)

Due to more cost effective technological advances a rapid rise in the production of olive oil has been observed (IOC, 2010). Approximately 97% of the total olive oil production comes from Mediterranean countries, while European Union produces 80–84%. Spain, Italy, Greece, and Turkey (~1.1, 0.4, 0.3, and 0.25 million tons in 2017, respectively), then Tunisia, Portugal, Morocco, and Algeria are the largest olive oil producing countries (Figure 2.2) (IOC, 2018). World consumption is similar with that of production (Figure 2.3). At present, olive oil is consumed in over 160 countries.

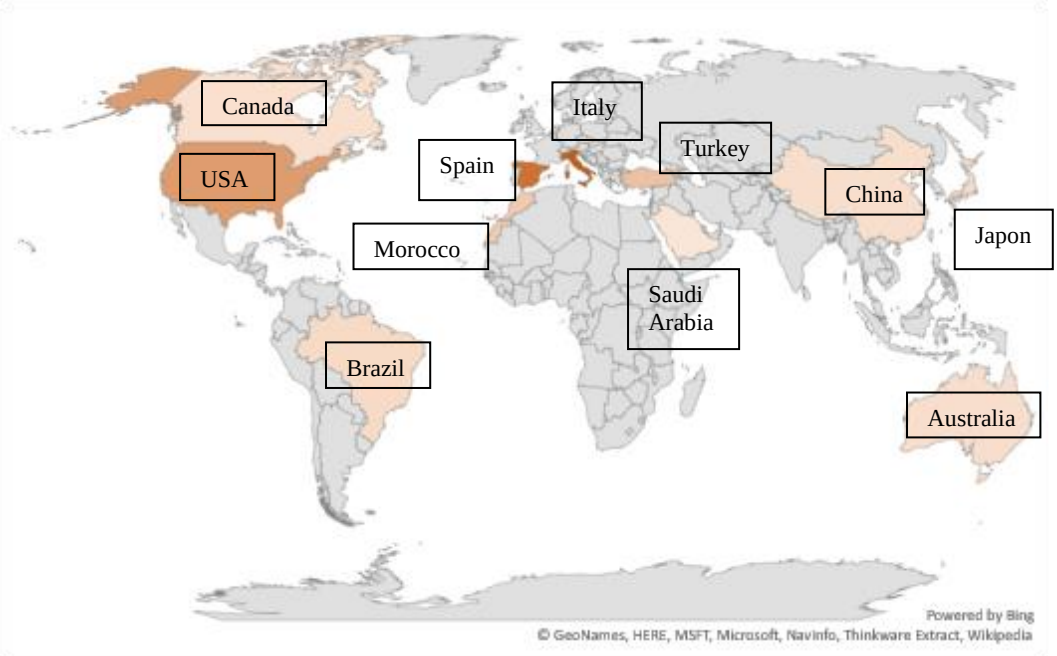


Figure 2.3. Major olive oil consumption locations

(Source: IOC, 2018)

2.1.2. Regulations and Classification of Olive Oil

Due to a global demand to olive oil, olive cultivation has also reached to the territories beyond the Mediterranean region such as Australia, Chile, United States and Argentina. Despite the issues related with the olive oil quality such as adulteration and mislabeling, global consumption has risen regularly. Mislabeling is labeling the olive oil misleadingly outside of its category of which it should belong, while adulteration is purposely blending olive oil with other oils or lower quality olive oil without informing the consumer.

Several measures could be followed for the authentication of olive oil. Firstly, major and minor components of olive oil could be identified taking into account of all the steps of production chain for the development of olive oil quality standards and related legislations. Secondly, origins of raw material and variety could be specified to fulfill in quality and safety assessments. Lastly, evaluation of authenticity could be made by

checking the presence or absence of adulteration, or confirming cultivar and geographical origin (Preddy and Watson, 2010).

Labelling has a an important function in clearly identifying the product contained within the package (Piscopo and Poi, 2012). Olive oils have to be labelled in accordance with the labelling requirements by Commission Directive 2000/13/EC and the label gives an idea about the product quality, origin and freshness (Koch, 2015). In the EU, all regulations have recently been revised to provide clear information to the consumer. Changes to all regulations such as how and where information appears have been determined to make the package content more clear (e.g. quality, country of origin, composition, method of production, etc.) and information regarding how to store the olive oil (e.g. information to 'store in cool, dark place') could be also placed on the label (Piscopo and Poi, 2012). Moreover, harvest year can only be specified on the label only if all olive oil comes from the current year harvest (Koch, 2015). Thus, consumers would be sure of the freshness of the olive oil before the purchase.

Legal classification of olive oil is primarily based on the European Union regulations (EUC, 2002). Besides, there are other organizations, such as the International Olive Council (IOC) and The Codex Alimentarius Commission which take part in development of regulations about olive oil. However, European legislation is internationally accepted as the first and fundamental reference for defining olive oil standards (Peri, 2014).

According to IOC categorization, EVOO has a free fatty acidity in terms of oleic acid not more than 0.8%, and the other characteristics are also defined in the IOC standard (Table 2.1). IOC standard with free fatty acidity of not more than 2% classifies oil as virgin olive oil (VOO) and ordinary virgin olive oil (OVOO) has a free fatty acidity of not more than 3.3%.

Table 2.1. The limits of free acidity for different categories of olive oils
(Source: IOC, 2010)

	EVOO	**VOO**	**OVOO**	**LVOO**	**ROO**
Free acidity (% oleic acid)	≤ 0.8	≤ 2.0	≤ 3.3	> 3.3	≤ 0.3

EVOO: Extra virgin olive oil, VOO: Virgin olive oil, OVOO: Ordinary virgin olive oil, LVOO: Lampante virgin olive oil, ROO: Refined olive oil(IOOC 2010).

2.2. Chemical Composition of Olive Oils

Lipids, under which oils and fats are grouped, consist of compounds that are typically soluble in organic solvents. 'Oil' term generally refers to liquid form, while 'fat' refers to specifically solids at room temperature. Olive oil, extracted from olive fruits, is primarily constituted of triacylglycerols (97-99% wt) and minor components (1-3% wt).

Triacylglycerols or triglycerides (TAGs/TGs) provide energy to body and also they serve as carriers of fatty acids (FAs) in liquid solutions. Glycerol esters of fatty acids constitute 99% of animal and plant-based lipids. TAGs form as a result of the esterification of three fatty acid molecules with a glycerol molecule as shown in Figure 2.4 (Perona et al., 2004). To keep the quality of olive oil until the end of storage, these three fatty acid molecules should be bound to TAG (Wiesman, 2017).

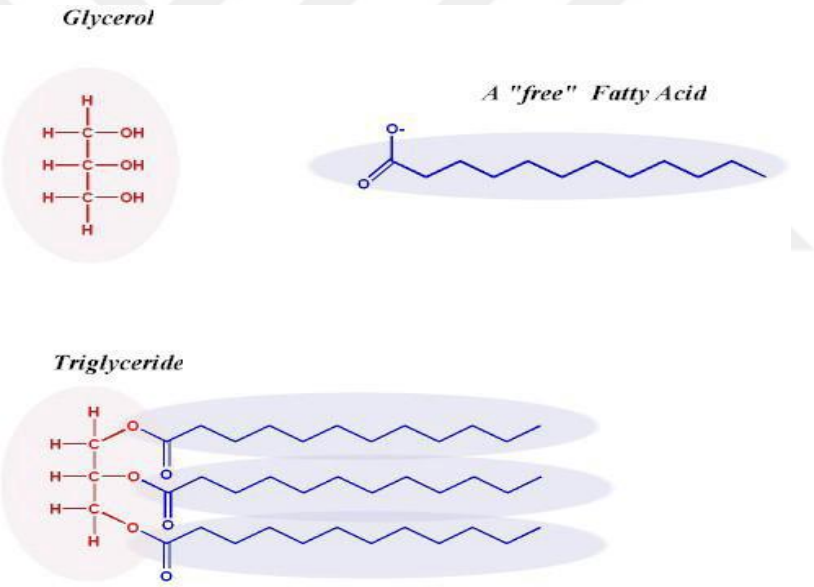


Figure 2.4. Glycerol, free fatty acid, and triglyceride (triacylglycerol) structures (Source: Olive Oil Source, 2019)

Fatty acid composition of olive oil, especially high content of oleic acid (monounsaturated fatty acid) and equally distributed saturated (palmitic and stearic) and polyunsaturated fatty acids (linoleic and α-linolenic acids) has been associated with its beneficial health effects (Ozyilkan et al., 2005; Gargouri et al., 2015) (Table 2.2).

Table 2.2. Fatty acid composition of olive oils according to regulation (Source: IOC, 2010)

Fatty acids		Content (%)
Myristic acid	C14:0	≤ 0.03
Palmitic acid	C16:0	7.50 - 20.00
Palmitoleic acid	C16:1	0.30 - 3.50
Heptadecanoic acid	C17:0	≤ 0.30
Heptadecenoic acid	C17:1	≤ 0.30
Stearic acid	C18:0	0.50 - 5.00
Oleic acid	C18:1	55.00 - 83.00
Linoleic acid	C18:2	2.50 - 21.00
Linolenic acid	C18:3	≤ 1
Arachidic acid	C20:0	≤ 0.6
Gadoleic acid (eicosenoic)	C20:1	≤ 0.40
Behenic acid	C22:0	≤ 0.20
Lignoceric acid	C24:0	≤ 0.20

The minor components of olive oils include polar, nonpolar and amphiphilic substances. Specifically, these components are hydrocarbons, tocopherols, phenolic compounds, sterols, chlorophyll, carotenoids, terpenic acids, monoglycerides and diglycerides, free fatty acids, esters and other volatiles and they are partially responsible for the sensory and health-promoting properties of EVOO.

2.2.1. Major Components

Extra virgin olive oils are produced by mechanical extraction without adding any chemicals and organic solvents at normal temperatures. Thus, all major and minor

components are generally preserved very well up to the end of the shelf life (Stefanoudaki - Katzouraki, 2004). Having these natural components, which are essential for the quality of olive oil, is the most important factor for the preference of olive oil over all other seeds and fruits oils. Olive oil constituents can be grouped into two according to their saponification ability. The saponifiable fraction of oil (97-99%) mainly consists of TAGs including mostly monounsaturated fatty acids (MUFA) varying from 55-85% (Boskou, 2008).

2.2.2. Minor Components

Oils can be produced from different sources which include fruits as olives, seeds and vegetables containing oils in their structures. In many ways, these oils have mostly similar properties; however, there are small amounts of different compounds which could have considerable positive effects on human health besides their effects on the quality of oil. These constituents are unsaponifiable fraction of olive oil, and free fatty acids, mono- and diacylglycerols**,** hydrocarbons, aliphatic alcohols, sterols, pigments, tocopherols, and also other volatile compounds are part of this fraction (Boskou, 2008). Unlike the other vegetable oils, olive oil provides sufficient preservation of these natural antioxidant components (Tsimidou et al., 2005; Angerosa, 2002). On the other hand, it has been noted that oxidative stability of oil was changed by different mechanisms in the presence of these minor components. However, there are still unclear points regarding the effects of these minor components on olive oxidation.

Free fatty acid (FFA) and peroxide levels of olive oil increase in the presence of enzymes as well as due to exposure to heat, light, and oxygen throughout three oxidation phases. It is difficult to detect some of the oxidation products by human senses over time, although, the rise in the concentration of these chemical constituents causes increase in the rate of oxidation. However, increase or decrease of the FFA percentage could be effective in determining the age of olive oil with respect to time passed during secondary oxidation phase (Freedman et al., 1986).

Small amounts of monoacylglycerols (MAGs) are also present in olive oil. There are two different types of MAGs and their contribution changes depending on olive oil acidity, but generally 1-monoglycerides are significantly higher than 2-monoglycerides (Boskou, 2008). In addition, minor fraction includes terpenes as squalene which

accumulates in the skin and help the production of reactive oxygen species by the reaction of ultraviolet radiation (Pérez-Rodrigo and Aranceta, 2015).

2.2.2.1. Fatty acid alkyl esters (FAAE)

Fatty acid alkyl esters (FAAEs) form by the esterification reaction between free fatty acids and short chain alcohols, methanol or ethanol (Aparicio, 2000). Alkyl esters in olive oil contain both fatty acid methyl esters (FAME) and fatty acid ethyl esters (FAEE). If olives are harvested and stored at inappropriate conditions before the olive oil extraction, lipolytic and pectolytic enzymes as well as microorganisms can easily interact with olive drupe. This situation results in increasing amounts of released free fatty acids and some other compounds including alkyl esters. Methanol and ethanol rise up throughout the fermentation of olives, although they are hard to found in olive oil since most are removed during pressing and washing steps of production (Biedermann et al., 2008). In order to prevent that kind of deterioration, refining and deodorization process can be effective to eliminate aliphatic compounds (Wiesman, 2017). High levels of alkyl esters have been linked to adulteration or mixing EVOO with soft-deodorized oils (Biedermann et al., 2008). The determination of FAAE and waxes can be performed with gas chromatography. There is an international standard for extra-virgin olive oil alkyl esters amounts, and this standard only provides a limit for FAEEs which should be ≤ 30 mg/kg (IOC, 2010). The most of the information in the literature on the measurement of FAME and FAEE has emphasized the importance of this parameter as a quality marker for olive oil (Oliveira et al., 2015; Biedermann et al., 2008).

Esterification of long chain fatty alcohols with fatty acids results in formation of waxes. Olive fruit skin generally contains more waxes than the other parts of the fruit. Therefore, the wax contents could be directly related to adulteration of EVOO with olive-pomace oil since pomace is the main part of the fruit skin (Wiesman, 2017). Various studies reported that the main waxes are C36, C38, C42, C44 and C46 esters (Boskou, 2008). Limits of an international standard about waxes are ≤150, ≤250, ≤350, ≤350, >350, and >350 mg/kg, for EVOO and VOO, ordinary virgin olive oil, refined olive oils, olive oils, refined olive-pomace oils and olive-pomace oils, respectively (IOC, 2010).

2.2.2.2. Diacylglycerols (DAGs)

Diacylglycerols (DAGs) in low amounts of between 1 and 10% could be found in vegetable oils (Boskou, 2012). Olive oil is much more acidic compared to other plant oils, thus it has a higher amount of DAGs (Shimizu et al., 2008). These compounds are formed as intermediate products in the biosynthesis of triacylglycerols (TAGs). DAGs could be also produced as a result of acidic and enzymatic hydrolysis of TAGs during olive oil processing and storage. At the same time, changes in the composition of the DAGs could take place due to isomerization reactions. Therefore, information regarding the composition and quantity of the DAGs is useful to evaluate the quality of the olive oil.

There are three different DAG fractions in olive oil chemical structure; however, C-34 and C-36 compounds are the commonly predominant ones (Boskou, 2008). On the other hand, storage conditions affect their distribution and 1,2-DAGs could be isomerized readily to their more stable version of 1,3-DAGs. This transformation is a good indicator of the age of olive oil as well as its storage conditions. It can be clearly concluded that even low DAG values can point out that olive oil has been oxidized or has possible sensory defects (Boskou, 2008). Therefore, these compounds give information about fruit quality and freshness of olive oil. Besides, a study in the literature suggested that the ratio of 1,2-DAGs to the total amount of DAGs or the ratio of 1,3 -DAGs/1,2-DAGs could be a meaningful indicator for the freshness of the oil (Pérez-Camino et al., 2001). Only Australian standards set a limit for DAG content for olive oils as 1.2 DAGs ≥35% (AS, 2011).

2.2.2.3. Pigments

Pigments determine the color of the olive oil and they could affect consumers' perception of the product quality. The color of the olive oil ranges from bright green (chlorophylls) to yellow (carotenoids) depending on olive type, cultivation area and the degree of ripening (Boskou, 2012). Considering all of the different substances that give a specific color to olive oil, the most important of them are the chlorophylls and carotenes. Moreover, pigments also contribute to antioxidant activity (Pérez-Rodrigo and Aranceta, 2015).

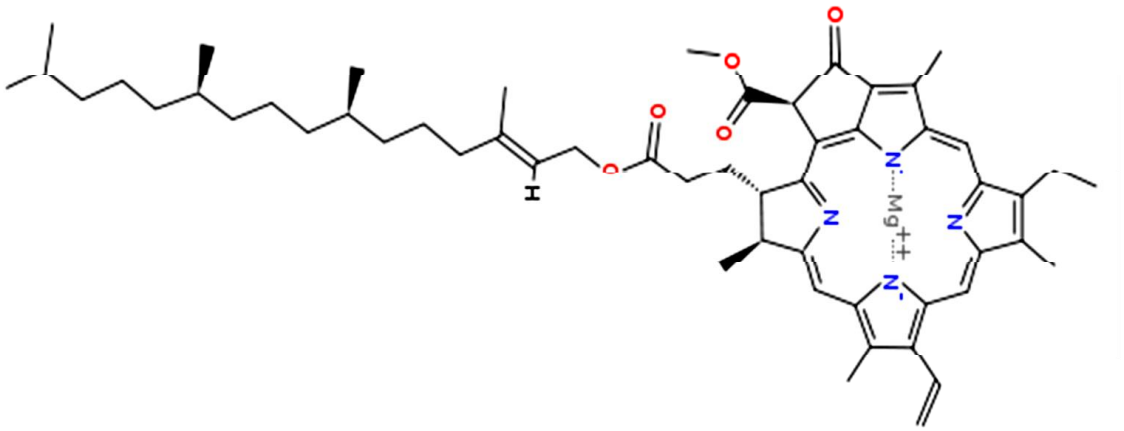


Figure 2.5. Structure of chlorophyll a
(Source: Morrolone, 2015)

The chlorophyllic and carotenoid profiles of olives are similar in general terms for all varieties. The chlorophyllic fraction is mainly composed of chlorophyll a and chlorophyll b forms, although allomerized chlorophylls, such as chlorophyllide, pheophytin and pheophorbide derivatives, are also detected in smaller quantities (Gandul-Rojas et al., 2013). Chlorophyll a chemical structure is shown in Figure 2.5 (Morrolone, 2015). There are various pathways which cause alteration of chlorophylls; and these are enzymatic, acid and heat catalyzed changes, photo-degradation and allomerization (Fennema, 1996). Enzymatic degradation of chlorophyll is shown in Figure 2.6. Chlorophyll derivatives formed during heating or thermal processing can be classified into two groups based on the presence or absence of the magnesium atom in the center of molecule. Mg-containing derivatives are green in color, while Mg-free derivatives are olive-brown in color (Fennema, 1996).

When the chlorophyll is exposed to heat the first changes observed are called as isomerization. Chlorophyll isomers are formed by inversion of the C-10 carbomethoxy group (Roca et al., 2007). The isomers are designated as a' and b'. They are more strongly absorbed on a C-18 reverse-phase HPLC column than the other derivatives and separation can be achieved clearly (Geitz and Fiebig, 2006). The magnesium atom in chlorophyll is easily displaced by two hydrogen ions, resulting in the formation of olive-brown pheophytin (Figure 2.7).

Pyropheophytins in olive oil are formed due to degradations of chlorophyll pigments. The pigments break down due to a process which involves the decarbomethoxylation of chlorophyll and pheophytins to form pyropheophytins (Guillamoue et al., 2014). Pyropheophytins formation as a result of heating may be spontaneous and irreversible and they could also form during extended storage at low temperature (Gallardo-Guerrero et al., 2005).

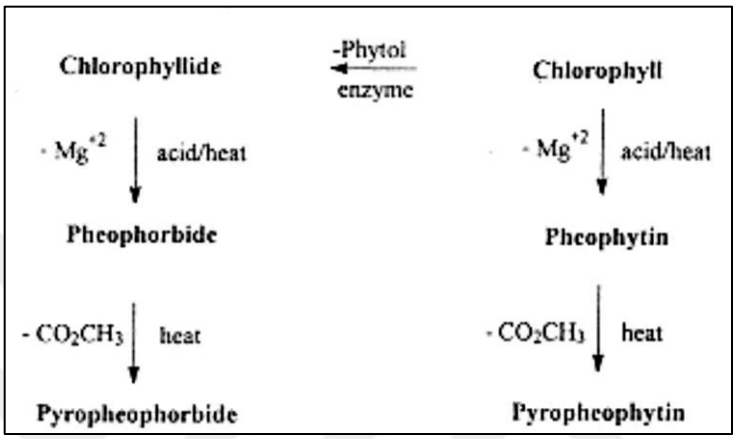


Figure 2.6. Chlorophyll and its derivatives
(Source: Fennema, 1996)

Pyropheophytins in olive oil are formed due to degradations of chlorophyll pigments. The pigments break down due to a process which involves the decarbomethoxylation of chlorophyll and pheophytins to form pyropheophytins (Guillamoue et al., 2014). Pyropheophytins formation as a result of heating may be spontaneous and irreversible and they could also form during extended storage at low temperature (Gallardo-Guerrero et al., 2005).

Biosynthetic pathway of carotenoids are shown in Figure 2.8 and they, specifically β-carotene and lutein, provide the typical green-yellowish color of olive oil (Figure 2.9). They act as inhibitors for photo-oxidation reactions (Boskou, 2008). Besides their role in the oxidation, previous researches demonstrated that β-carotene has the anti-cancer activity together with other carotenoids (Boskou, 2012).

The other carotenoids present in olives belong to the β series and include β-carotene, violaxanthin, neoxanthin, antheraxanthin, and β-cryptoxanthin (Figure 2.8) and they constitute more than 95% of the carotenoids including lutein present in olives (Gross, 1987).

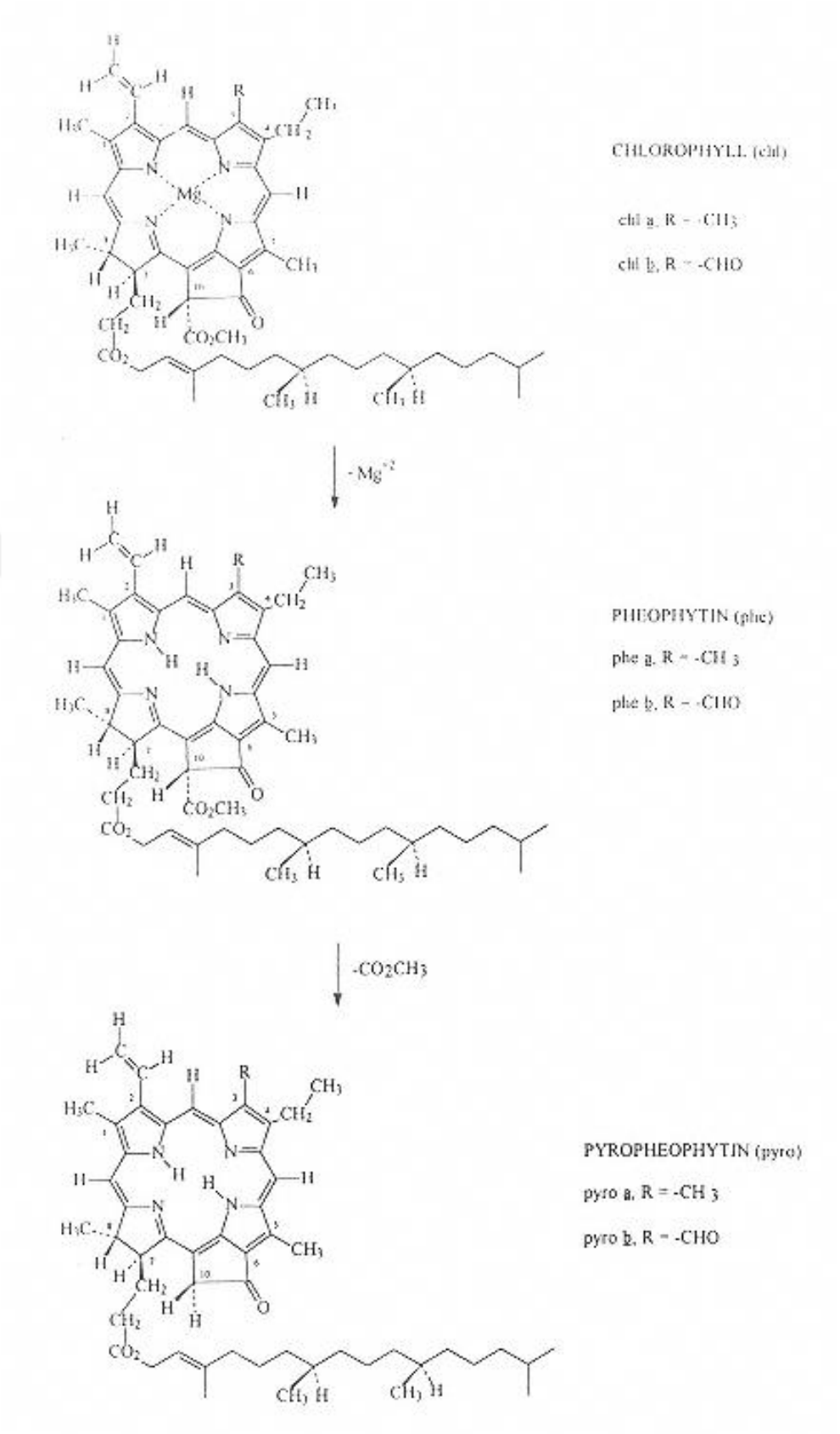


Figure 2.7. Formation of pheophytin and pyropheophytin from chlorophyll (Source: Fennema, 1996)

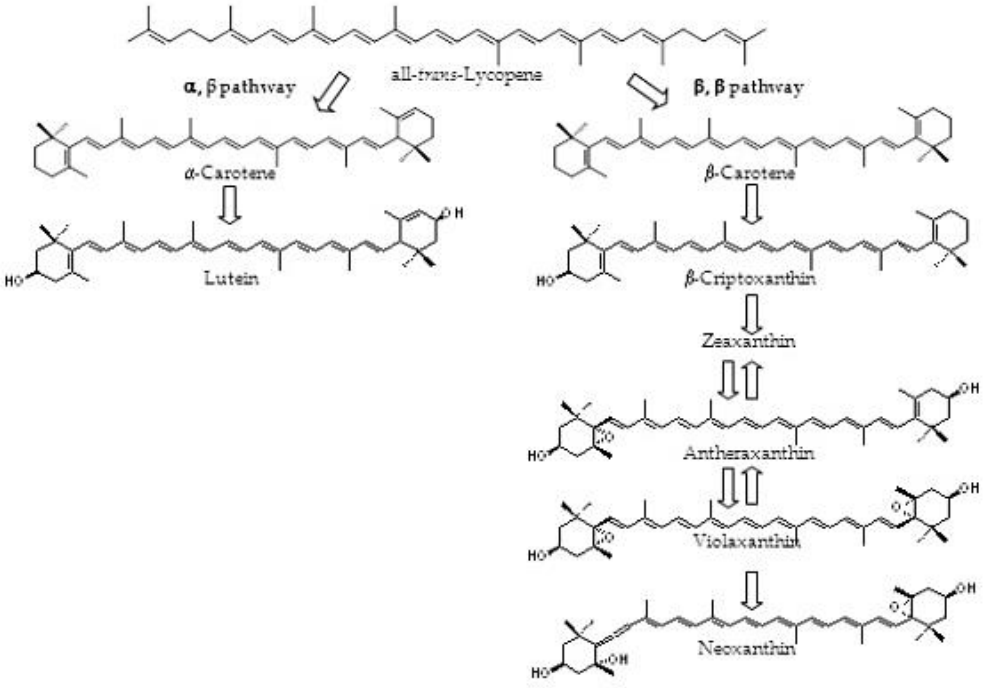
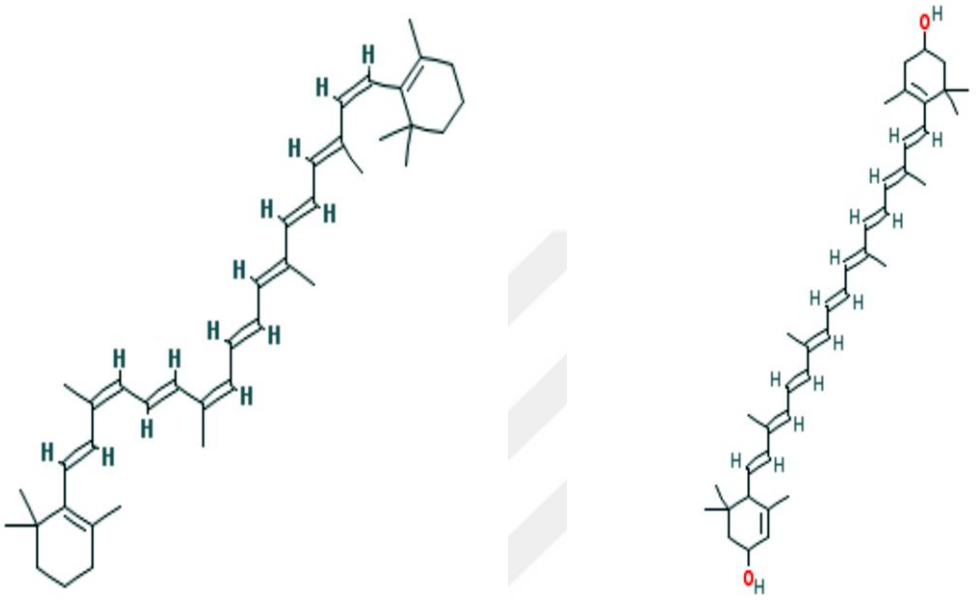


Figure 2.8. Biosynthetic pathway of carotenoids presents in olive oil
(Source: Gandul-Rojas et al., 2013)

The olive oil extraction process can cause a decrease in total pigment content, and chlorophyll pigments are more affected than the carotenoids (Aparicio, 2000). During the production of olive oil, release of acids can cause structural transformation of pigments. These transformation cause removal of the Mg^{2+} ion in chlorophylls as a result pheophytin forms (Giuffrida et al., 2011). Inappropriate processing and storage conditions, agricultural and technological factors can change the pigment profile and also can cause deterioration of olive oil natural color compounds. Artificial colors could be added to the olive oil to misled the consumers. These pigment compounds do not only give color to olive oil, but their importance has been also demonstrated by several researchers in terms of their nutritional benefits. Besides, a few studies have found that the pigment profile could indicate the authenticity of monovarietal olive oils (Giuffrida et al., 2007; Boskou, 2008).

Oxidation reactions that take place in olive oil result in the development of undesirable compounds. Deterioration of olive oil could be through different mechanisms depending on the presence (photo-oxidation) or absence (auto-oxidation) of light,

(Pristouri et al., 2010). The oxidation reactions of chlorophyll are inhibited by carotenoids especially β-carotene, for that reason all pigments work together in balance to protect olive oil oxidative stability (Morrolone, 2015). There are various methodologies to measure the pigment amounts of olive oil, but ultraviolet-visible (UV-vis) spectrophotometry is a simple and rapid technique directly related to color measurements (Boskou, 2012).



(a) (b)

Figure 2.9. β -carotene (a) and lutein (b) chemical structures
(Source: Pérez-Rodrigo and Aranceta, 2015)

Oxidation reactions that take place in olive oil result in the development of undesirable compounds. Deterioration of olive oil could be through different mechanisms depending on the presence (photo-oxidation) or absence (auto-oxidation) of light, (Pristouri et al., 2010). The oxidation reactions of chlorophyll are inhibited by carotenoids especially β-carotene, for that reason all pigments work together in balance to protect olive oil oxidative stability (Morrolone, 2015). There are various methodologies to measure the pigment amounts of olive oil, but ultraviolet-visible (UV-vis) spectrophotometry is a simple and rapid technique directly related to color measurements (Boskou, 2012).

2.3. Quality of Olive Oil

Olive oil, which is the symbol of healthy Mediterranean diet, has been also a part of the world cuisine as one of the main source of vegetable oils. The increasing popularity of olive oil has caused this product to be one of the most adulterated among the agricultural commodities. As a result, economic value of olive oil has gained importance, while the quality of it has begun to be questioned.

Olive oil production usually depends on the traditional methods in the industry and this may cause problems at the end of the production stage with respect to its quality (Wiesman, 2017). Due to a variety of economic, environmental, and sociological reasons, consumers generally may not be familiar with the grades of olive oil. As a result, it could be difficult for them to distinguish its attributes which vary from one quality level to the next. International olive oil grading standards were designated and the International Olive Council (IOC) standards are used by the most countries in European Union. The IOC focuses on not only regulating the legal sides of the olive oil industry but also thwarting unfair competition between countries. Some other countries like Australia and Germany also have strict criteria such as pyropheophytin (PPP) and diacylglycerols (DAGs) limits which reflect the particular attributes of olive oils (Frankel et al., 2011).

Olive oil has contributing factors which are important in defining its quality. One of these factors is the high content of polyunsaturated fatty acids which could cause a short life, whereas higher proportion of saturated fatty acids promise a longer shelf life. However, olive oil is also rich in terms of monounsaturated fatty acids which are not as sensitive to oxidation as polyunsaturated fatty acids. High levels of peroxide value, free fatty acids and moisture cause enzymatic oxidation (hydrolysis) and reduce the olive oil shelf life. On the contrary, natural antioxidants such as phenols, carotenoids and tocopherols extend shelf life of olive oil (Choe and Min, 2006).

2.3.1. Storage of Olive Oil

Chemical composition of olive oil that determines its quality are affected by genetic, environmental and varietal factors. In addition to these factors, storage time and conditions influence its attributes. It has been demonstrated that authenticity parameters of an oil are stable even after 12 months of storage at 15°C in darkness—the usual

industrial conditions for oil that is not marketed immediately following its production. The variables distinguishing between oil varieties also remain stable during the same period of storage (Gandul-Rojas et al., 2013). On the other hand, there could be some changes in olive oil minor components and sensory attributes that are caused by important reactions such as hydrolysis, oxidative reactions and esterification during storage (Dabbou et al., 2003).

In general view, two major types of degradation reactions occur in the olive oil during olive oil processing and storage. These are lipid hydrolysis and lipid oxidation reactions. Lipid hydrolysis is basically the breakdown of ester bonds of lipid, and this could be the result of the activity of enzymes (lipolysis) or the reaction of glycerides with water (hydrolysis) causing the formation of diglycerides, monoglycerides, free fatty acids and off-flavors and odors. Olive fruits could be broken or damaged when they are harvested, transported and stored prior to processing, and then enzymes mainly lipases, lipoxygenases and phenolases could be released; consequently, under high temperatures and high moisture content, a rapid increase in free fatty acids could occur (Figure 2.10). After the olive oil extraction, lipolytic reactions could not take place due to the inactivation of these enzymes by thermal treatment; however, reaction products produced before olive oil processing could have an effect on the final quality of olive oil. Hydrolytic reactions could take place under some certain conditions; presence of water, high temperatures (180-200ºC) and low molecular weight fatty acids (Stefanoudaki-Katzouraki, 2004). Water reacts with minor components of olive oil causing formation of precipitates. On the other hand, if water levels are below 0.1%, hydrolytic reactions may not be so important for the finished product.

Lipid oxidation is much more complex than hydrolysis. This process involves various reactions which generally occur simultaneously and causes chemical and physical changes of lipid molecules. Unsaturated fatty acids in olive oil are oxidized during that process. As a consequence, shelf life and quality of olive oil could decrease after these reactions. Saturated fatty acids could also involve in the oxidation; however, they are more resistant to oxidation than unsaturated fatty acids. Thermal oxidation of saturated fatty acids can start at high temperatures when heated in the presence of air, or with the help of trace metals. At storage below 50ºC, oxidation of saturated fatty acids is difficult to measure and quite slow (Harwood and Aparicio, 2000; Aparicio, 2000).

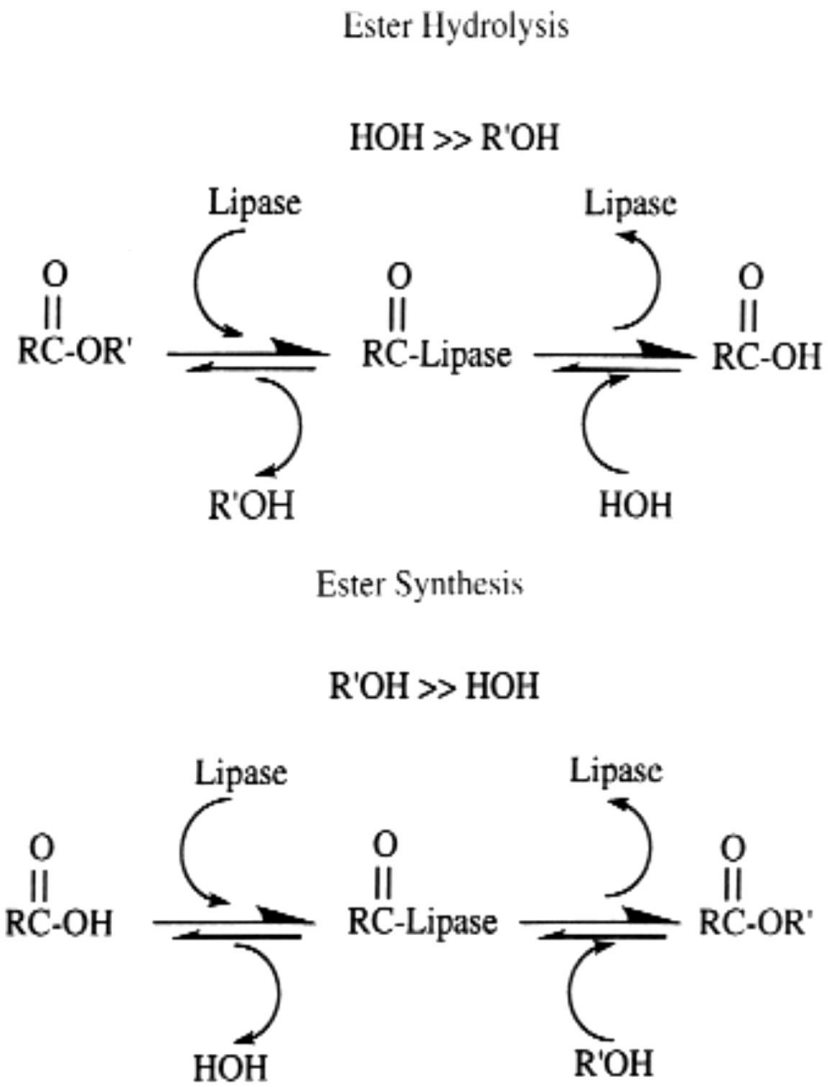


Figure 2.10. Lipase-catalyzed ester hydrolysis and synthesis
(Source: Stefanoudaki - Katzouraki, 2004)

Figure 2.11 demonstrates how the olive oil oxidation (autoxidation) process evolves over time. In the initiation stage, oxidation increases slowly in contact with oxygen or trace metals and reactive oxygen free radicals damage double bonds of unsaturated fatty acids resulting minor quantity of peroxides (Harwood and Aparicio, 2000). After this, peroxides increase gradually up to certain levels and this stage is called propagation, but at some point peroxides break down into other substances which do not raise quickly. During the termination stage, peroxides further break down releasing different off-flavor compounds which can be easily detected by human senses. As a result of the formation of these undesirable compounds, the olive oil might have a bitter rancid

taste. In order to maintain the stability and quality of olive oil which is mainly determined by its fatty acid, polyphenol and tocopherol composition, these reactions should be prevented as much as possible (Dabbou et al., 2003).

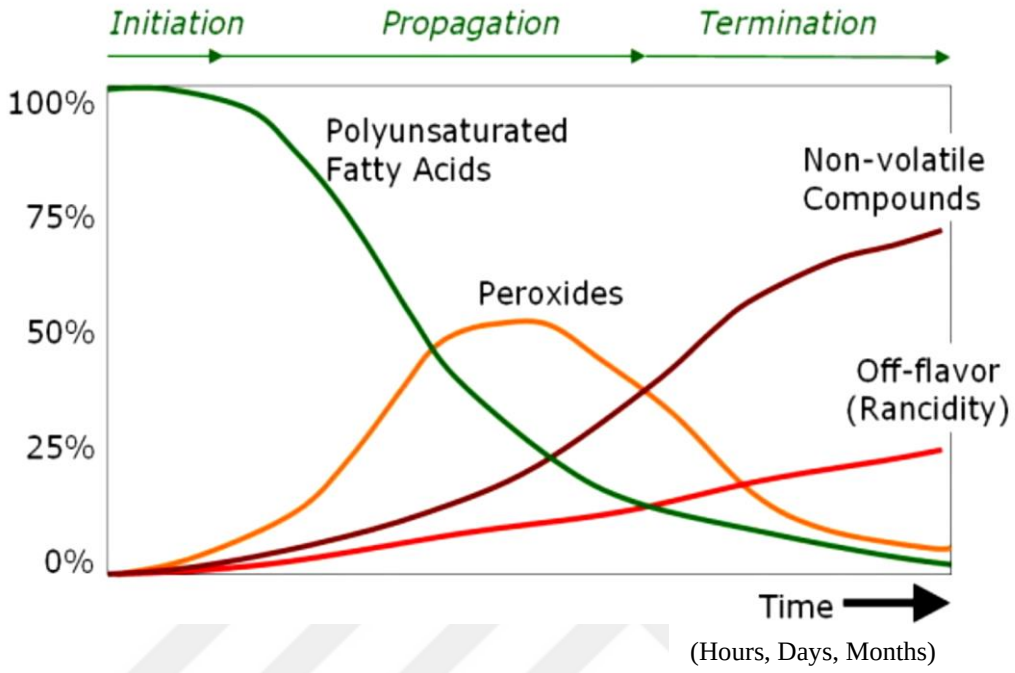


Figure 2.11. Olive oil oxidation process (autoxidation)
(Source: Aparicio, 2000)

Autoxidation process starts with initiation step; the reaction can start to produce free radicals with the aid of a catalyst. There are many different ways for the formation of free radicals, but these pathways are still unclear. At the propagation step, radicals interact with oxygen to yield in peroxyl free radicals. During the termination stage, free radicals react with each other or produce non-radical products (Figure 2.12).

Photo-oxidation mechanism differs from autoxidation pathway. Photo-sensitizers, which have the ability to capture and intensify the energy of light, should be present in lipid. These kind of oxidation reactions occur in the presence of light and photo-sensitizers. When sensitizer (Sen) is formed, there are two pathways for photo-oxidation as shown in Figure 2.13 (Kanavouras et al., 2006).

Several studies investigated different factors related to the quality of olive oil during production. Percent contribution of each factor affecting the quality properties of oil were determined (Aparicio, 2000). According to this research, the most significant

factors were found as ripeness (30%), olive variety (20%), extraction systems (20%), storage (10%) and storage times (10%).

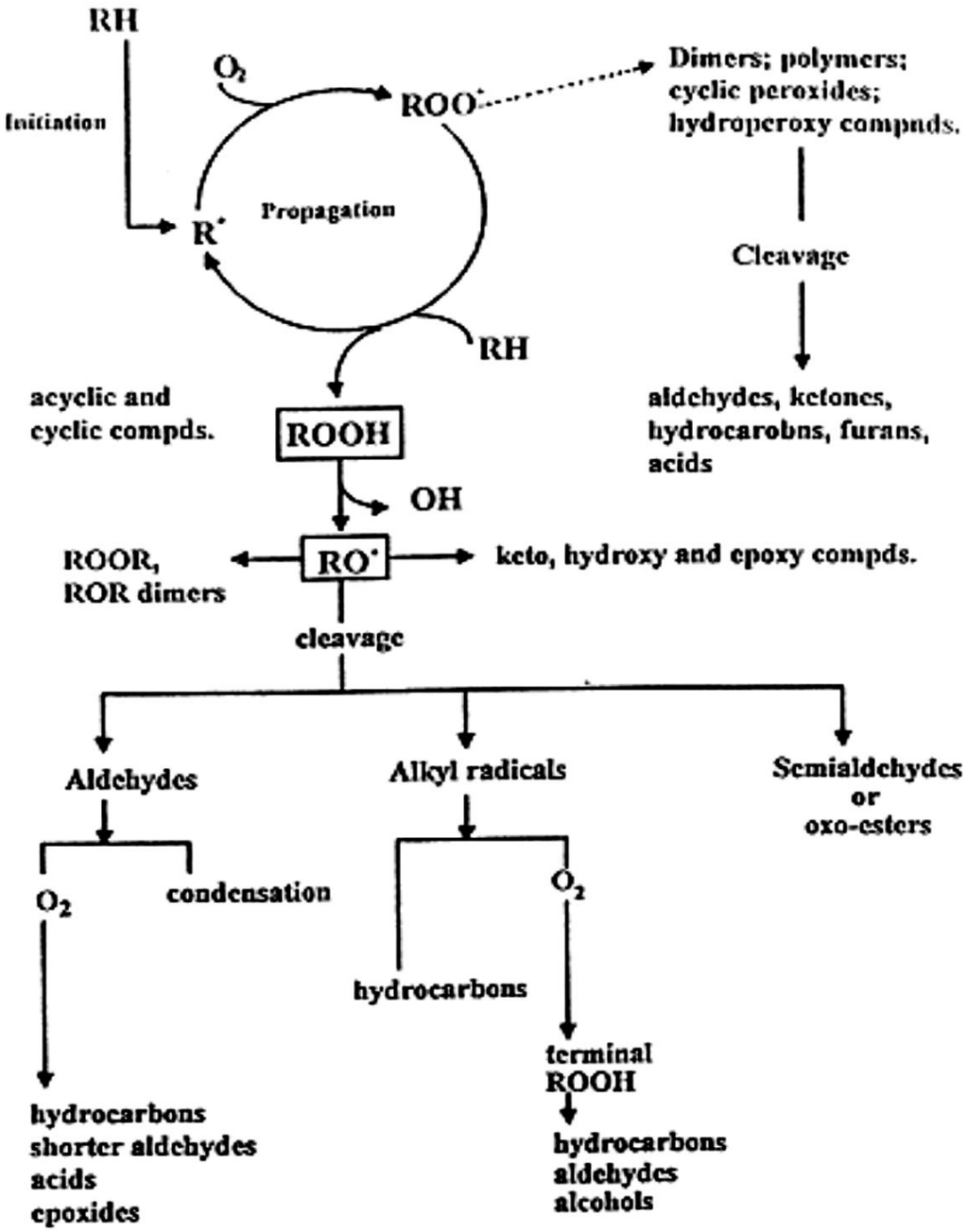


Figure 2.12. Generalized design for autoxidation of lipids

(Source: Fennema, 1996)

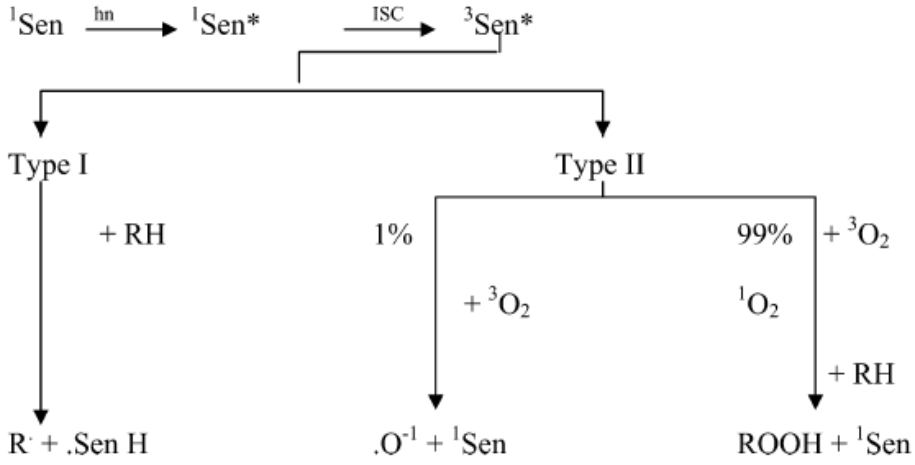


Figure 2.13. Type I and Type II pathways of photo-oxidation process
(Source: Kanavouras et al., 2006)

2.3.2. Adulteration

High-cost ingredients could be replaced with lower grade substances and cheap ingredients to adulterate the food products. Due to the potential of making profit, the extra-virgin olive oil is often adulterated with cheap oils. EVOO has unique sensory properties and also nutritional benefits. On the other hand, other vegetable oils and lower grade olive oils may undergo further processing in order to remove many undesirable flavors (Yang and Irudayaraj, 2001). The most common adulterants found in EVOO are refined olive oil, seed oils and nut oils because of their similar physical and chemical characteristics (Frankel et al., 2011; Kostik et al., 2013). When an EVOO and edible oil are mixed at any rate, the resulting oil consists of the fatty acids and other compounds from both oils (Ruiz-Samblás et al., 2012).

The typical consumer may not be able to determine whether a product that is labelled as extra virgin, is really extra-virgin or refined or mixed to some degree with lower quality oil. The high value of EVOO and the potential difficulty in detecting adulteration provides opportunities for fraudsters in the olive oil industry.

The best quality among all grades of olive oil is EVOO, and EVOO can be purposely mixed with inferior vegetable oils or lower grade olive oils. Major consequences of this mixing is economic and health related (Aparicio et al., 2013; Tekaya et al., 2018). In order to assure EVOO quality, the different international organizations

(EEC/2568/1991; EUC/796/2002; International Olive Council, 2013 and Codex Alimentarius (FAO-OMS,1981)) determined critical values of some parameters for each olive oil grade. Despite these legal standards, adulterations have been reported in the olive oil industry (Frankel et al., 2011).

2.4. Analytical Methods for Quality Evaluation of Olive Oil

Various analytical methods are available for determining the different quality parameters of olive oil.

2.4.1. High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography is a powerful technique which provides the separation of a whole into individual parts. The samples can be separated on the basis of solubility and polarity of their components (Nollet, 2000). This technique allows both qualitative and quantitative evaluation of the samples.

In recent years, HPLC technique has been improved and various food components have been isolated with this technique (Mueller, 2012). Many studies which investigated the olive oil chemical composition including phenolic compounds and pigments are available in literature.

Various studies have been published on pigment content of olive oils determined by HPLC using different extraction systems (Mínguez-Mosquera et al., 1992; Gimeno et al., 2000; Cichelli and Pertesana, 2004; Giuffrida et al., 2007). In a study, researchers investigated the development of an HPLC method for monitoring the pigment concentrations in virgin olive oils during oxidation (Psomiadou and Tsimidou, 1998). On the other hand, there are limited studies performed with Turkish olive oils and also most of the current literature do not pay attention to the quantification of olive oil pigments during long term storage.

2.4.2. Gas Chromatography (GC)

Gas chromatography offers a high potential for separation of the components of volatile mixtures (Jennings et al., 1997). As a result of many technical improvements and

innovations, the reliability, accuracy, and versatility of modern capillary GC improved in the last decade (Wittkowski and Matissek, 1990). The analysis of lipids, especially TAGs and FAMEs, is an active area of research for the GC application (Jennings et al., 1997). Many methods for the esterification of fatty acids have been published. In spite of the advancements in HPLC, preferred method for determining the fatty acid composition of fats and oils is still the GC analysis of FAMEs (Wittkowski and Matissek, 1990).

Fatty acids are also important compounds in the authentication studies (Aparicio et al., 2015). Diraman and Dibeklioglu (2009) determined the cis-trans fatty acid contents and fatty acid methyl esters (FAMEs) by GC for the characterization of Turkish virgin olive oils. A research investigated the influence of different storage conditions on the quality of Tunisian olive and olive oils by determining FAAE and wax content with GC analysis (Jabeur et al., 2015). Several studies reported that GC combined with chemometric analysis is a powerful technique to determine the composition and quality of olive oil obtained from different cultivars (Bouchaala et al., 2014; Bouarroudj et al., 2016).

Analysis of DAG isomers using a GC method have been a widely accepted analysis (Zhu et al., 2013). A number of authors have considered the investigation of DAG isomers in EVOOs under the different storage conditions and different cultivars (Pérez-Camino et al., 1996; Caponio et al. 2013). However, there is a relatively few literature that is concerned with DAG composition of olive oil during storage (Guillaume et al., 2014).

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

Extra virgin olive oil (EVOO) samples were supplied by Tariş Olive and Olive Oil Agriculture Sales Cooperatives Union (İzmir, Turkey) and also by local producers.

3.1.1. Olive Oil Samples

Olive oil samples were grouped as North and South Aegean samples depending on their production area (Figure 3.1). Samples from North were produced mainly from Ayvalık variety and Memecik variety is the most common olive type for South Aegean olive oils.

Ten olive oil samples from South Aegean Region which is referred as 'S' were used in this study and North Aegean Region olive oil samples (N) consisted of 11 samples. Origins of all samples are shown in Table 3.1 and 3.2. North and South Aegean olive oil samples belong to 2015/16 harvest year. In addition, an olive oil sample (O) was prepared by mixing three olive oils from 2014/15 harvest year (Table 3.2)

Figure 3.1. Map showing the locations where oil samples are obtained from (Source: Google Map, 2017)

Table 3.1. Contents of South and North olive oil mixtures from 2015/2016 harvest year

Sample No	Geographical origin	Sample Code	Amount (mL) (% of total)	Acidity (%)
1	Muğla	S	400 (18.2)	0.4
2	Koçarlı	S	200 (9.1)	0.6
3	Tepeköy	S	200 (9.1)	0.6
4	G. Ege Selçuk	S	200 (9.1)	0.6
5	Horsunlu	S	200 (9.1)	0.2
6	Ortaklar	S	200 (9.1)	0.6
7	Bayındır	S	200 (9.1)	0.4
8	Aydın	S	200 (9.1)	0.6
9	Selçuk	S	200 (9.1)	0.4
10	Sultanhisar	S	200 (9.1)	0.4
			Total	**Average**
			2200	0.48

Sample No	Geographical origin	Sample Code	Amount (mL) (% of total)	Acidity (%)
11	Ezine Ayvalık	N	200 (9.1)	0.4
12	Edremit	N	200 (9.1)	0.5
13	Burhaniye	N	200 (9.1)	0.5
14	Altınoluk	N	200 (9.1)	0.4
15	K.kuyu	N	200 (9.1)	0.3
16	Burhaniye	N	200 (9.1)	0.4
17	Edremit	N	200 (9.1)	0.6
18	Altınova	N	200 (9.1)	0.6
19	Ezine	N	200 (9.1)	0.6
20	Ayvalık	N	200 (9.1)	0.6
21	Havran	N	200 (9.1)	0.6
			Total	**Average**
			2200	0.5

Table 3.2. Contents of old olive oil mixtures from 2013/14 harvest year

Sample No	Geographical origin	Sample Code	Amount (mL) (% of total)	Acidity (%)
22	Urla Gemlik	O	250 (14.3)	3
23	Ayvalık	O	1000 (57.14)	0.9
24	Memecik Muğla	O	500 (28.57)	0.7
			Total	**Average**
			1750	1.53

3.1.2. Preparation of Olive Oil Mixtures

Fresh olive oil samples were blended with an old (from previous harvest year) oil sample at 5 different percentages from 10 to 50% (v/v) (Table 3.3) and prepared samples were stored at room temperature during 15 months of storage period in the dark. Samples were analyzed after 6, 12 and 15 months of storage.

Table 3.3. Abbreviations of olive oil mixtures*

Olive oil sample name	Abbreviations
South fresh oil	S
North fresh oil	N
Old mixture	O
10% old + 90% north	N10
20% old + 80% north	N20
30% old + 70% north	N30
40% old + 60% north	N40
50% old + 50% north	N50
10% old + 90% south	S10
20% old + 80% south	S20
30% old + 70% south	S30
40% old + 60% south	S40
50% old + 50% south	S50

*Numbers coming after adulteration level refer to storage time of 6, 12, 15. e.g. N10 6 shows 10% adulterated North olive oil sample after 6 months of storage.

3.1.3. Chemical Reagents

Analytical grade chemical reagents were used in the experiments and they were purchased from Riedel-de Haen, Sigma-Aldrich and Merck. Standards used in pigment analysis were chlorophyll a, b and lutein and they were also obtained from Sigma-Aldrich. Pheophytin a and b were derived from chlorophyll a and b, respectively according to a procedure in the literature (Sievers and Hynninen, 1977).

3.2. Methods

Analytical methods and chromatographic techniques were used in order to evaluate the impact of storage time and adulteration level.

3.2.1. Free Fatty Acid (FFA) Value Determination

European Official Methods of Analysis (EEC 1991) was used in the determination FFA contents of the oil samples as % oleic acid (Commission European Union, 2015). Approximately 1 g of potassium hydrogen phthalate ($C_8H_5KO_4$) was dried in an oven at 110 ± 0.5ºC for 2 hours and then cooled in a desiccator. 0.5 g of phenolphthalein was mixed with 50 mL of 95% ethanol (v/v). 0.1 N potassium hydroxide (KOH) was prepared in deionized water. For the standardization of potassium hydroxide (KOH), 0.4 g of dried potassium hydrogen phthalate was dissolved in 75 mL of deionized water. About three drops of phenolphthalein were added to the solution. Then, standardization of the solution was done by titrating with KOH solution until the formation of the first persistent faint pink color. The initial and final titrated volumes of KOH were used in calculation.

The mixture of diethyl ether and 95% ethanol-water (95:5, v/v) was neutralized just at the time of use with potassium hydroxide solution by adding 0.3 mL phenolphthalein solution per 100 mL mixture until the occurrence of a change in the color. A sample of olive oil (10 g) was mixed with neutralized mixture and 3 drops of phenolphthalein indicator was added. Then, titration of the mixture was done with 0.1 mol/L solution of KOH until the pink color remained for 30 seconds and the volume of solution spent was measured. The content of FFA was calculated in terms of the amount of oleic acid equivalent and expressed as a percentage with the equation shown below:

$$V \times c \times \frac{M}{1000} \times \frac{100}{m} = \frac{V \times c \times M}{10 \times m} \quad (3.1)$$

V : the volume of titrated KOH (mL)

c: exact concentration of the titrated solution of KOH (mol/L)

M: the molar weight of the oleic acid (282g/mole)

m: weight of the sample (g)

3.2.2. Specific Extinction Coefficients at 232 (K232) and 270 nm (K270)

Extinction coefficients were determined spectrophotometrically using a procedure of International Olive Council (COI/T20 Doc.no. 19/Rev.3) (International Olive Council, 2010).

An accurately weighed (0.25 ± 0.05 g) olive oil sample was placed into a 25 mL volumetric flask and then filled up with cyclohexane as the solvent. The solution was gently mixed for 30 seconds to obtain a homogeneous solution.

A standard 10 mm quartz cuvette cell having an optical length of 1 cm was used in spectrophotometric measurements. The specific extinction coefficients of the clear solution were obtained at 232 and 270 nm (Shimadzu UV-Visible spectrometer, UV-1601, Japan). Pure cyclohexane was used as the blank.

3.2.3. Fatty Acid Profile Determination

After alkaline treatment, fatty acid profiles of the oil samples were determined as fatty acid methyl esters (FAMEs) by a gas chromatography (GC) method. The procedure was adapted from European Official Methods of Analysis (Commission European Union, 2015). Briefly, approximately 0.1 g of oil sample was mixed with 10 mL of n-hexane in a 15 mL centrifuge tube and subjected to alkaline saponification by adding 0.1 mL of 2N methanolic potassium hydroxide solution (5.6 g/50 mL). Then, the sample solution was shaken by a vortex for 30 s and centrifuged for about 15 min at 5000 rpm. Filtration of clear supernatant was done by using 0.45 μm syringe filter and then transferred into 2 mL vial and injected to a GC device.

The composition of fatty acids was expressed as percentages of FAMEs. FAME identification was carried out by Agilent Technologies 6890 GC having a Flame Ionization Detector (FID) and Agilent 7683 auto-sampler. The configuration of the instrument and analytical conditions are provided in Table 3.4.

Table 3.4. Instrumental conditions of GC chromatographic analysis of fatty acid methyl esters (Source: Commission European Union, 2015)

Chromatographic system	Agilent 6890 GC
Inlet	Split/splitless
Detector	FID
Automatic sampler	Agilent 7683
Liner	Split liner (p/n 5183-4647)
Column	100 m x 0.25 mm ID, 0.2 μm HP-88 (J&W 112-88A7)
Inlet temperature	250^{o}C
Injection volume	1μL
Split ratio	1/50
Carrier gas	Helium
Head pressure	2 mL/min constant flow
Oven temperature	175^{o}C, 10 min, 3^{o}C/min, 220^{o}C, 5 min
Detector temperature	280^{o}C
Detector gas	Helium make-up gas:30mL/min Hydrogen:40mL/min; Air:450mL/min

Fatty acid peaks were designated using a 14 component FAME mixture (Supelco, Bellefonte, PA, USA). After peak identification fatty acid percentages were determined on a peak area basis. The results were calculated in terms of a relative area percentage of total FAMEs by applying equation below.

$$FA\ composition\ (\%) = \frac{A_S}{A_T} \times 100 \quad (3.2)$$

where: A_s= area of the specific fatty acid peak,

A_T = total area of the main fatty acid peaks.

3.2.4. Fatty Acid Methyl Esters and Fatty Acid Ethyl Esters and Wax Content Determination

A standard method by European Commission Regulation (EU No.61/2011) was used in simultaneous determination of alkyl esters and waxes (Commission Regulation, 2011). The method includes a procedure for the separation by column chromatography and then a GC analysis.

3.2.4.1. Preparation of reagents prior to analysis

Before analysis, an accurate amount of Silica Gel 60 (60-200 µm mesh) was dried in a muffle furnace at 500ºC for a period of at least 4 h. Silica gel was cooled and then mixed with 2% water which is proportional to the quantity of silica gel and was homogenized. The silica gel-water mixture was shaken to homogenize slurry and placed in the desiccator for at least 12 h.

A solution of methyl heptadecanoate (C17:0 ME, 0.02% (w/v), 0.02 g/100 mL of n-hexane) was used as an internal standard for determining esters. Dodecyl arachidate (0.05% (w/v), 0.05 g/100 mL of n-hexane) was also added to hexane and used as an internal standard for waxes. Sudan I solution (1-phenylazo-2-naphthol, 0.1/100 mL of n-hexane) was used as an indicator. n-hexane/ethyl ether (99:1, v/v) mixture was used as a mobile phase and prepared freshly every day.

3.2.4.2. Olive oil sample preparation and analytical conditions

First**,** approximately 5 mm high topper of cotton wool was placed into the lower part of 5 mL pipette tip, then 15 g of silica gel 60 (2% hydrated) and a tea-spoon full of

sodium sulfate (Na_2SO_4) was added for the protection against moisture. Another same height stopper of cotton wool was inserted on top of the silica gel and glass column was clamped on a retort stand. The silica gel was conditioned with 30 mL of n-hexane to keep stationary phase-silica gel from drying and to remove any impurities.

500 mg of olive oil sample was added to 200 µL of dodecyl arachidate, 250 µL of methyl heptadecanoate and 100 µL sudan 1 (2-3 drops) solutions. Thereafter, the mixture was transferred to a column using 2x2 mL of n-hexane. The beaker was rinsed into the column completely with mobile phase. The column was eluted with solvent mixture (hexane/ethyl ether) until the dye flowed to the lower part of the column since Sudan I dye and TAGs have similar retention times. The resultant fraction was evaporated in a rotary evaporator at 45ºC for 10 min. The fraction having the alkyl esters and waxes was diluted with 2 mL of n-heptane, and then filtered through 0.45 µm PTFE syringe filter into dark brown vial. 1.5 µL of dissolved eluent was injected into the GC.

Esters and waxes identification was carried out with a 7890A Agilent Technologies Gas Chromatograph System (Santa Clara, CA, USA) equipped with a flame ionization detector (FID). The column was a capillary HP-5 with dimensions of length 30 m, id 0.32 mm and film thickness 0.25µm. The operating conditions were as follow: oven temperature was kept at 80ºC for 1 min and then increased from 20ºC/min up to 140ºC, then raised from 5ºC up to 335ºC and maintained for 20 min; injector was programmed from 70 to 300ºC; detector temperature was kept at 350ºC. Carrier gas was helium with a flowrate through the column of 1 mL/min and 1:50 split ratio.

3.2.4.3. Peak Evaluation and Quantitative Analysis

The methyl and ethyl esters of the major fatty acids in olive oil (C16 ME, C16 EE, C18 ME and C18 EE, respectively) were identified by their positions with respect to internal standard peaks. The quantification of each peak was done on the basis of the area corresponding to the C17:0 ME IS. The results were calculated in terms of the sum of the of the methyl and ethyl esters from C16 to C18, and the total of the two, expressed to the nearest mg/kg (ppm).

$$\sum ME+\sum EE= (100- \text{Area of ISTD/Area of ISTD})/ \text{weigh of oil (g)} \text{ X } 100 \text{ X } 0.5 \quad (3.3)$$

$$\sum ME+\sum EE=MEE \quad (3.4)$$

For wax content, the wax internal standard was determined and 4 other wax peaks after two identical peaks had been identified. The result was obtained in ppm wax.

Total wax content = (100- Area of ISTD/Area of ISTD)/ weigh of oil (g) X 100 (3.5)

3.2.5. Diacylglycerol Determination

The GC determinations of DAG provide a separation into 1,2- and 1,3-DAG forms. The analysis was performed according to International Organization for Standardization (ISO) 29822:2009 method (International Standardization Organization, 2009).

3.2.5.1. Preparation of reagents and silica gel chromatography column

Accurate amount of Silica Gel 60 (60-200 μm mesh) was activated in the muffle furnace by heating overnight at 160±5ºC. Then, the silica gel was cooled in a desiccator and transferred into a glass bottle. Before the analysis, 5% mass fraction of water was mixed with silica gel and shaken until no lumps could be seen so that the powder was flown freely. The conditioned silica gel was stored overnight.

Approximately 2 mm high cotton was put into the lower part of 5 mL pipette tip followed by placement of 1 g of silica gel 60 (previously dried and hydrated). Another 2 mm high stopper of cotton wool was inserted on top of the silica gel. The filling was slightly pressed down on the cotton wool with the end of a pipette tip.

An internal-standard solution of 1,2 and 1,3-dipalmitin mixture (1 mg/mL) was prepared for the analysis of oil samples. An internal-standard solution of 1,2 and 1,3-distearin mixture was also used.

Each solution was prepared by dissolving standards in toluene with respect to the accurate amount of internal standard solution. Isooctane was added to diisopropyl ether in the volumetric ratio of 85:15 as the solvent mixture. Silylating reagent was prepared by mixing 50 μL of 1-methylimidazole with 1 ml of N-Methyl-N-(trimethyl-silyl) heptafluorobutyramide (MSHFBA) (1-methyl imidazole:MSHFBA, 1:20, v/v).

3.2.5.2. Separation of the non-polar lipids fraction

A 100 mg oil sample was mixed with 1 mL toluene. The solution was flowed through a silica column followed by addition of 1 mL of isooctane/diisopropyl ether (85:15, v/v). The column was washed with 2x3.5 mL of the solvent mixture to elute the relative hydrophobic compounds including triacylglycerols. The pipette tip was washed with solvent mixture and the solvent was thrown away. DAGs were eluted with 2.5 mL portions of diethyl ether and the eluate was kept in a pointed flask. The solvent was evaporated from the eluate with a rotary evaporator at 20ºC.

As the next step, 200 µL of the silylation reagent was placed into the pointed flask. The solution was kept sealed and reaction continued for 20 minute at room temperature. Following the silylation, 1 mL of acetone was added into this solution. Clear solution at the top was taken into a GC vial before analysis with the GC.

3.2.5.3. Peak evaluation and calculation

The analyses were performed using a GC equipped with a Restek RTX-5 column (France), having dimensions of 60 m length, 0.25 mm inner diameter (i.d.) and 0.10 µm film thickness and an injector on-column and a flame ionization detector (FID). The temperature program was as followed: from 180 to 360ºC, 6ºC/min, isotherm at 360ºC for 10 min; FID temperature 400ºC. Injection volume was 1 µL (split ratio 1:50) and hydrogen at 2 ml/min was the carrier gas. Hydrogen at 33 mL/min and air at 420 mL/min flow rates were the gases for FID. Injector, detector and oven temperatures were set to 340ºC, 340ºC, 240ºC; respectively.

A standard solution of dipalmitin (1,2-C32, 1,3-C32) and distearin (1,2-C36, 1,3-C36) were prepared in toluene. 100 µL of each of these standard solutions were transferred to derivatization vials. The solvent was blown off in the reaction vials with a stream of nitrogen and silylated.

Calculation of the area % of 1,2-diacylglycerols (W) with respect to the total of the areas of the individual 1,2- and 1,3-diacylglycerols (C32, C34, C36) were done with the equation below:

$$W = {Ay * 100}/{Ax} \quad (3.6)$$

Ay = Peak area of all 1,2-diacylglycerols (C32, C34, C36) in the olive oil sample
Ax = Sum of the peak areas of the individual 1,2 and 1,3-diacylglycerols (C32, C34, C36)

3.2.6. Quantification of Chlorophylls and Carotenoids

A modified procedure from literature (Mateos and García-Mesa, 2006) was used in the determination of pigments.

3.2.6.1. Preparation of standards, reagents and samples

Stock solutions of chlorophyll a, b and lutein were prepared in acetone. Amount of pheophytin a and b were calculated using the standard curve of chlorophyll a and chlorophyll b at that specific wavelength, respectively.

To prepare mobile phase A (water-ion-pair reagent-methanol (1:1:8 v/v)), firstly, ion pair reagent was mixed with purified/deionized water and methanol in volumetric ratio of 1:1:8. Deionized water was passed through a 0.45 µm nylon membrane filter. The ion-pair reagent solution was prepared by mixing of tetrabutylammonium bromide (0.05 M) and ammonium acetate (1 M) in deionized water. For the preparation of other mobile phase B (acetone-methanol (1:l, v/v), HPLC grade solvents (methanol-acetone(1:1, v/v)) were used without further purification. The mobile phases were degassed before use.

The analyses were performed using a HPLC (Agilent 1200 HPLC, USA) having a Spherisorb ODS-2 column (Supelco, Germany) (25 cm x 4.6 mm i.d.; 5-µm particle size) protected with a guard cartridge (3.2-4.6 mm i.d.) (Supelco, Germany) packed with the same material as the column and diode-array detector (DAD). Gradient scheme used in HPLC analysis is shown in Table 3.5.

3.2.6.2. Procedure for pigment extraction

Solid-phase extraction (SPE) were performed with octadecyl (C_{18}) disposable extraction columns (Agilent, AGT-12256001 Mega BE-C18)**.** First 6 mL of methanol and then 6 mL of hexane were used in the conditioning of the column. An oil sample, 1 g, dissolved in 4 mL of hexane. This solution was then placed into the C_{18} cartridge. The beaker was then washed twice with 3 mL of hexane, using these washings back on the

cartridge again. Finally, after checking any remaining lipids, the non-polar substances/pigments were eluted with 5 mL of acetone soon after draining of the solvent from the top of the cartridge. This fraction was placed in a heart-shaped flask and kept out of light. The obtained sample and solvent were dried in a rotary evaporator at a maximum temperature of 30ºC. The residue was taken into 0.3 mL of acetone. This solution was injected to the HPLC.

Table 3.5. Gradient scheme used for the HPLC analysis of the olive oil pigments (Source: Mateos and García-Mesa, 2006)

	Mobile Phase	
Time (min)	**A (%)**	**B (%)**
0	75	25
10	50	50
12.5	50	50
14.0	20	80
16.0	20	80
21.0	0	100
35.0	0	100
40.0	75	25

3.2.6.3. Peak evaluation and calculation

Chromatograms were obtained at 410, 430, 435, 446 and 466 nm and 11 different pigments were identified by comparison with respect to retention times of commercial standards. Amounts of pigment as mg/kg were calculated from their respective calibration curves.

Total xanthophyll was obtained relatively as the sum of the peaks on the left side of the lutein in HPLC chromatogram. β-carotene and a part of pheophytin a were measured by using UV-vis spectrophotometer in a quartz cuvette with the hexane as blank and concentration was calculated according to equation given below:

$$\text{Concentration (g/ml)} = E \div (E^{1\%}_{1\ cm} \times 100) \quad (3.7)$$

where E is the absorbance and $E^{\%1}$ is the extinction coefficient in hexane. E= 2592 and $E^{\%1}$=454 nm for β-carotene and E= 613 and $E^{\%1}$= 660 nm for pheophytin a.

3.2.7. Spectral Measurements

All infrared spectra of the samples were collected between the ranges of 4000-650 cm^{-1} wavenumber with ZnSe-horizontal ATR accessory of a FTIR spectrometer (Spectrum 100, Perkin Elmer, USA) having a DTGS detector. Number of scans and resolution were set to 64 and 4 cm^{-1} for the collection of each spectrum. Air spectrum was obtained as the background.

UV-visible spectra of the samples were obtained in a plastic cuvette within the ranges of 200-800 nm with a UV-vis spectrometer (Shimadzu, Japan). Measurement parameters were 2.0 nm sampling interval and 5.0 nm slit width.

3.2.8. Statistical Analysis

Measurements of at least 3 randomly chosen samples were repeated 3 times and standard deviations of these measurements were calculated for each analysis.

Multivariate analysis offers a series of tools for management of a very large number of data and variables from different analytical methods. Convenient multivariate model recognition techniques to process the data are required, especially for the spectral data. The low-set data from combination of various instruments could be also combined into a single matrix before multivariate model development. The mid-set data combination firstly derives characteristics from individual matrices and then integrates the obtained variables into a 'scores matrix' which is used to create the final model. These new features are generally obtained by techniques which differentiate/separate the samples such as Principal Component Analysis (PCA) or Partial Least Squares-Discriminant Analysis (PLS-DA). In this study, the multivariate analyses were performed by SIMCA 13.0.3 software (Umetrics, Sweden).

PCA is a widely known unsupervised multivariate technique which is used to reduce the number of observed variables into smaller number of artificial variables which explain the most of the variances in the data set. Choosing the most useful variables, visualization of multidimensional data, identification of underlying variables, and identification of groups of objects or of outliers are PCA's general purposes.

PLS is a supervised regression method which aims at predicting Y variables (basic quality parameters included free fatty acid and absorbance values, fatty acid content, pigment composition, fatty acid alkyl esters) from X variables (FTIR spectra) by maximizing the correlation between them. The general principle of PLS-DA is to find a model that separates classes of observations on the basis of their X-variables.

Results of PCA and PLS-DA can be given in two supplementary plots as scores and loading plots. (Euerby & Petersson, 2003). Score plot indicates how the observations are scattered and which of them are clustered to differentiate principal groupings among observations while loading plots are focused on variables to reveal which variables are responsible from the groupings among the observations. For example, observations close to each other in the space of principal components have similar characteristics in the score plots. Similarly, the variables whose unit vectors are close to each other are said to be positively correlated, meaning that their influence on the positioning of objects is similar in the loading plots. However, variables far away from each other will be defined as being negatively correlated.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Evaluation of Basic Quality Parameters During Storage Time

Extra virgin olive oils (EVOO) from North and South Aegean Region were mixed with old olive from previous harvest year at various ratios (10-50%, v/v). Several chemical parameters including free fatty acid value, specific extinction coefficients, fatty acid profile, fatty acid methyl and ethyl esters, diacylglycerols and pigment contents of fresh oils and fresh oils adulterated with old olive oil were determined during 15-months storage period and the results are presented in this section. In this study, samples were kept in dark at room temperature to mimic domestic use. Same chemical characteristics of old olive oil determined throughout storage and the data are presented in the Appendix A (Table A.6, A.7 and A.8).

4.1.1. Specific Extinction Coefficients (K_{232} and K_{270} Values)

Extinction coefficients, K_{232} and K_{270}, are useful and simple parameters for indicating the status of olive oil oxidation. Autoxidation reactions are associated with the formation of conjugation double bonds (Boskou, 2008). In addition, the configuration of fatty acids could be changed easily by refining process and this causes formation of conjugated dienes and trienes (Angerosa, 2006). K_{232} is related with the primary oxidation products, conjugated dienes, which are the result of a shift in one of the double bonds (Ran, 2014; Vekiari et al., 2007) and K_{270} is indicative of conjugated trienes (the main oxidation products of linolenic acid) and secondary products of oxidation, such as aldehydes and ketones (Ran, 2014). In EU regulation, the maximum permitted values for K_{232} and K_{270} are 2.50 and 0.22 for EVOO, and 2.60 and 0.25 for virgin olive oil (VOO), respectively (EU Regulation, EEC No.2568/91, 2015). Changes in K_{232} and K_{270} values of the olive oil samples are listed in Appendix A and they are graphically shown in Fig. 4.1 and 4.2.

The initial K_{232} values of all samples except one (N50) were within the legal limit at the beginning of the storage. Most of the oil samples were above the upper limit of 2.5 of K_{232} for EVOO and 2.6 for VOO during 15-months storage. K_{232} values of all samples increased at the end of six months of storage and then they decreased on the 12th month of the storage time and they continued to decrease at the last part of the storage (Figure 4.1). This decrease is probably related to the conversion of primary autoxidation products to secondary products during storage. There was no difference in the change trend of the oils with respect to their origin and pure and adulterated samples also followed the same type of trend for K_{232} value.

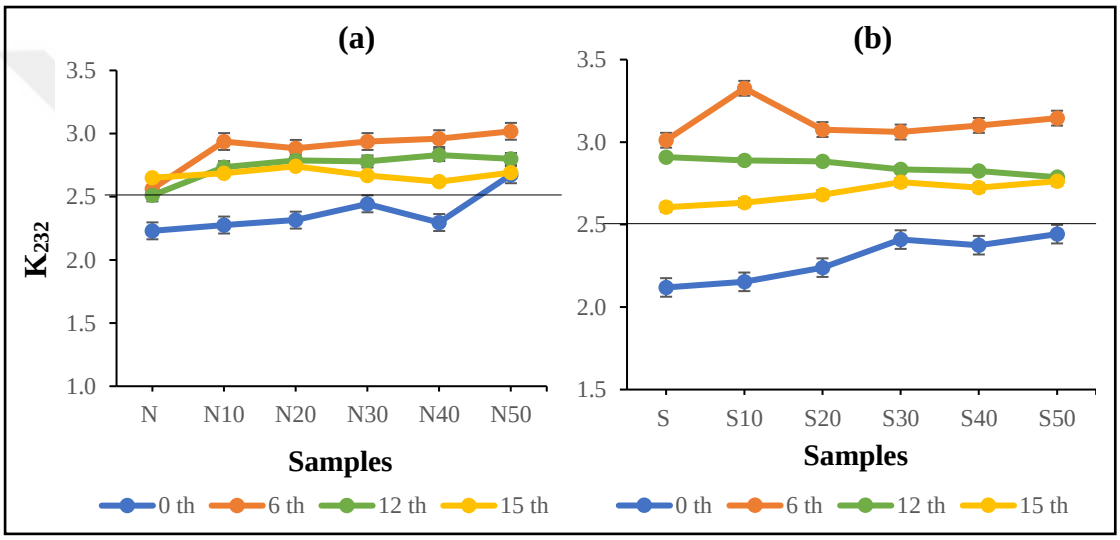


Figure 4.1. K_{232} values of the samples during 15 months of storage period (a) North Aegean olive oil samples (b) South Aegean olive oil samples

Our results agree with the studies in the literature in spite of the fact that slightly different storage times, different packaging materials and storage parameters were used in those studies (Caponio et al., 2005; Vekiari et al., 2007; Méndez and Falqué, 2007).

Figure 4.2 shows the changes in K_{270} values of fresh and adulterated oil samples with respect to storage time. A sharp increase in K_{270} values was observed at 6th months of storage and all of the olive oil samples exceeded the limit at the end of 6 months; therefore, the samples used are not considered as “extra-virgin” anymore. As it happened in K_{232} value, K_{270} values decreased continuously after 6 months. This decrease again can be associated with conversion of measured oxidation products into different products.

With regards to origin of oil and adulteration, same trends were observed in the change of K_{270} values during the storage.

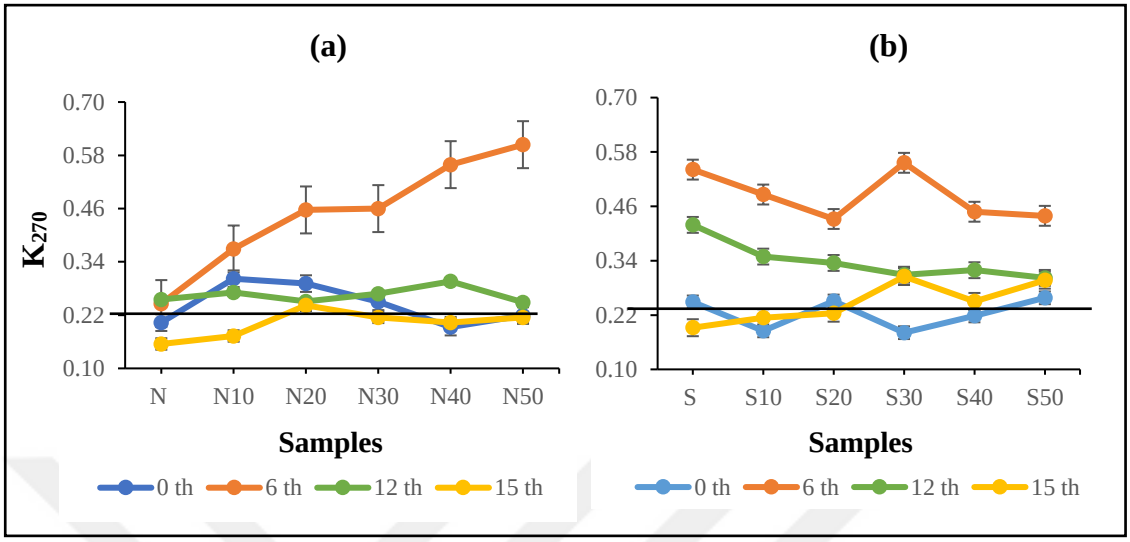


Figure 4.2. K_{270} values of the samples during 15 months of storage period (a) North Aegean olive oil samples (b) South Aegean olive oil samples

Both K_{232} and K_{270} indices of fresh and adulterated samples first increased in 6 months of storage time but then had a decreasing trend; therefore, these indices might not be good indicators of quality during storage at longer periods. Specific extinction coefficients measure the primary and secondary oxidation products and some of these compounds formed repeatedly and were converted into different products depending on the storage time and conditions (Dabbou et al., 2003). Therefore, these fluctuations are expected.

4.1.2. Free Fatty Acidity (FFA)

FFA is an important and essential chemical property which has been used particularly in classifying olive oils. The release of fatty acids from TAGs results in the formation of FFAs in the olive due to the enzyme activity, and further reactions are stimulated by heat, water and light (Tena et al., 2015). Consequently, FFA content shows how olives were handled prior to extraction and is a sign of TAG hydrolysis (Tena et al., 2015).

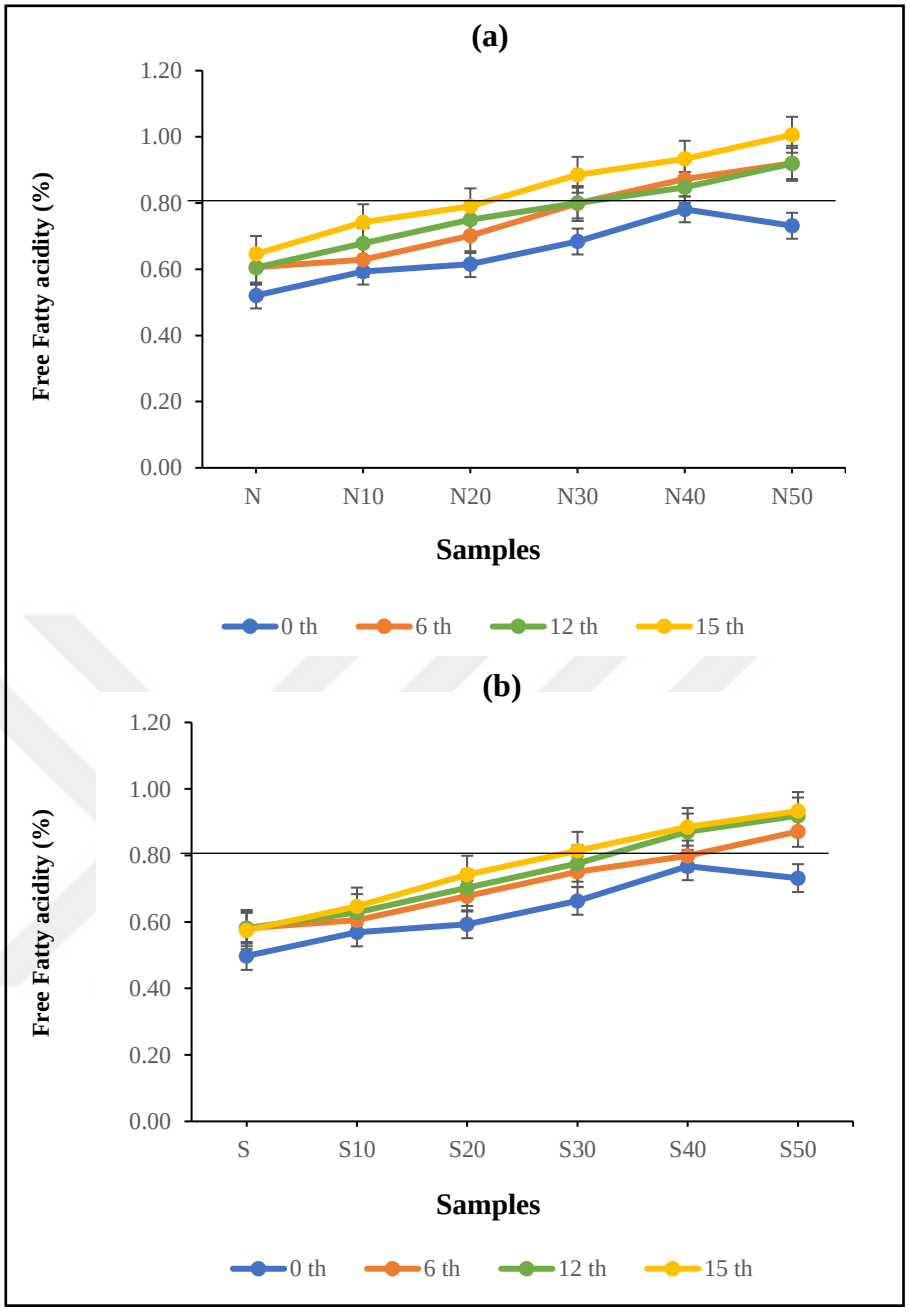


Figure 4.3. FFA values of EVOO samples during 15 months storage period (a) North Aegean olive oil samples (b) South Aegean olive oil samples

FFA values of the oil samples increased linearly with respect to increasing adulteration percentage during the storage period (Figure 4.3). In addition, FFA also increased with respect to time for all samples. While fresh oil samples and adulterated oil samples at 10-30% levels had FFA below 0.8%, which is a limit for EVOO grade, during 15 months of storage, higher adulteration levels reached to this level of FFA after 6 months. FFA values of non-adulterated samples increased 24.1% for North oils and

15.5% for South oils after 15 months of storage. 37.6% and 27.6% rise in FFA values were observed at the end of 15 months for 50% adulterated North and South oils, respectively.

Indeed, 30% adulteration is a critical point for the samples from both regions because a progressive increase in FFA level as a quality attribute was observed as shown in Figure 4.3. While samples containing up to 30% old olive oil have FFA lower than 0.8% during 15 months, FFA reached to levels higher than 0.8% at and above 40% adulteration in first 6 months of storage.

In a previous study, the quality indices of olive oils produced from different olive varieties grown in India and stored in different container types at dark for 180 days were determined (Gargouri et al., 2015). It was found out that the FFA values of the olive oil samples sustained their initial acidity values for 75 days, and acidity increased with increasing storage time.

4.1.3. Fatty Acid Profile (FAP)

The individual fatty acid composition is specific to each particular type of fats and oils. Major fatty acids in olive oils were palmitic (C16:0), oleic (C18:1) and linoleic (C18:2) acids (Figure 4.4). The minor fatty acids were, on the other hand, palmitoleic (C16:1), stearic (C18:0), linolenic (C18:3) and arachidic (C20:0) acids. Changes in the composition of major fatty acids of fresh and adulterated oils are shown in Figures 4.5-4.9.

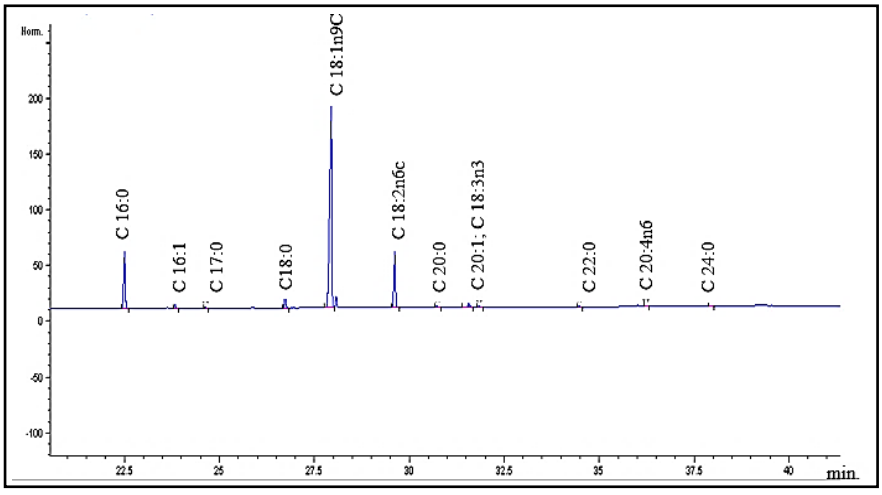


Figure 4.4. GC chromatogram of olive oil sample from North region

Oleic acid as a monounsaturated fatty acid constitutes the major portion of all fatty acids in olive oil. Oleic acid composition of South Aegean olive oil samples (70.20-73.44%) is higher than North Aegean olive oil samples (68.79-70.41%) while the samples from North have higher linoleic (10.49-11.38% of North and 9.04-12.09% of South) and palmitic (13.44-14.49% of North and 12.28-13.05% of South) acid concentrations. Fatty acid compositions, especially oleic acid content, are mainly affected by growth location, variety and maturity stages of the olives (Salvador et al., 2000) and olive oils from South Aegean Region were generally reported to have higher oleic acid content (Gurdeniz et al., 2008).

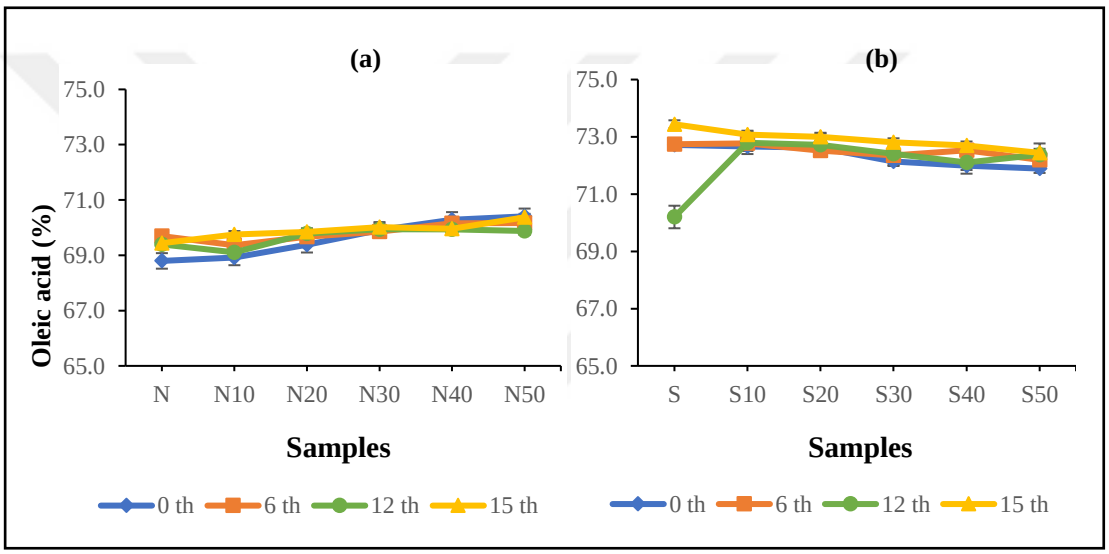


Figure 4.5. Oleic acid contents of EVOO samples during 15 months of storage period (a) North Aegean olive oil samples, (b) South Aegean olive oil samples

There are not any major changes in the concentrations of major fatty acids of fresh oils and old oil containing fresh oils with respect to storage time. After 15 months, percent changes of oleic acid content of non-adulterated oils from North and South Aegean Region were 0.93% and 0.99%, respectively. 15 months of storage resulted in 0.05% and 0.76% difference in oleic acid content of 50% adulterated North and South oils, respectively. Even the polyunsaturated linoleic and linolenic acid contents of old olive oil containing samples were not affected much during storage. For example, differences in linolenic acid content of both non-adulterated South and 50% adulterated South oil samples were approximately 5% at the end of 15 months of storage. This could be related to high phenolic content of olive oils since these compounds have antioxidant activity.

Therefore, it could be stated that fatty acids in olive oil are quite stable components and they are not good indicators of quality change during storage.

A slight change in fatty acid composition of olive oils stored for 12 months was also reported in the literature (Morelló et al., 2004). In addition, no significant variations in fatty acid compositions of olives stored for 6 months in different containers including glass, plastic and metal packages were observed in another study (Mendez A.I. & Falque, 2007).

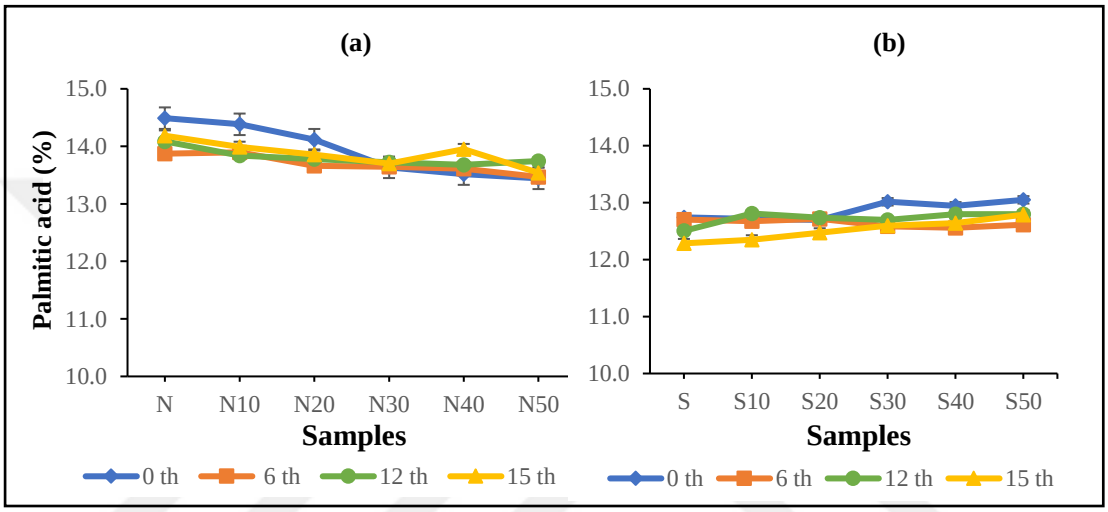


Figure 4.6. Palmitic acid contents of EVOO samples during 15 months of storage period (a) North Aegean olive oil samples, (b) South Aegean olive oil samples

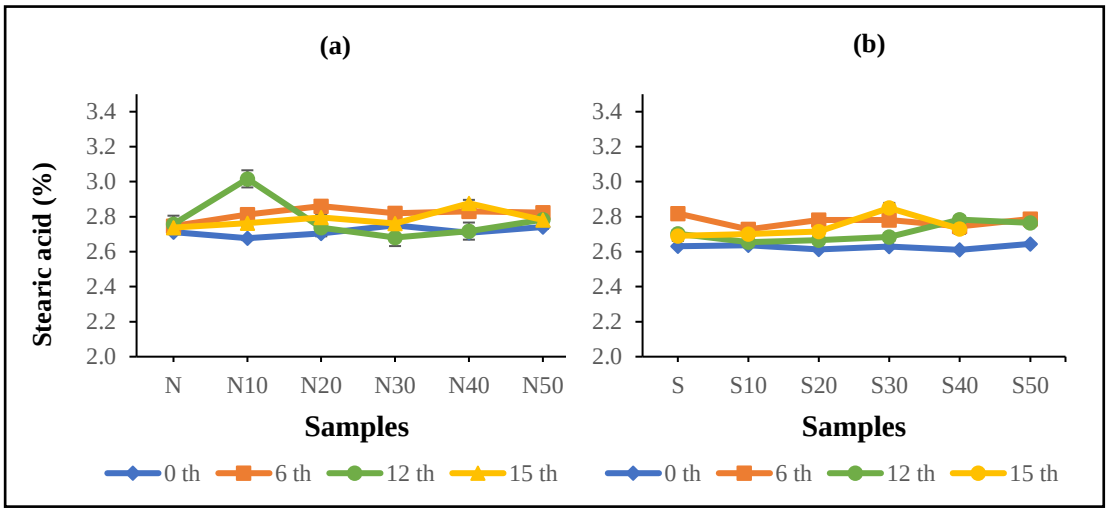


Figure 4.7. Stearic acid contents of EVOO samples during 15 months of storage period (a) North Aegean olive oil samples, (b) South Aegean olive oil samples

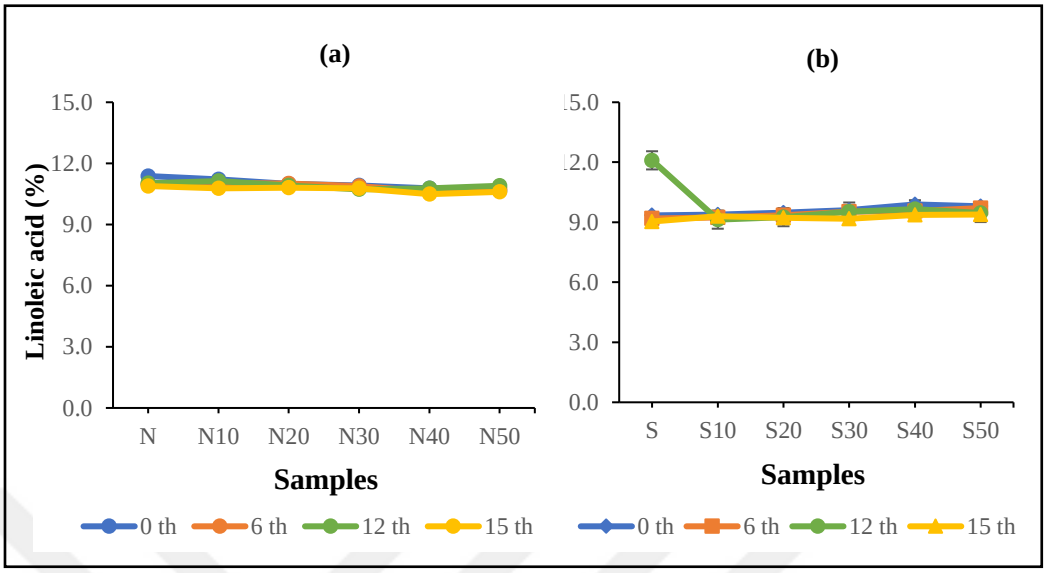


Figure 4.8. Linoleic acid contents of EVOO samples during 15 months of storage period (a) North Aegean olive oil samples, (b) South Aegean olive oil samples

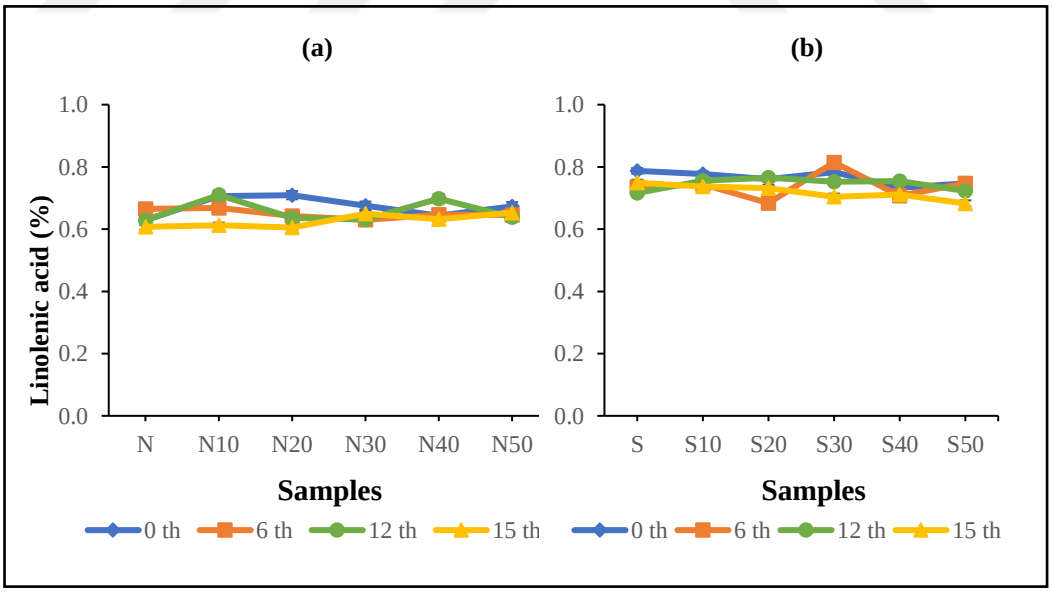


Figure 4.9. Linolenic acid contents of EVOO samples during 15 months of storage period (a) North Aegean olive oil samples, (b) South Aegean olive oil samples

4.1.4. Fatty Acid Ethyl Esters (FAEEs)

FAEE concentration is one of the most effective quality parameters that could be directly associated with the fruit quality at the beginning of the extraction (Gómez-Coca et al., 2016). FAEEs also form as a result of the inappropriate practices during the harvesting and storage of the olives before extraction of the olive oil due to the damage of the olive fruit and its contact with yeasts and lipolytic and pectolytic enzymes (Aparicio et al., 2015). Typical FAAE chromatogram of a sample olive oil is presented in Figure 4.10.

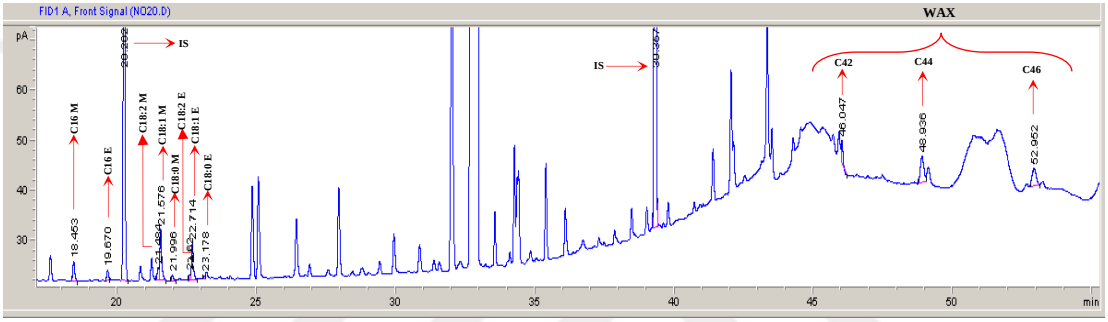


Figure 4.10. GC chromatogram of olive oil sample from North region

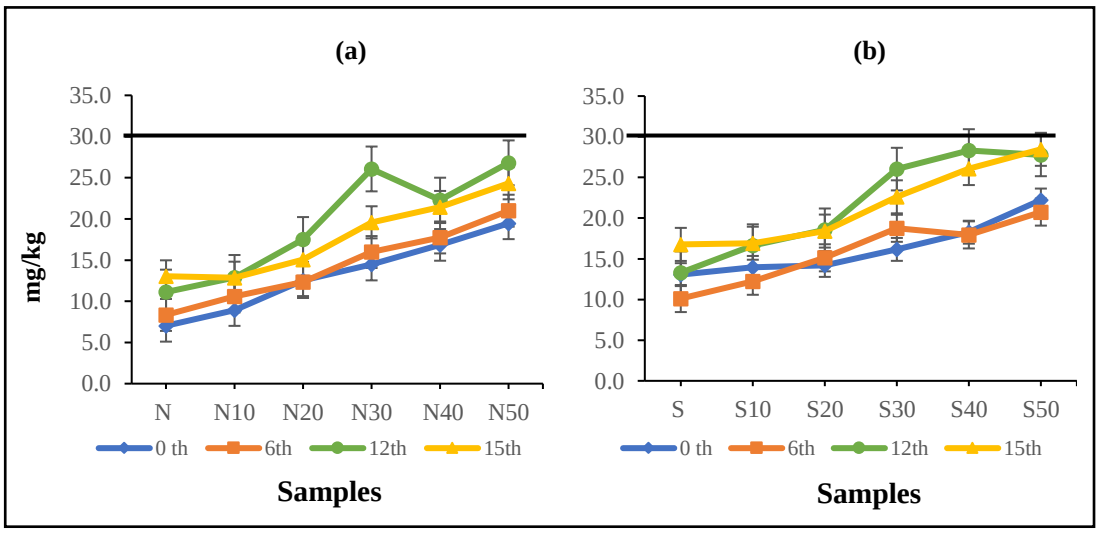


Figure 4.11. FAEEs of EVOO samples during 15 months of storage period (a) North Aegean olive oil samples, (b) South Aegean olive oil samples

Figure 4.11 shows the changes in FAEE concentrations of fresh and adulterated oil samples during storage. FAEE content of adulterated samples increased with increasing adulteration level and increasing trend was similar for North and South oil samples. For both fresh and adulterated samples there is not much change in FAEE content in the first 6 months; however, FAEE amount increased during 12 months of storage. After 15 months, FAEE content of non-adulterated and 50% adulterated North oils increased 85.44% and 24.95%, respectively. Increase in FAEE content of non-adulterated and 50% adulterated South oil samples were 28.46% and 28.05% in order. Despite this increase none of the samples were above 35 mg/kg limit set for FAEEs at the end of 15 months of storage; however, FAEE content of 40 and 50% adulterated samples were quite close to the limit at the end of 12 and 15 months of storage. As in our study, it was determined that FAEE content of olive oils increased with storage time (Gomez-Coca et al., 2016). Furthermore, temperature of storage is an important factor in the formation of FAEE according to the same study. While FAEE levels increased above 30 mg/kg limit in 7.5 months at 40°C, it remained below legal limit at a storage temperature of 20°C for the same storage period. In the current study, samples were kept at room temperature ranging from 15 -27.6ºC. Although FAEE content have an increasing trend during storage it was not very successful as an adulteration indicator for identifying the mixtures of old and fresh olive oils since FAEE contents were still under limits for these samples during 15 months of storage at room temperature.

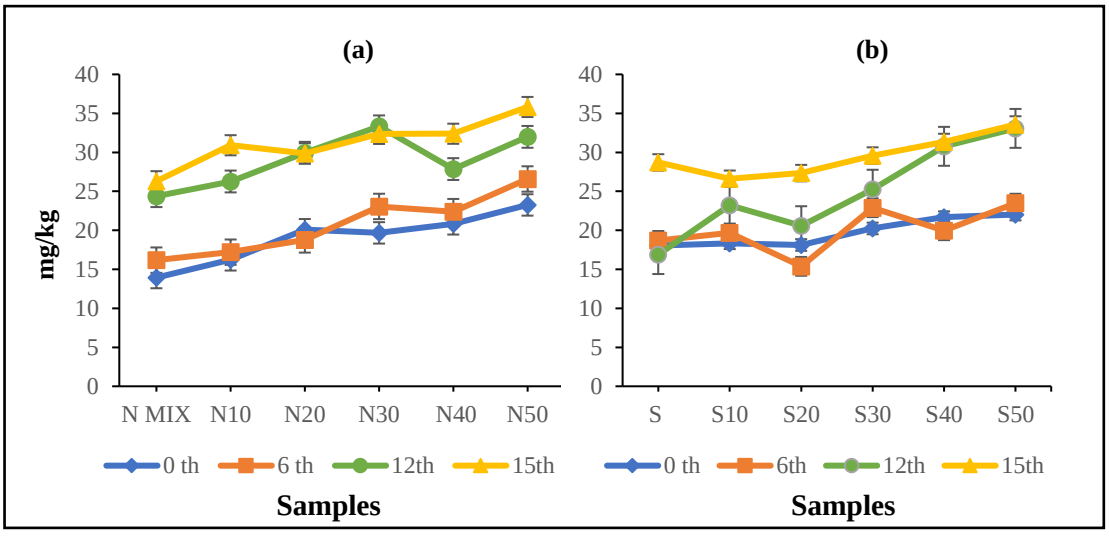


Figure 4.12. FAMEs of EVOO samples during 15 months of storage period (a) North Aegean olive oil samples (b) South Aegean olive oil samples

Changes in fatty acid methyl ester (FAME) contents of the oil samples are shown in Figure 4.12. As could be seen from these graphs trend related to changes of FAME content of non-adulterated samples are quite similar to FAEE. There is no change in FAME content in first 6 months for all oils. However, an increase in the amount of FAME was observed after 12 months of storage. There is also not much change in FAME content of the samples between 12 and 15 months' period for all North oils. However, FAME content increased further in 15 months with respect to 12 months for South oils. After 15 months, FAME content of non-adulterated and 50% adulterated North oils increased 88.63% and 54.02%, respectively. Increase in FAME content of non-adulterated and 50% adulterated South oil samples were 59.03% and 54.42% in order.

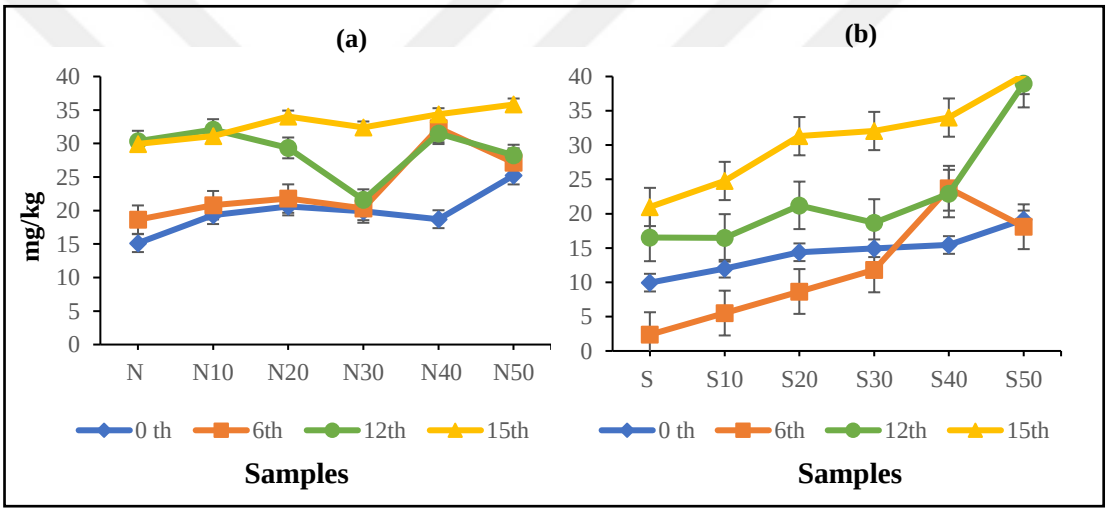


Figure 4.13. Total wax contents of EVOO samples during 15 months of storage period (a) North Aegean olive oil samples, (b) South Aegean olive oil samples

Trend regarding the change in wax content of non-adulterated and adulterated oil samples are quite similar with rise in FAME content (Figure 4.13). Considerable increases were observed especially after 12 months. After 15 months, wax content of non-adulterated and 50% adulterated North oils increased 97.83% and 41.96%, respectively. Increase in wax content of non-adulterated and 50% adulterated South oil samples were 110.80% and 109.80% in order.

4.1.5. Diacylglycerols

1,2-diacylglycerols (DAGs) in olive oil are produced during the biosynthesis or as a result of hydrolysis of triacylglycerols (TAGs). DAG profile of one of the olive oil samples is shown in Figure 4.14. Figure 4.15 shows the change in 1, 2 DAG content of the fresh and adulterated olive oils depending on storage time. Although individual 1,2- and 1,3 DAG profiles of the samples were determined only the results of the total 1,2- and 1,3 DAGs are presented here since more meaningful results are obtained with total DAGs. For olive oils from North, there were no considerable differences in the percentages of 1,2-DAGs during storage for the first 12 months. Change in 1,2 DAG amount in terms of percentage for non-adulterated samples was 2.35% while sample mixed with old oil at 50% ratio have negligible difference at the end of 15 months. For non-adulterated oils from South Aegean Region there was no difference in 1,2 DAG content throughout the storage; however, there was a 3.1% increase for 50% old oil containing sample at the end of 15 months. Therefore, 1,2 DAG content does not look like a good indicator of oil quality during storage.

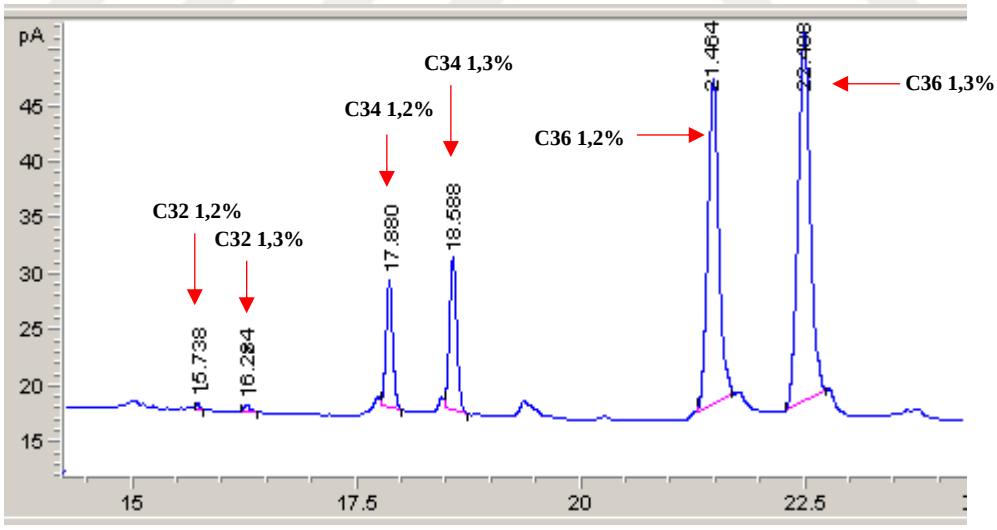


Figure 4.14. GC chromatogram of DAG content of olive oil samples from North region

The change in 1,3 DAG content of the fresh and adulterated olive oils depending on storage time are shown in Figure 4.16. For olive oil samples from South region, 1,3 DAG content increased during 12 months of storage. However, any changes in 1,3 DAG

concentration was not observed especially for adulterated samples from North throughout storage. As a result, 1,3 DAG/1,2 DAG ratio was not also affected from storage time (Figure 4.17). Change in the contents of 1,3 DAG (%) for non-adulterated samples from North Aegean Region was 5.21% while sample mixed with old oil at 50% ratio have inconsiderable difference at the end of 15 months.

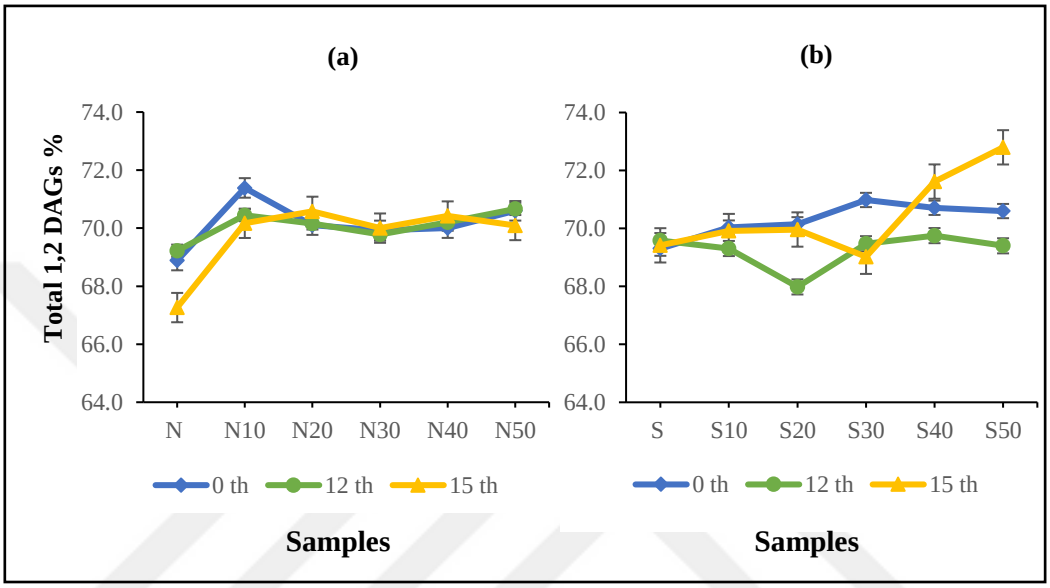


Figure 4.15. Total 1,2 DAGs of EVOO samples during 15 months of storage period (a) North Aegean olive oil samples, (b) South Aegean olive oil samples

A study in the literature indicated that there were remarkable differences on the DAG contents of olive oils stored in various temperature and time combinations (Cossignani et al., 2007). According to the results of this study, the highest contents of sn-1,2-DAG and also the lowest contents of sn-1,3 DAG and sn-2,3 DAG were found at the beginning of the storage. Furthermore, it was observed a strong relationship between storage temperature and DAG contents. When olive oils were stored at warmer temperature than room temperature (30ºC), the isomerization processes of sn-1,2-DAG to the more stable sn-1,3-DAG could occur more easily (Cossignani et al., 2007). Effect of time and temperature on DAG content of olive oils were also investigated in another study and the results of this study in literature is more consistent with the current research (Perez-Camino, Moreda and Cert, 2001). According to this study, changes in 1,2-DAG, 1,3-DAG and their ratios were not notable for oils having low FFA value (0.12%) stored

at low temperatures (5, 25C). However, higher FFA values and higher temperature resulted in more decrease/increase in these contents.

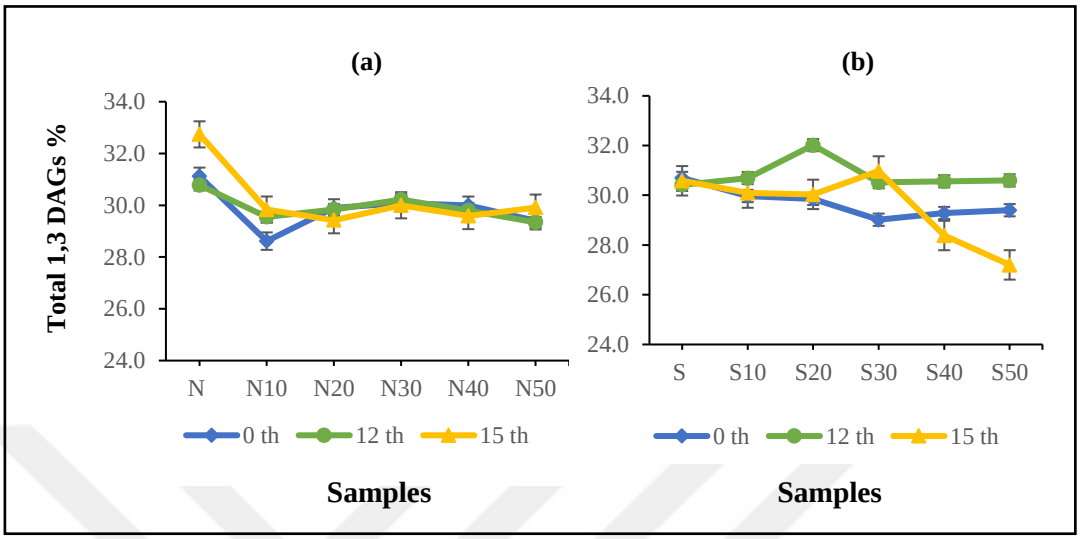


Figure 4.16. Total 1,3 DAGs of EVOO samples during 15 months of storage period (a) North Aegean olive oil samples, (b) South Aegean olive oil samples

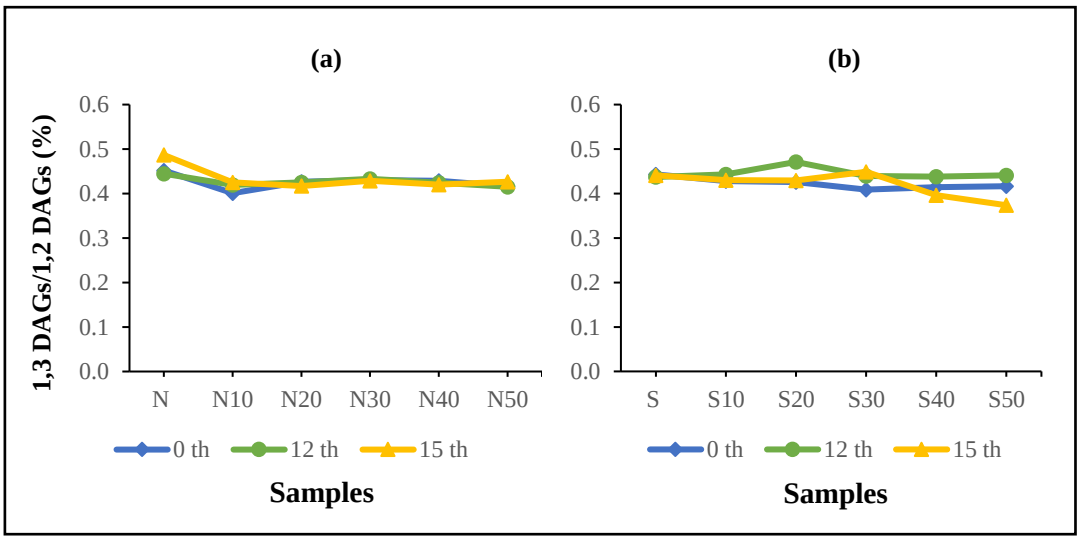


Figure 4.17. Ratio of total 1,3 DAGs to total 1,2 DAGs of EVOO samples during 15 months of storage period (a) North Aegean olive oil samples, (b) South Aegean olive oil samples

4.1.6. Pigments

Pigments are strongly related to color of olive oil and also use of some of the pigments has been suggested as quality and authentication indicators (Geitz and Fiebig, 2006). Chlorophylls and carotenoids are the major pigments of olive oils and these compounds exist in various epimeric and isomeric form. A total of 13 pigments separated from olive oil samples and 8 of them were chlorophylls, and the rest of them were identified as carotenoids in this study. HPLC chromatogram of an olive oil sample could be seen in Figure 4.18. Lutein and β-carotene as major carotenoids and chlorophyll a and b as well as pheophytin a and b as significant chlorophylls were identified and they are discussed in more detail in this part.

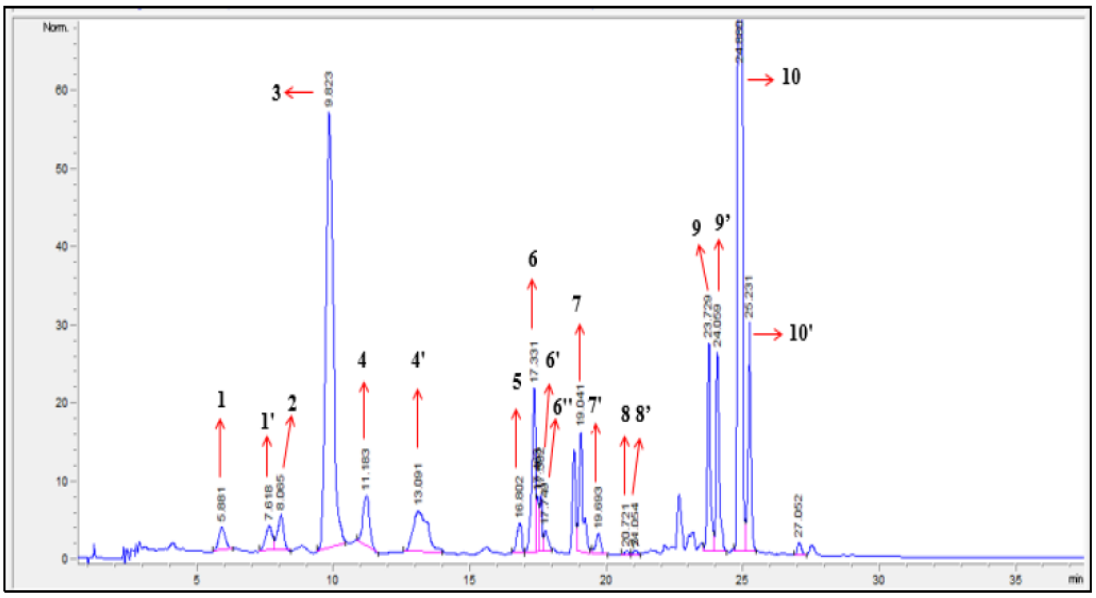


Figure 4.18. Sample HPLC chromatogram of olive oil from South region (1: neoxanthin + 1': neoxanthin isomer + 2: violaxanthin + 3: luteoxanthin + 4: antheraxanthin + 4': antheraxanthin isomer + 5: mutatoxanthin as all together as total xanthophyll; 6: lutein; 6': lutein isomer; 6'': lutein isomer; 7: chlorophyll b; 7': chlorophyll b isomer; 8: chlorophyll a; 8': chlorophyll a isomer; 9: pheophytin b; 9': pheophytin b isomer; 10: pheophytin a; 10': pheophytin a isomer)

Lutein is a major carotenoid found in olive oil and provides a yellowish color. Change in lutein concentrations for non-adulterated and old oil adulterated samples throughout 15 months of storage are provided in Figure 4.19. Non-adulterated South olive oil has almost twice the amount of lutein compared to pure North oil at the beginning of the storage. There is not much change in lutein content in first 6 months for both fresh and adulterated samples except South oil containing 40% (15.6% decrease) and 50% (12.2% decrease) old oil; however, lutein amount decreased considerably especially for all South oils during 12 months of storage (Figure 4.19). After 15 months, lutein content of non-adulterated and 50% adulterated North oils decreased 18.8% and 15.1%, respectively. On the other hand, decrease in lutein content of non-adulterated and 50% adulterated South oil samples were 42.3% and 55.5% in order.

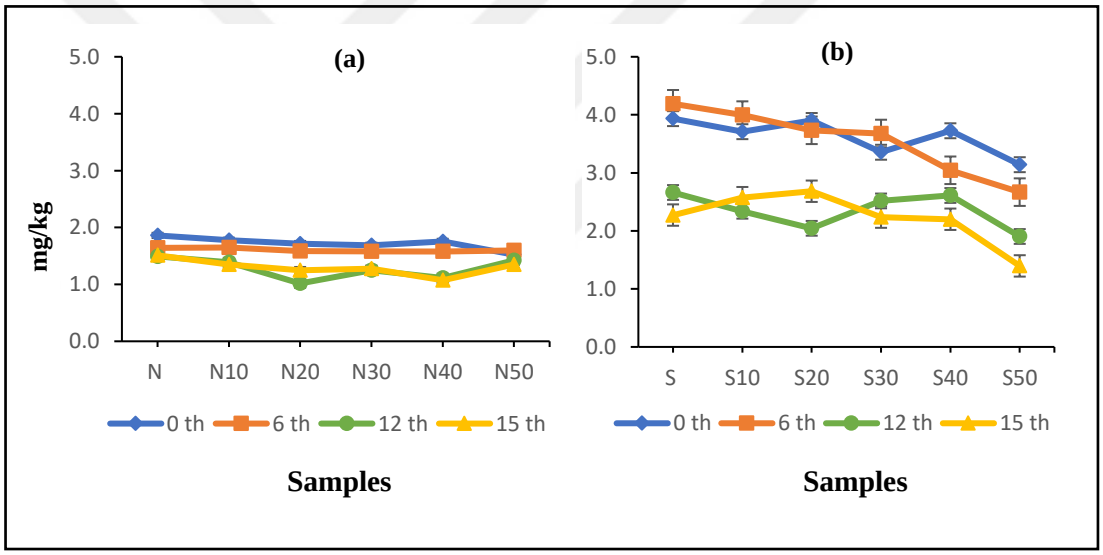


Figure 4.19. Lutein contents of EVOO samples during 15 months of storage period (a) North Aegean olive oil samples, (b) South Aegean olive oil samples

In this study, pheophytin a content of the oils constantly decreased during storage of oils from both regions. Some olive oils, such as SO20 and SO30, did have a slightly higher pheophytin a content at the beginning of storage; however, this was not consistent across 15 months of storage (Figure 4.20). Pheophytin a content of non-adulterated samples decreased 45.8% for North oils and 31. 9% for South oils after 15 months of storage. 41.6% and 64% decreases in pheophytin a content were observed at the end of 15 months for 50% adulterated North and South oils, respectively.

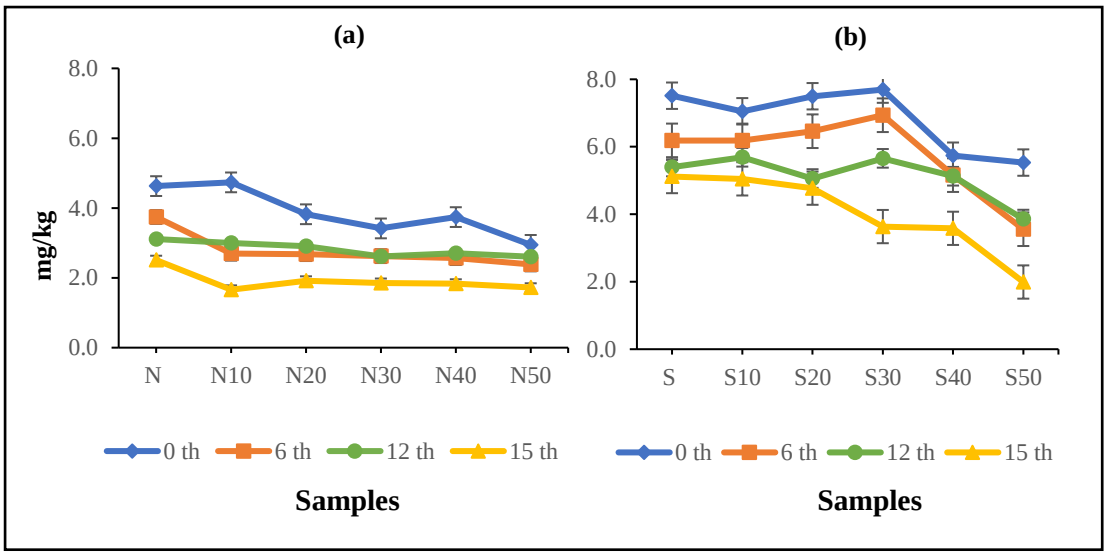


Figure 4.20. Pheophytin a contents of EVOO samples during 15 months of storage period (a) North Aegean olive oil samples (b) South Aegean olive oil samples

Changes in the concentrations of another major chlorophyll, chlorophyll a are shown in Figure 4.21. There are not any significant changes in the concentration of chlorophyll a of non-adulterated oil (N) from North during storage, but decreasing trend in the content of chlorophyll a was observed for 50% adulterated North oil with respect to storage time. For both fresh and adulterated South oil samples, 6 months of storage resulted in a sharp decrease in chlorophyll a content (Figure 4.21). After 6 months of storage, firstly an increasing and then again a decreasing trend were observed for South oil samples. This situation could be related to conversion of chlorophyll a to different derivative forms (mainly pheophytin, pyropheophytin, and pheophorbide) (Boskou, 2012). Percent changes of chlorophyll a content of non-adulterated oils from North and South Aegean Region were 29.9% and 25.2% and for 50% adulterated North and South oils, 33.8% and 14.6%, respectively after 15 months.

Chlorophyll b contents of non-adulterated and adulterated oils from North and South Aegean Region are shown in Figure 4.22. According to literature, chlorophyll a content is generally higher than that of chlorophyll b, another major chlorophyll component, in olive oil (at a ratio of 3 to 1) (Giuliani, 2011). Results of this study regarding chlorophyll a to b ratio do not match with the previous observation. This could be due to differences in the variety or cultivation practices of the olives (Gandul-Rojas and Minguez- Mosquera, 1996). In general, chlorophyll b content of adulterated and non-

adulterated oils after 15 months were lower than their initial amounts. Percent changes of chlorophyll b content of non-adulterated oils from North and South Aegean Region were 29.9% and 25.2% and for 50% adulterated North and South oils, 33.8% and 14.6%, respectively after 15 months.

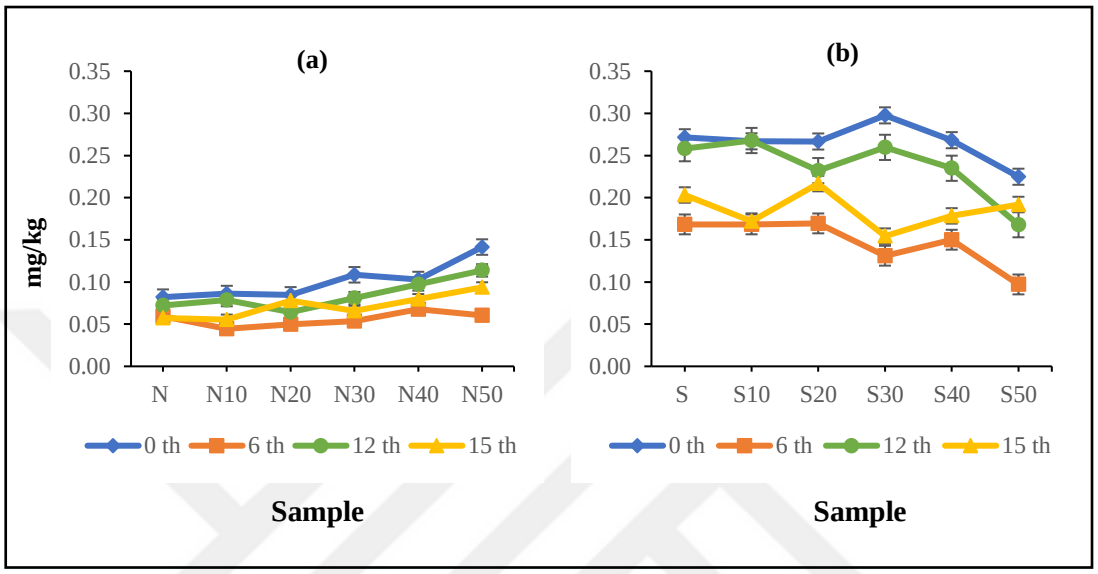


Figure 4.21. Chlorophyll a contents of EVOO samples during 15 months of storage period (a) North Aegean olive oil samples, (b) South Aegean olive oil samples

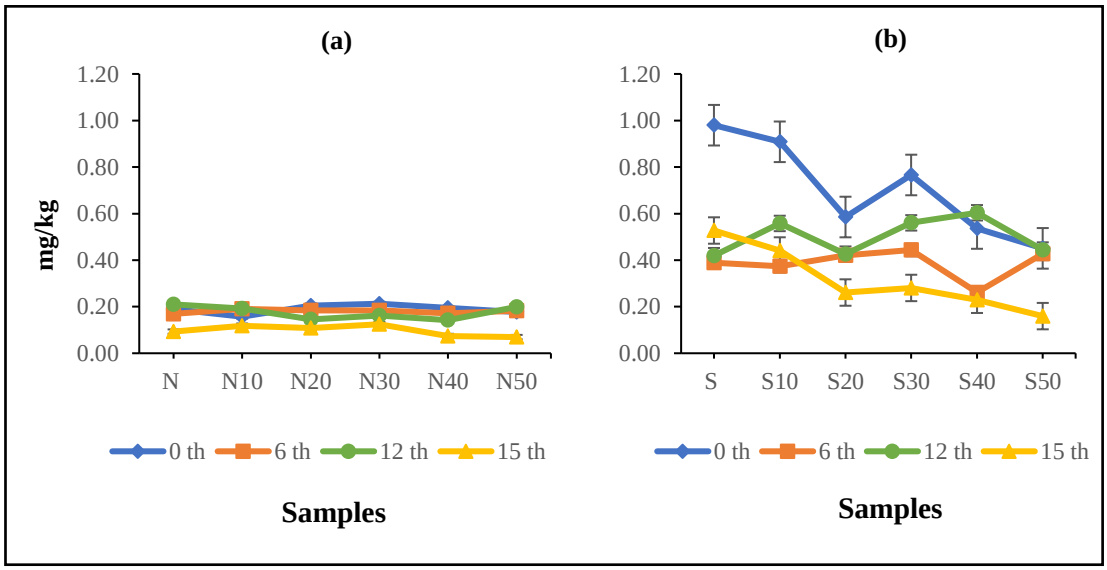


Figure 4.22. Chlorophyll b contents of EVOO samples during 15 months storage period (a) North Aegean olive oil samples, (b) South Aegean olive oil samples

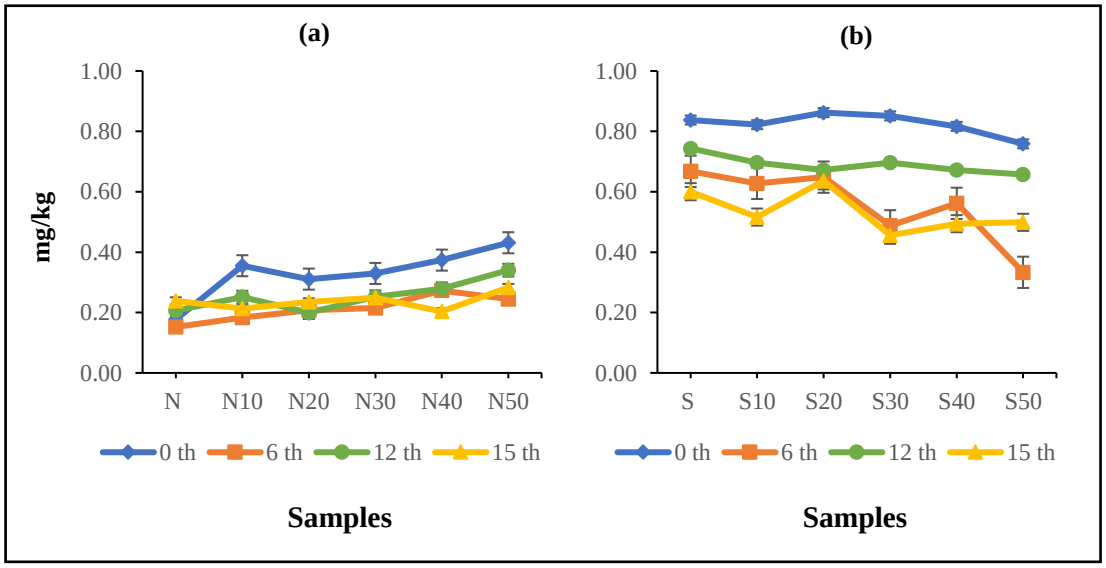


Figure 4.23. Changes in pheophytin b of EVOO samples during 15 months of storage period (a) North Aegean olive oil samples, (b) South Aegean olive oil samples

Although there might be fluctuations in the amount of pheophytin b due to unstable nature of chlorophylls pheophytin b amount reached to its lowest level at the end of 15 months (Figure 4.23). Percent changes of pheophytin b content of non-adulterated oils from North and South Aegean Region were 34.78% and 28.32% and for 50% adulterated North and South oils, 34.33% and 34.26% respectively after 15 months. It was reported that the most significant change in chlorophyll is due to pheophytinization reaction, which is initiated during the oil extraction process as a consequence of the released free acidity (Roca et al., 2003). Therefore, fluctuations in the amount of different types of chlorophylls are expected due to conversion of one form to another.

Changes in the concentration of the other major carotenoid, β-carotene, throughout storage are shown in Figure 4.24. Amount of this pigment gradually decreased with respect to time for North oil samples while it fell down in the first 6 months of the storage and then did not change much for the rest of the storage for South samples. Decreases were 78.2% for North non-adulterated oil and 48% for 50%adulterated North oil. 25.5% and 45.6% decreases were observed for non-adulterated and 50% adulterated samples from the South, respectively. Therefore, quite considerable losses were determined in the amount of this pigment for both non-adulterated and adulterated samples with respect to time.

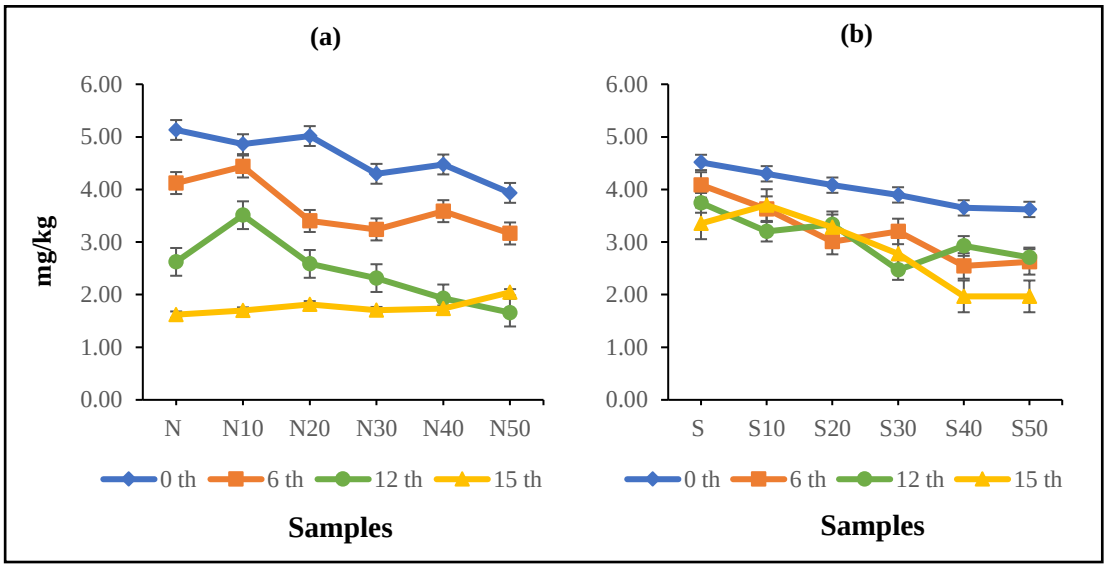


Figure 4.24. β-carotene contents of EVOO samples during 15 months of storage period (a) North Aegean olive oil samples (b) South Aegean olive oil samples

Total amounts of chlorophylls and carotenoids were also calculated to investigate the changes in pigments during storage (Figure 4.25 and 4.26). Total chlorophyll concentration decreased in first 6 months then a further decrease in 15 months were observed for samples from both regions.

Changes in total chlorophylls were 42.3% for non-adulterated and 31.6% for 50% adulterated North oils. 32.5% and 59.1% lower total chlorophyll were determined for non-adulterated and 50% adulterated South oils, respectively. Significant amounts of losses were also determined in total carotenoids. Losses were 50% and 25% for non-adulterated North and South oils, respectively and there were 32.3% and 47.9% decrease, in order, for 50% adulterated North and South samples.

Overall, South oils had higher total chlorophyll and carotenoids contents compared to North samples; however, same trend was observed in terms of their loss during the storage. In a study in literature, quality changes in olive oils stored in light and dark conditions for 12 months were investigated (Caponio et al., 2005). According to this study chlorophyll and carotenoid contents of olive oils decreased gradually throughout 12 months of storage and the decreases were especially more significant when the oils were kept in the light.

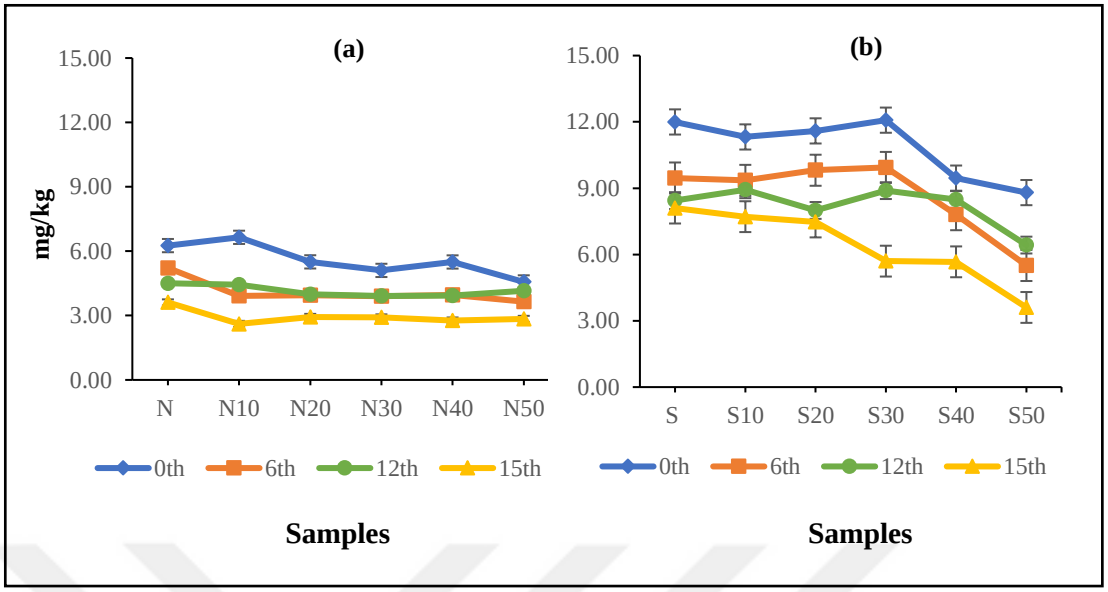


Figure 4.25. Changes in total chlorophylls of EVOO samples during 15 months of storage period (a) North Aegean olive oil samples, (b) South Aegean olive oil samples

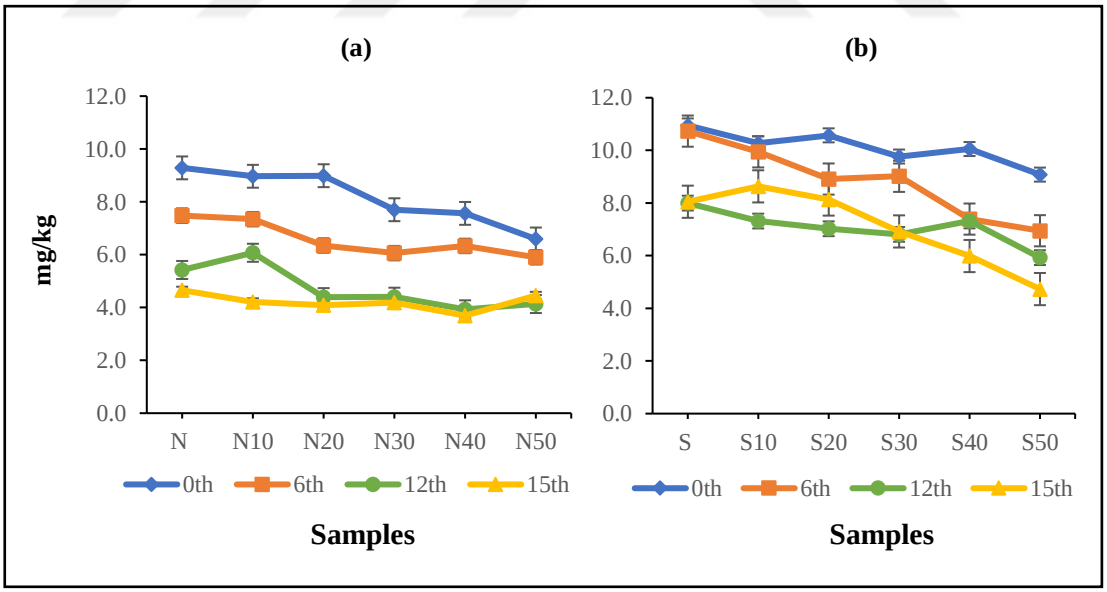


Figure 4.26. Changes in total carotenoids of EVOO samples during 15 months of storage period (a) North Aegean olive oil samples, (b) South Aegean olive oil samples

4.2. Differentiation of Olive Oil Samples with respect to Adulteration Level and Storage Time

4.2.1. Differentiation Using Basic Quality Parameters (Free Fatty Acidity (FFA), Specific extinction coefficients (K_{232}, K_{270}))

For the purpose of examining the effect of basic quality parameters on storage time, a multivariate data set of 3 chemical variables (FFA; K_{232}, K_{270}) with 24 observations was used for olive oil samples from each region. These data set was investigated with OPLS-DA to observe the changes in the quality of these olive oils over time. Models have R^2= 0.49 and R^2= 0.47 for North and South samples, respectively. The score and loading plots of OPLS-DA models are shown in Figure 4.27 and Figure 4.28.

On the basis of the score plot of North oils, some separation with respect to storage time is observed. In each storage period, samples are also located in the order of their adulteration level. Non-adulterated and adulterated oils are quite separately placed in 6^{th} and 12^{th} months. Samples from 15^{th} month are close to the ones at the beginning of the storage because K values of these samples are similar. High FFA and high K values are the parameters which separate out the samples of 6 and 12 months from initial and 15 month oils as the loading plots indicated. Similar observations were also true for South region oils. Although the combination of these quality parameters are good indicators of quality in the first 12 months they became unreliable with increasing storage time. These parameters might be also useful as adulteration indicators although this observation needs to be confirmed by analyzing more samples.

4.2.2. Differentiation Using Fatty Acid Profile

In order to investigate the effect of fatty acid profiles during storage, a multivariate data set of 11 fatty acids as variables and 24 observations were constructed for both region samples and this data set was analyzed with OPLS-DA to observe the changes with respect to storage time and adulteration level. Figure 4.29 and Figure 4.30 are the score and loading plots of OPLS-DA models with R^2= 0.30 and R^2= 0.54 for North and South region samples, respectively.

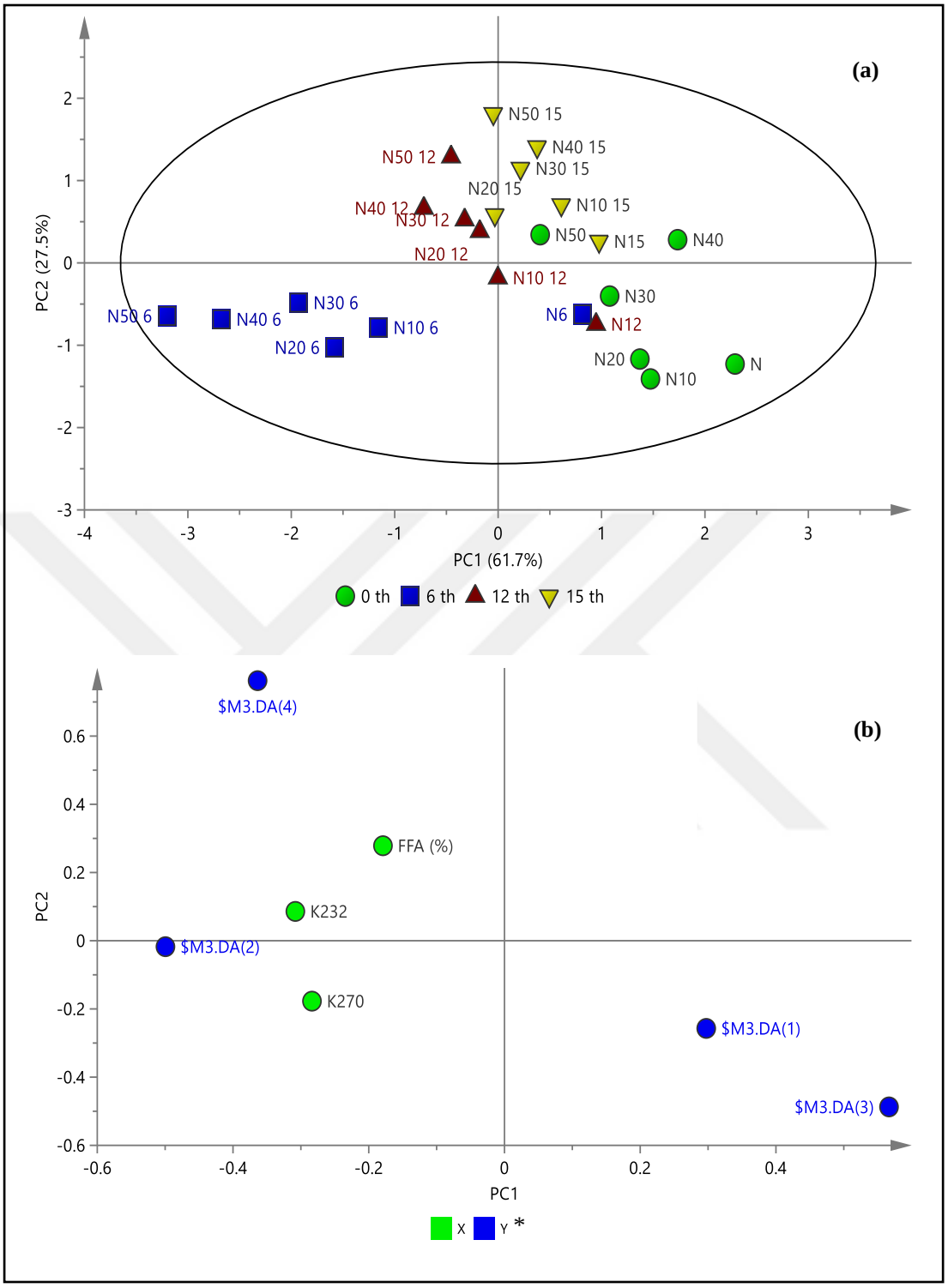


Figure 4.27. OPLS-DA (a) score and (b) loading plots of basic quality parameters of olive oils from North region with respect to storage time (N: North, first two numbers after letters: percentage of adulteration, last two numbers: storage time)*X: Variables Y:Classes

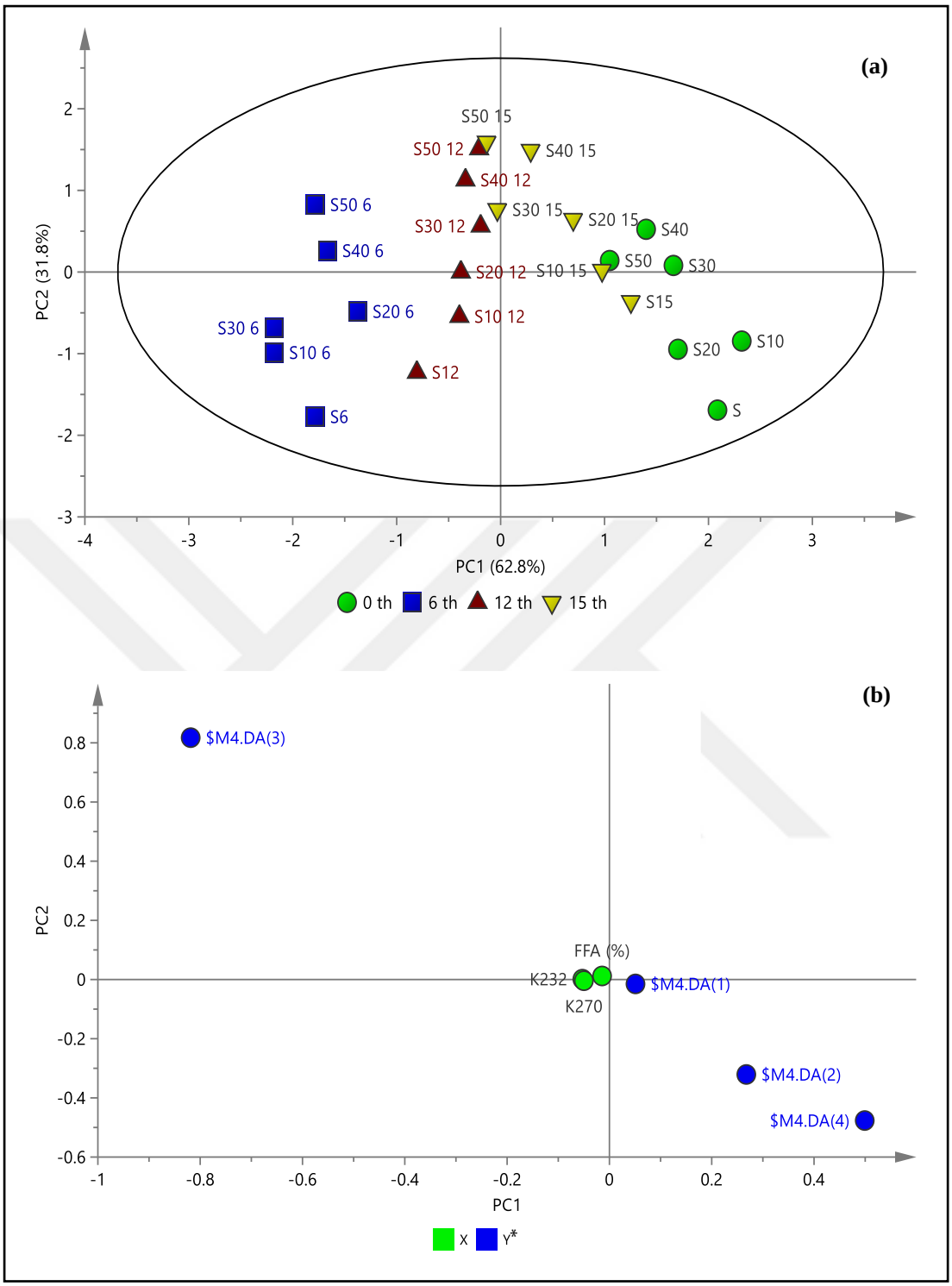


Figure 4.28. OPLS-DA (a) score and (b) loading plots of basic quality parameters of olive oils from South region with respect to storage time (S: South, first two numbers after letters: percentage of adulteration, last two numbers: storage time)*X: Variables Y:Classes

In terms of fatty acid composition, there is a quite rough separation of the samples from both regions with respect to storage time. There are overlaps especially between the samples belonging to 6 and 12 months for both regions. For South oils, only clear differentiation is between initial samples and samples stored for 15th months. However, higher adulteration concentrations of North oils before storage overlap with lower adulteration concentration oils from 15th month. As far as the adulteration is concerned, non-adulterated North olive oils separated from 30, 40 and 50% old oil containing samples with respect to the first principal component during first 12 months. However, differentiation is not that clear for 15 months of storage. Although there are some differences between non-adulterated and adulterated South samples these differences are not very clear cut. Since changes in the fatty acid composition of adulterated and stored oils are small clear separations with respect to storage time and adulteration level are not observed from score plots also.

4.2.3. Differentiation Using Fatty Acid Alkyl Esters

In order to determine the effect of fatty acid alkyl esters on storage time, a multivariate data set of 5 fatty acid alkyl ester variables and 24 observations was used in constructing OPLS-DA models for each region samples. Two principal components containing North and South Region OPLS-DA models have R^2= 0.95. The score and loading plots for these models are shown in Figure 4.31 and Figure 4.32.

Figure 4.28 indicated that there is no separation between the initial samples and samples from 6 months (except 40 and 50% adulterated) as well as oils from 12 and 15 months for North oils. Samples containing high amounts of old oils from 6th month are also located close to 12 months. This means that alkyl esters of the samples did not change much in the first 6 months but then increased within 12 months. Samples from the start of storage and 6 months form a group while the samples belonging to 12 and 15 months form another group. According to the loading plot, longer storage time group separates from the other due to its higher FAAE and FAEE contents along with FAAE/FAEE (Figure 4.31 (b)). Non-adulterated olive oils at the start of storage period and after 6 months are separated from 20-50% adulterated samples with respect to second principal component. However, this separation limit increased to 30% adulteration level after 12 months and adulterated and non-adulterated samples are closer at the end of 15 months.

Therefore, the level of adulteration detection depends on the storage time and it is getting more difficult to separate adulterated and non-adulterated samples with increasing time.

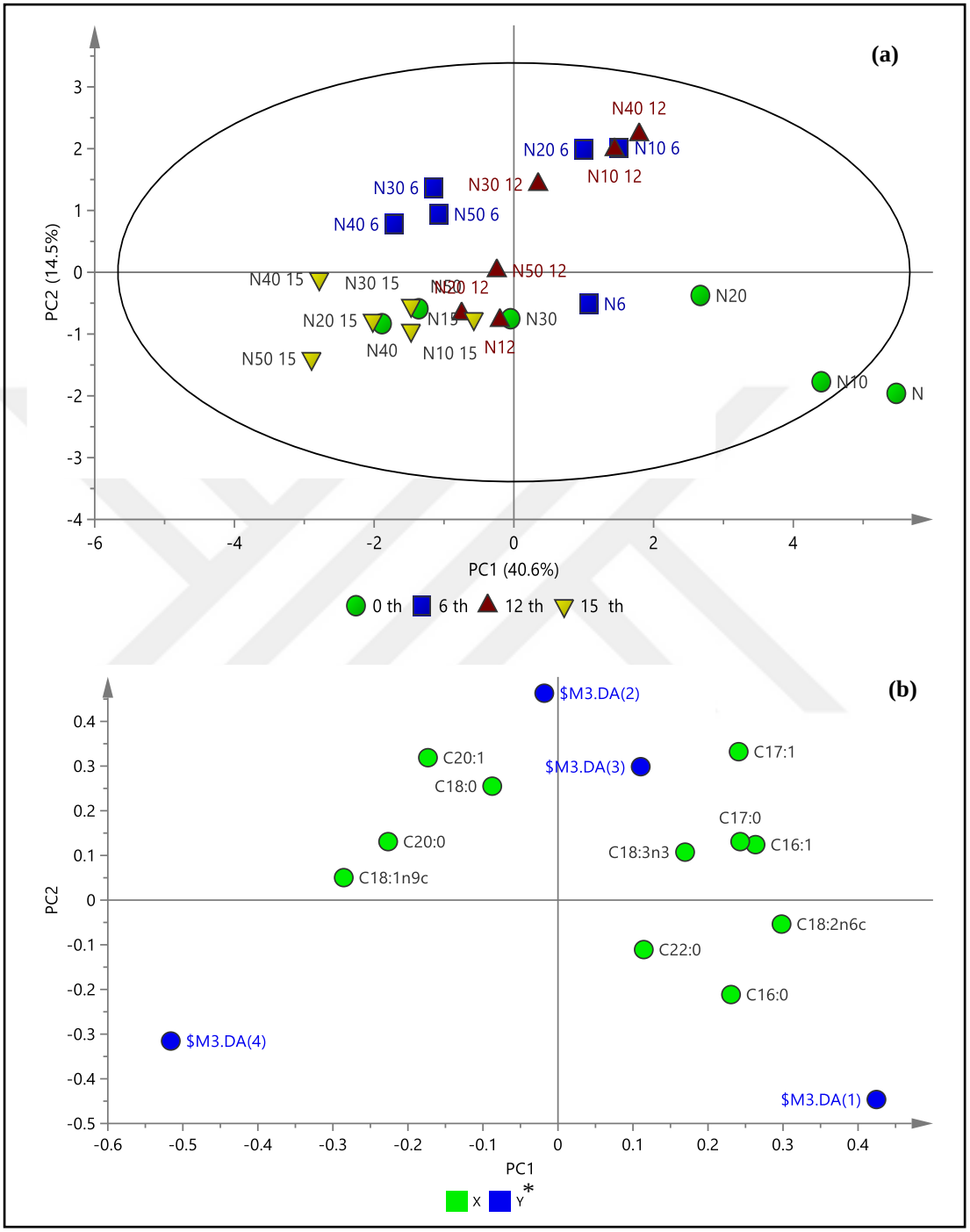


Figure 4.29. OPLS-DA (a) score plot and (b) loading plots of fatty acids of olive oils from North region with respect to storage time (N: North, first two numbers after letters: percentage of adulteration, last two numbers: storage time)*X: Variables Y:Classes

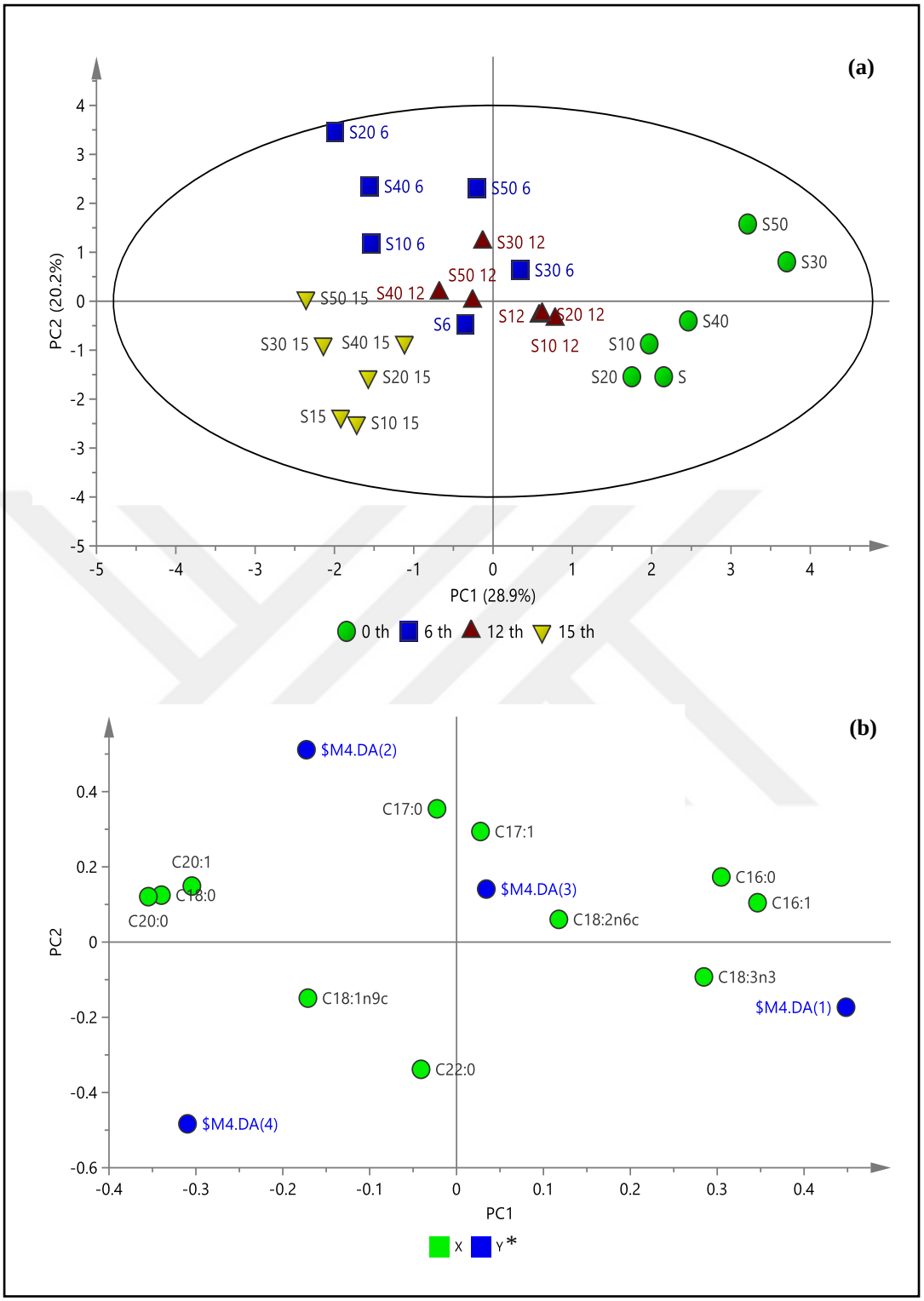


Figure 4.30. OPLS-DA (a) score plot and (b) loading plots of fatty acids of olive oils from South region with respect to storage time (S: South, first two numbers after letters: percentage of adulteration, last two numbers: storage time) *X: Variables Y:Classes

For South oil samples, one cluster in score plot (Figure 4.32 (a)) includes the samples before storage and from 6 months as well as some adulterated samples from 12th month with lower adulteration levels (10 and 20%). While the rest of the samples of 12th month and 15th month form another group. This finding is somewhat similar to the one for North oils. Differences between these two clusters are defined by the higher amounts of FAAE, FAEE and their ratios.

Separation between non-adulterated and adulterated oils with 30 to 50% concentrations are achieved with respect to second principal component in the first 12 months of the storage. However, differentiation of non-adulterated oils from adulterated ones are not that clear for 15th month samples. Therefore, similar conclusions hold true for South and North oil samples as far as FAAE and FAEE amounts are concerned.

OPLS-DA method was also applied in order to determine the effect of fatty acid alkyl esters on adulteration level. The score plots for these models grouped according to adulteration levels are shown in Figure 4.33. Non-adulterated North olive oil samples are located far from the origin of the first PC, to the right in the score plot in Figure 4.33 (a), which means that they are differentiated very well from adulterated samples of 40-50% (NO40, NO50, N0406, NO506, etc.). On the other hand, adulterated North samples up to 30% stored for 6 months are placed close together.

For South region olive oil samples, similar results are obtained (Figure 4.33 (b)). The interpretation of the scores' plots indicates an arrangement of the samples into two groups: the first group includes non-adulterated and 10-20% adulterated samples while the second group includes higher adulteration levels of 40 and 50% and longer storage periods' (12 and 15 months) of 30% adulterated oils.

Since differentiation is achieved at higher adulteration levels data from two regions are also combined to test the efficiency of fatty acid alkyl esters in terms of adulteration level regardless of geographic origin. Figure 4.34 indicated that there is a separation between the initial samples and samples containing 50% old oil with respect to the first principal component. Lower adulteration levels cannot be differentiated with respect to the first principal components. The combined reading of score and loading plots show, that the samples located on the left side of the ellipse (SO5015, NO4012, etc.) have higher FAEE and FAME (Figure 4.34 (a) and (b)).

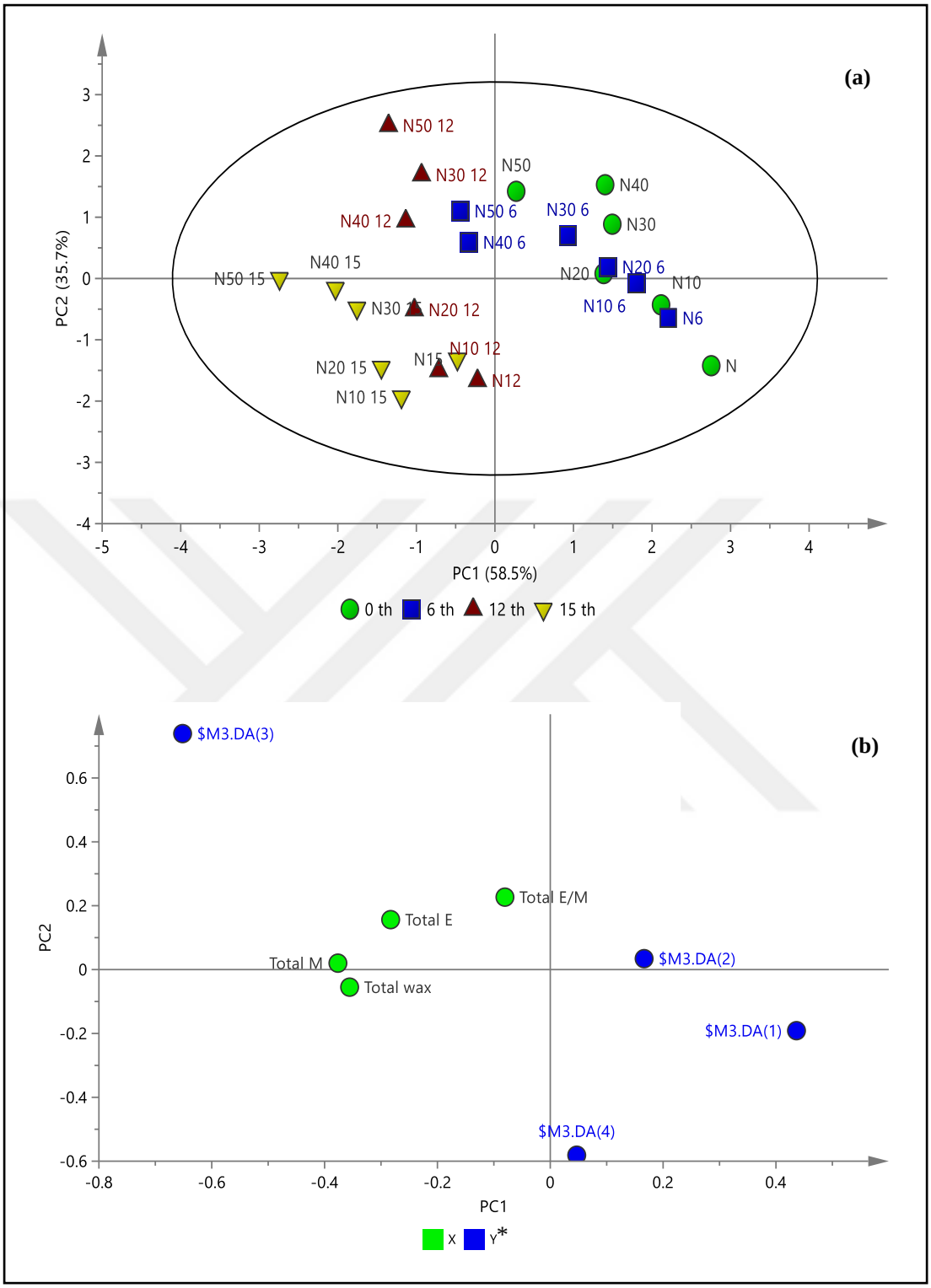


Figure 4.31. OPLS-DA (a) score and (b) loading plots of fatty acid alkyl esters of olive oils from North region with respect to storage time (N: North, first two numbers after letters: percentage of adulteration, last two numbers: storage time) *X: Variables Y:Classes

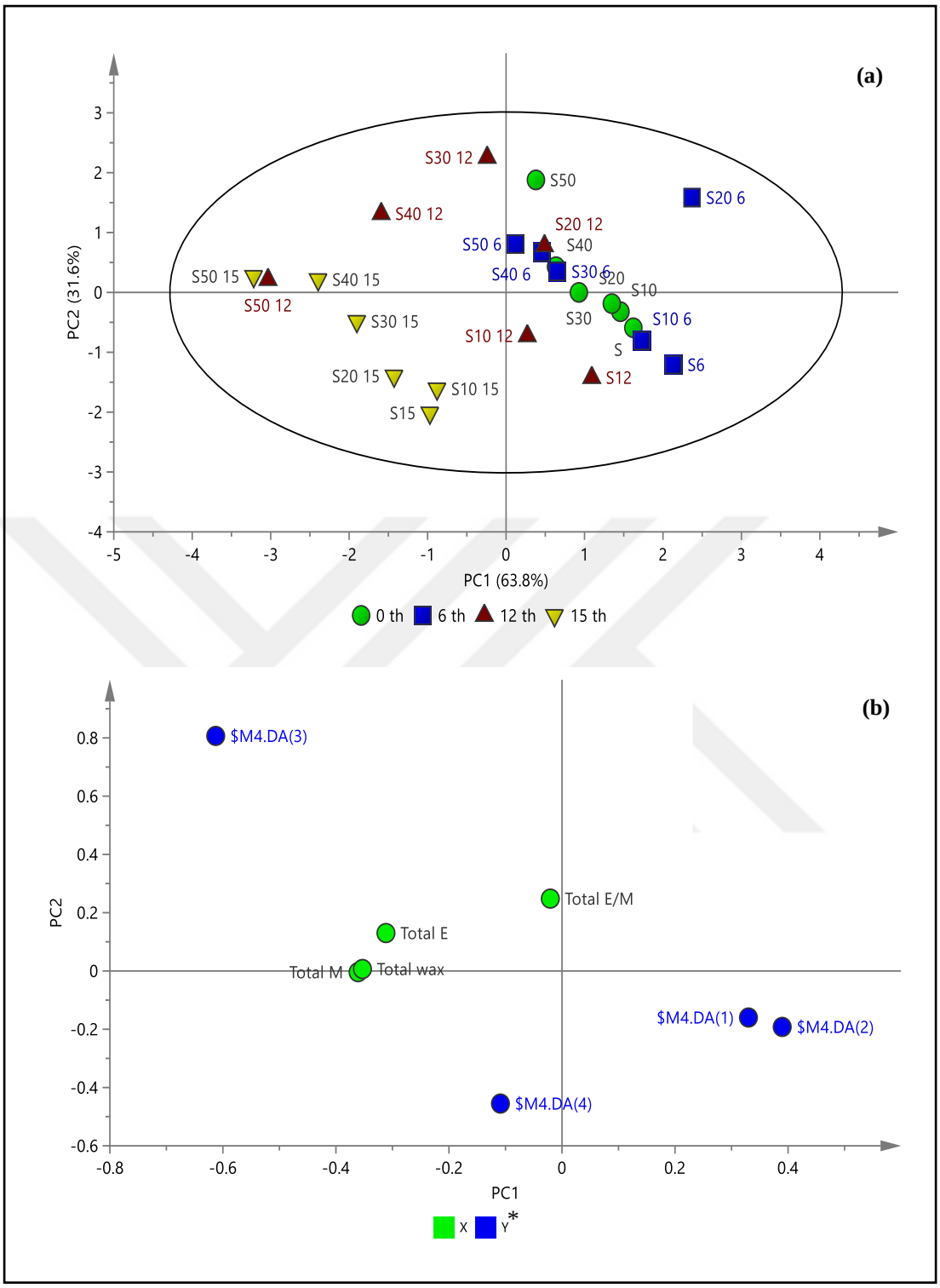


Figure 4.32. OPLS-DA (a) score and (b) loading plots of fatty acid alkyl esters of olive oils from South region with respect to storage time (S: South, first two numbers after letters is percentage of adulteration and last two numbers is storage time) *X: Variables Y:Classes

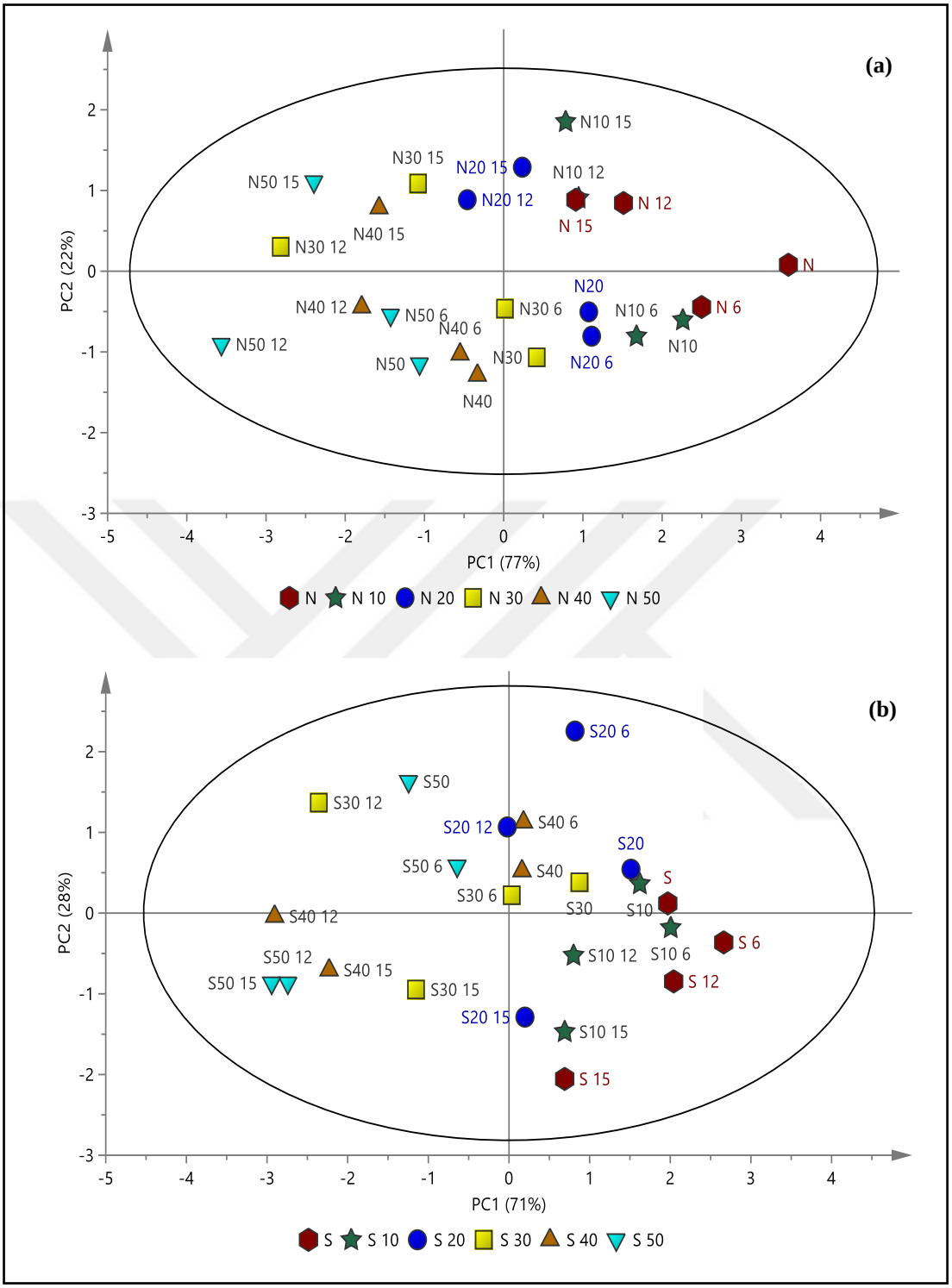


Figure 4.33. OPLS-DA score plots of fatty acid alkyl esters of olive oils from North (a) and South (b) region with respect to adulteration level (N: North, S: South, first two numbers after letters is percentage of adulteration and last two numbers is storage time)

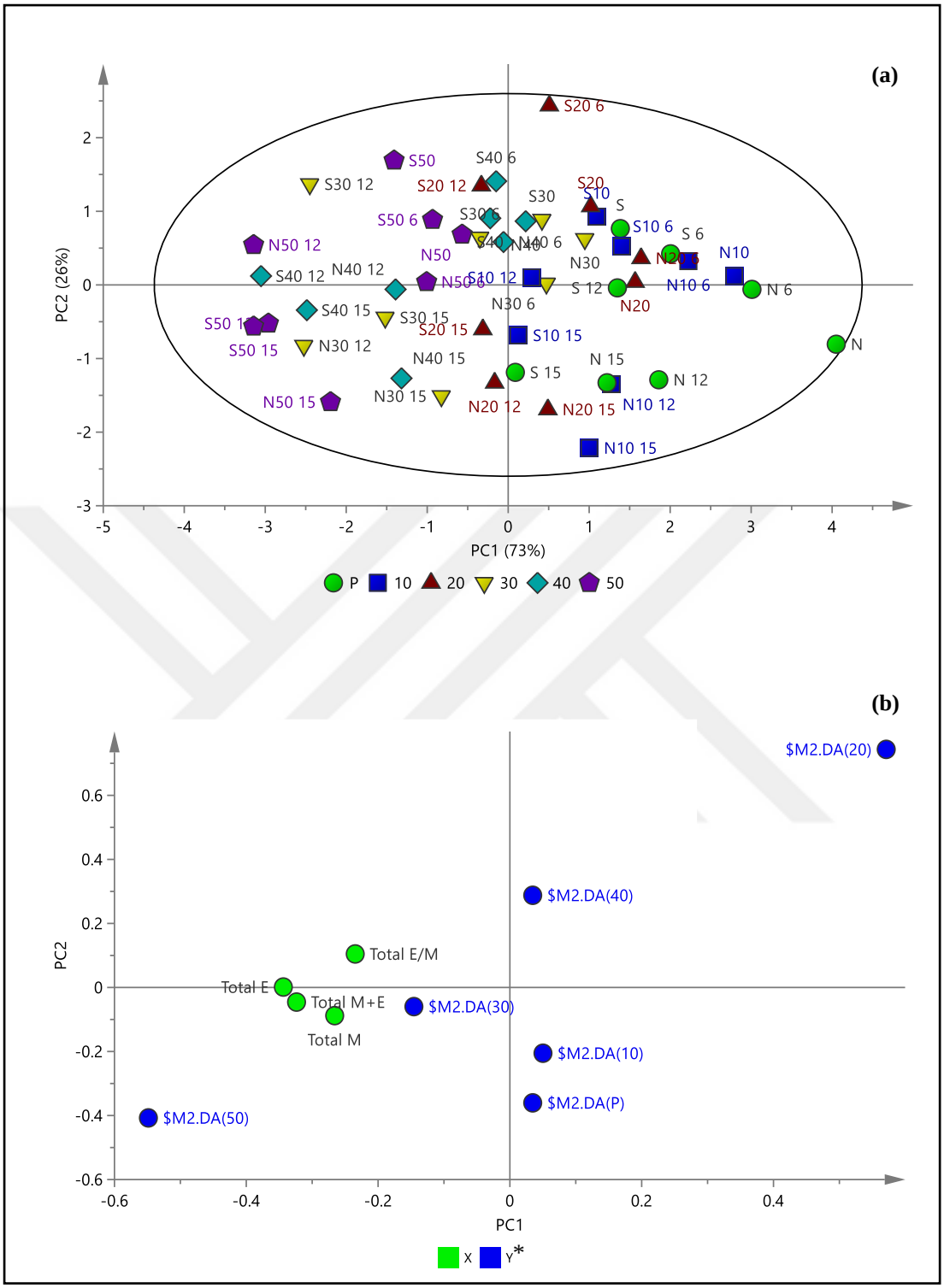


Figure 4.34. OPLS-DA score (a) and loading (b) plots of fatty acid alkyl esters of olive oils from North and South region together with respect to adulteration level (N: North, S: South, first two numbers after letters is percentage of adulteration and last two numbers is storage time) *X: Variables Y:Classes

4.2.4. Differentiation Using Diacylglycerols

In order to investigate the effect of diacylglycerols (DAG) on storage time with old olive oil a data set of 10 diacylglycerol variables and 18 observations was used for each region olive oil samples. Then, OPLS-DA models having two components are created with R^2= 0.82 and R^2= 0.93 for North and South Region samples, respectively. The score and loading plots of these models are shown in Figure 4.35 and Figure 4.36.

According OPLS-DA score plot of North Region oils, there are two separate clusters (Figure 4.35 (a)). Samples at the beginning of storage is one group while the samples stored for 12 and 15 months formed the other class. This separation is due to higher 1,2 C32 DAG content of initial samples and also higher amounts of 1,2 C34, 1,3 C34, total 1,3 DAG and ratio of total 1,3 DAG to 1,2 DAG (Figure 4.35 (b)). Non-adulterated samples after 12 and 15 months of storage separated from adulterated ones with respect to the second principal component. However, especially the separation for 12 months is very close; therefore, more samples need to be analyzed before reaching to a conclusion.

Initial samples from South Region are mostly located on the upper right quartile of score plot while the samples belonging to 12^{th} and 15^{th} months are placed on the lower half (Figure 4.36 (a)). Although some of the stored samples from different times are close they are still separated from each other. Loading plot indicate that samples before storage have higher 1,2 C32 DAG content while oils of 12 months contain higher amounts 1,3 C36 DAG and total 1,3 DAG/1,2 DAG (Figure 4.36 (b)). In addition, adulterated samples are not very well separated from non-adulterated ones using DAG contents of South oils. As a result, it could be concluded that DAG provides differentiation of stored and initial oil samples. However, adulteration detection might change depending on the origin of the oil. Although there are small changes regarding the amounts of individual DAG components evaluating their combination in multivariate analysis results some differentiation with respect to storage time.

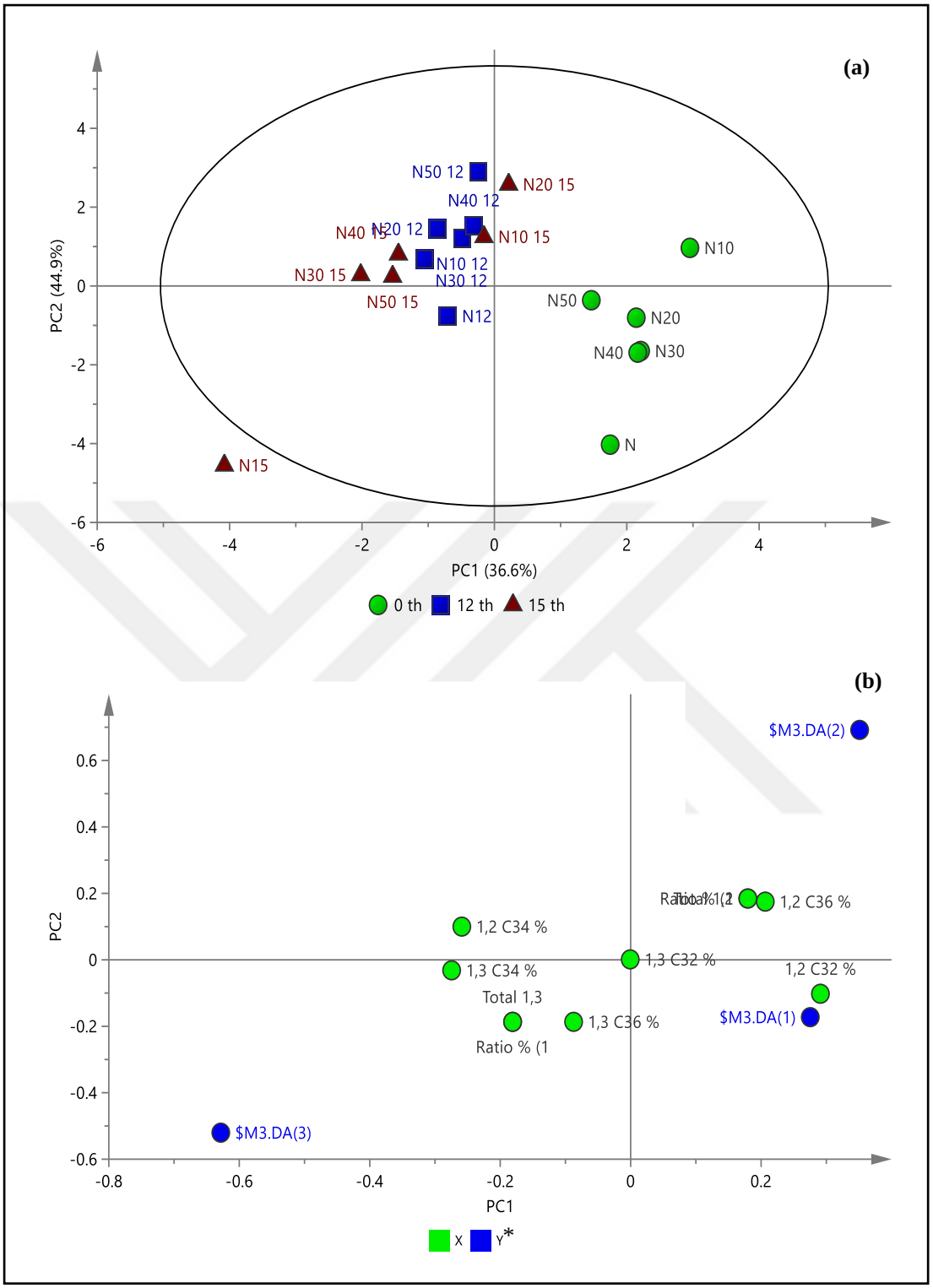


Figure 4.35. OPLS-DA (a) score and (b) loading plots of diacylglycerols of olive oils from North region with respect to storage time (N: North, first two numbers after letters: percentage of adulteration, last two numbers: storage time) *X: Variables Y:Classes

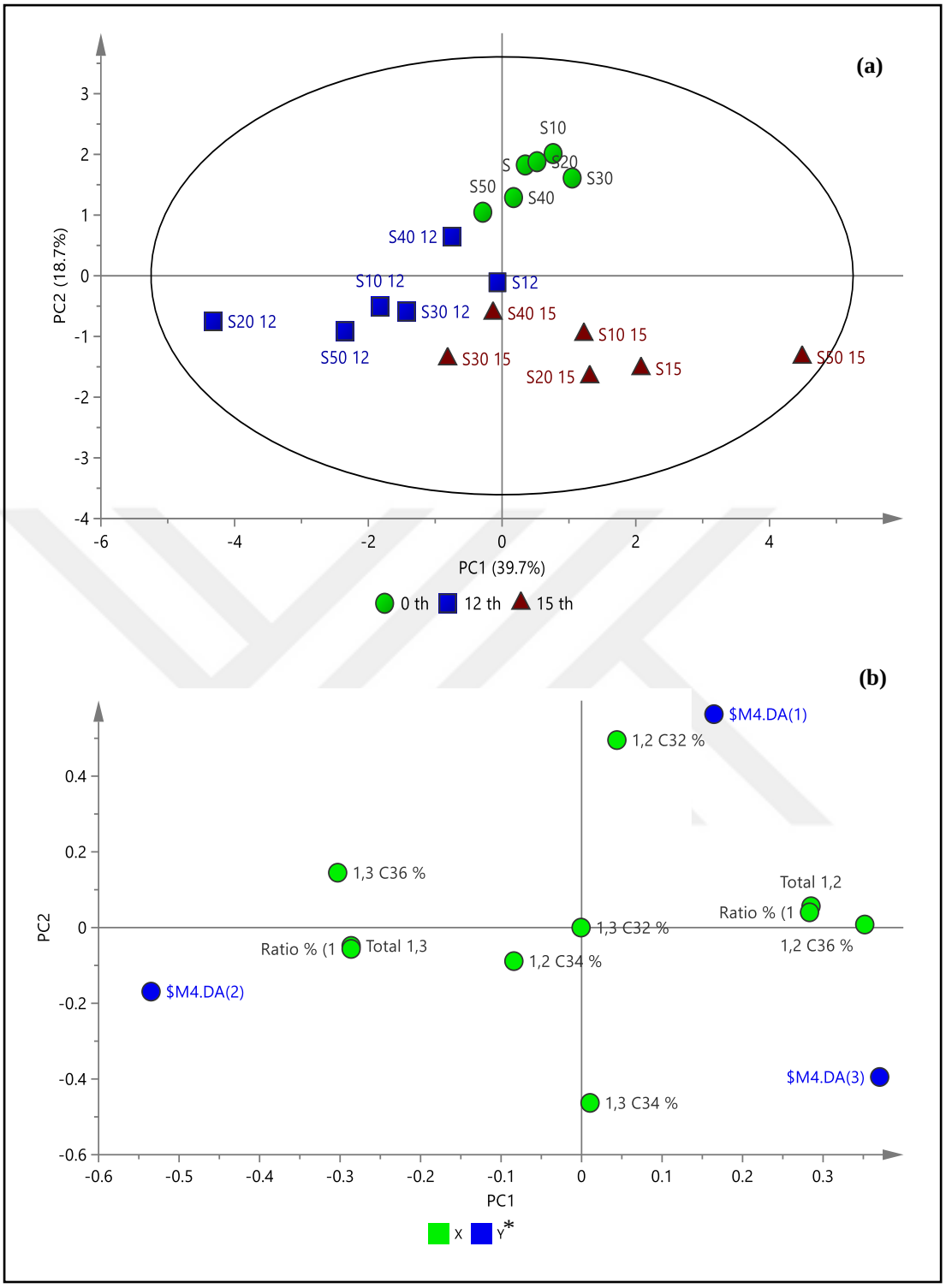


Figure 4.36. OPLS-DA (a) score (b) loading plots of diacylglycerols of olive oils from South region with respect to storage time (S: South, first two numbers after letters: percentage of adulteration, last two numbers: storage time) *X: Variables Y:Classes

4.2.5. Differentiation Using Pigments

A multivariate data set of 13 pigment variables and 24 observations was used for each region olive oil samples to construct an OPLS-DA model for differentiation with respect to storage time. Score and loading plots of these models with R^2= 0.95 for North and R^2= 0.92 for South region samples are shown in Figure 4.37 and Figure 4.38, respectively.

According to score plot of North oils, samples at the start of storage are separated from the rest and mostly located in the right bottom quarter (Figure 4.37 (a)). Samples belonging to 6 and 12 months, although do not overlap, are quite close to each other in the upper half of the score plot. Samples of 15 months form a separate cluster from the rest. Initial samples are differentiated from stored samples due to their higher content of almost every pigment measured. No separation is observed with respect to adulteration percentages using pigment profile throughout the storage. Quite similar results are obtained with South oils (Figure 4.38). Only difference is that samples are separated better with respect to their storage period since they form different clusters for each storage time. Consequently, pigment profile provides good differentiation regarding the storage time regardless of the origin of the oil.

Since good separation was achieved with respect to storage time OPLS-DA model according to adulteration level was also created with pigment data. Initial samples from North are mostly located on the right of score plot while samples belonging to 12^{th} and 15^{th} months are placed on the left (Figure 4.39 (a)). Samples containing 50% old oil are separated from fresh oils with respect to the first principal components while other percentages are not. On the other hand, fresh South oil could not be differentiated from South oil containing 50% adulterant at the start of storage; however, better separation is observed with storage with and over 6 months of storage (Figure 4.39 (b))., As a result, it could be concluded that pigment content provides differentiation of stored and initial oil samples. Despite that, detection of adulteration at high concentration of 50% could be possible but it depends on the origin of the oil and storage time.

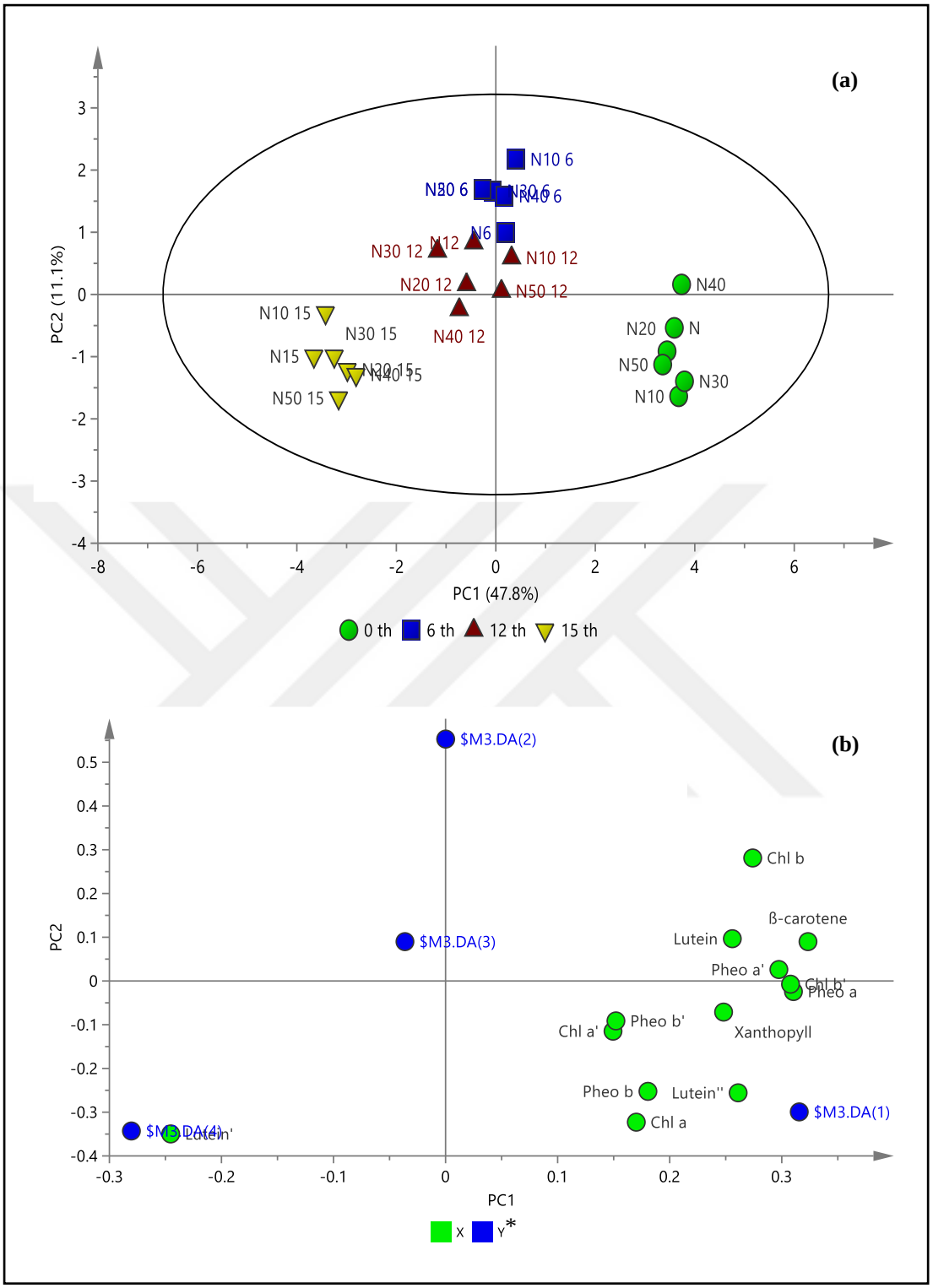


Figure 4.37. OPLS-DA (a) score and (b) loading plots of pigment profile of olive oils from North region with respect to storage time (N: North, first two numbers after letters: percentage of adulteration, last two numbers: storage time) *X: Variables Y:Classes

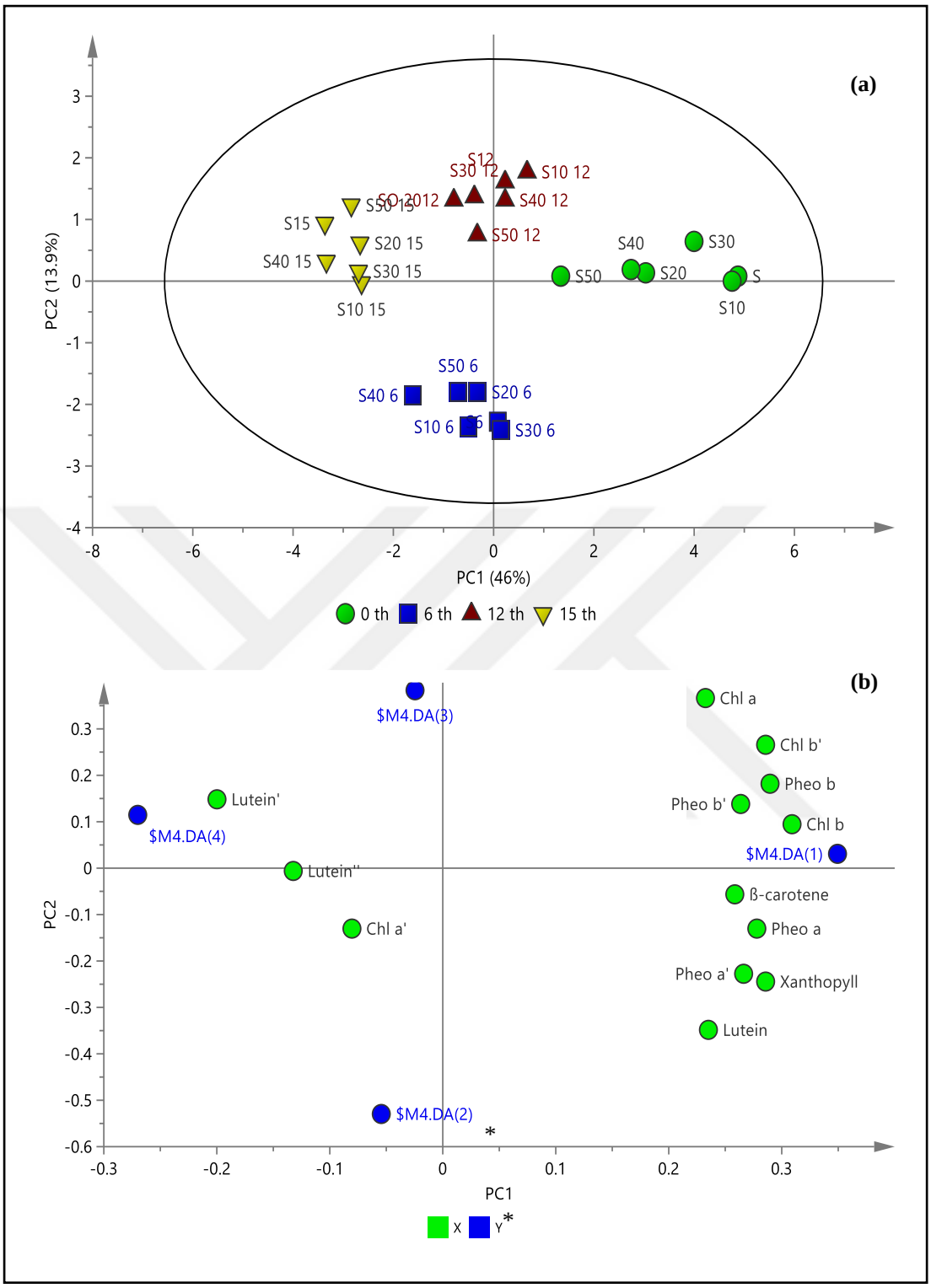


Figure 4.38. OPLS-DA (a) score plot and (b) loading plots of pigment profile of olive oils from South region with respect to storage time (S: South, first two numbers after letters: percentage of adulteration, last two numbers: storage time) *X: Variables Y:Classes

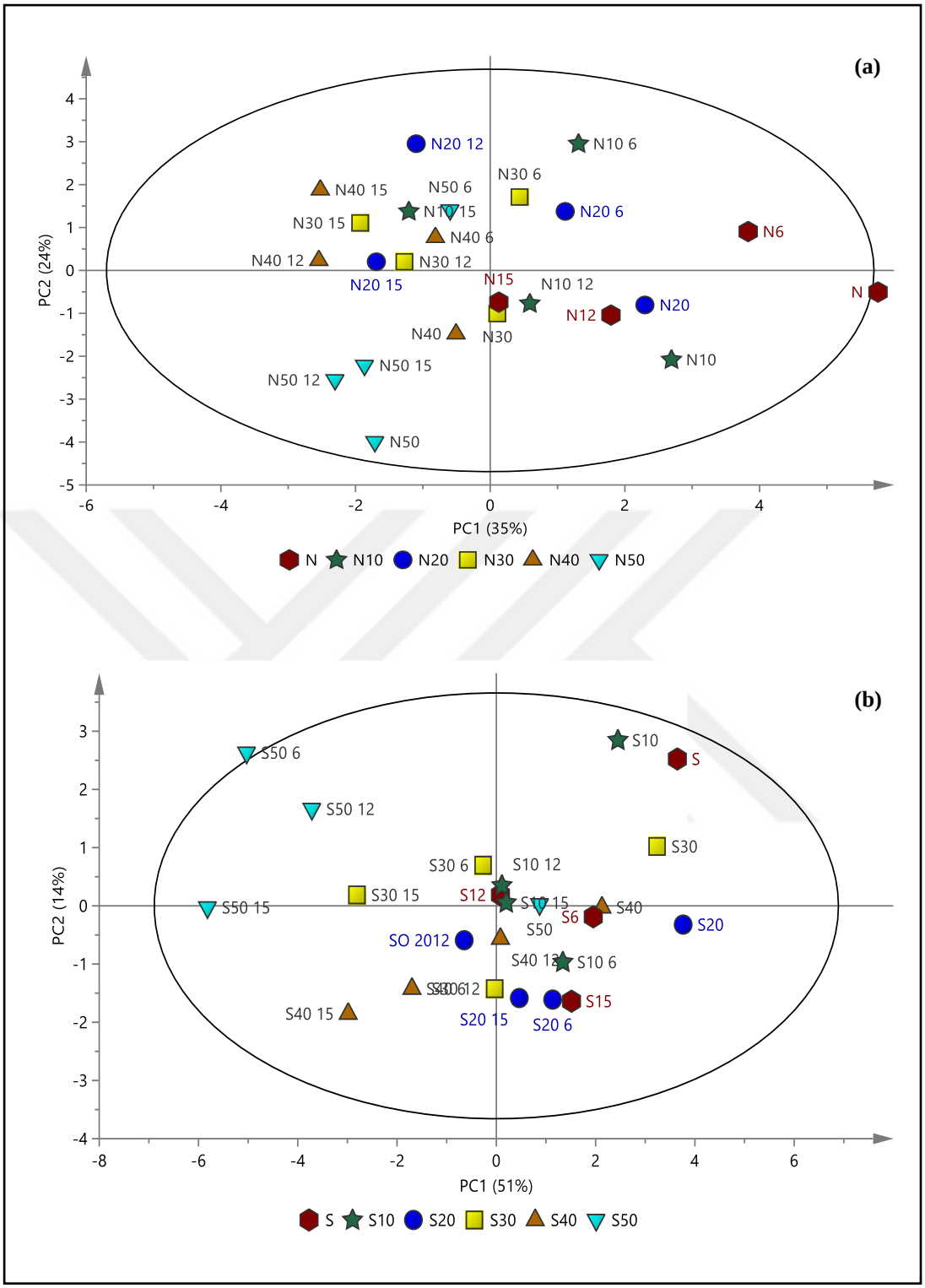


Figure 4.39. OPLS-DA score plots of pigment profile of olive oils from North (a) and South (b) region with respect to adulteration level (N: North, S: South, first two numbers after letters is percentage of adulteration and last two numbers is storage time)

4.2.6. Use of Mid-infrared spectroscopy (MID-IR) and UV-Vis Spectroscopy in Differentiation of Adulterated Samples and Monitoring of Their Storage

Two instrumental techniques, mid-infrared spectroscopy (MID-IR or FTIR) and UV-visible (UV-vis) spectrophotometry, were used to differentiate adulterated oils stored for 15 months. UV-Visible and FTIR profiles of one of the analyzed olive oil samples are shown in Figure 4.40-4.41, respectively. Peaks at wavelengths of 2924, 2852, 1743, 1463, 1377, 1238, 1163, 1114, 1099 and 721 cm−1 of the FT-IR spectra (Figure 4.40) are quite significant for olive oils (Sinelli et al., 2007). Peaks at 2924 and 2852 cm−1 wavelengths could be associated with -CH2 asymmetric and symmetric stretching vibrations, respectively. C=O stretching, CH2 and CH3 scissoring vibrations are attributed to the major peaks at 1743 cm−1, 1463 and 1377 cm−1, respectively. The rest of the peaks at 1238, 1163, 1114, 1099 cm−1 are due to C-O stretching vibration and an absorption at 721 cm−1 are associated with CH2 rocking mode (Sinelli et al., 2007). Main differences in UV-spectra are in the peaks which could be attributed to carotenoids (400-500 nm) and chlorophylls (670 nm) (Figure 4.41). Both infrared and UV-vis spectral data were analyzed with a multivariate statistical analysis technique, OPLS-DA. Before analysis with OPLS-DA, data were transformed using the first and the second derivative. The second derivative provided better results; therefore, only the results of this pre-treated data are discussed here.

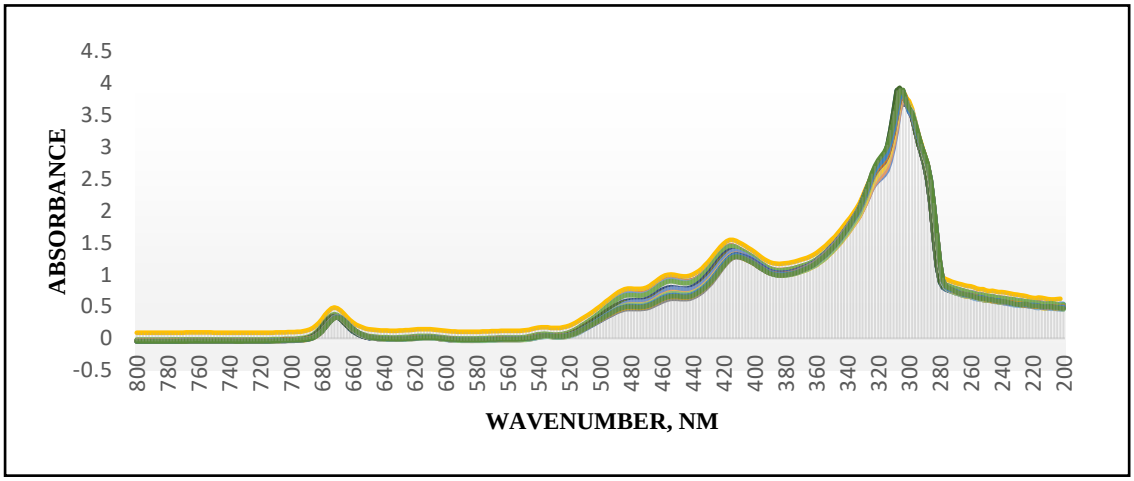


Figure 4.40. UV-Vis spectra of all North region samples during storage

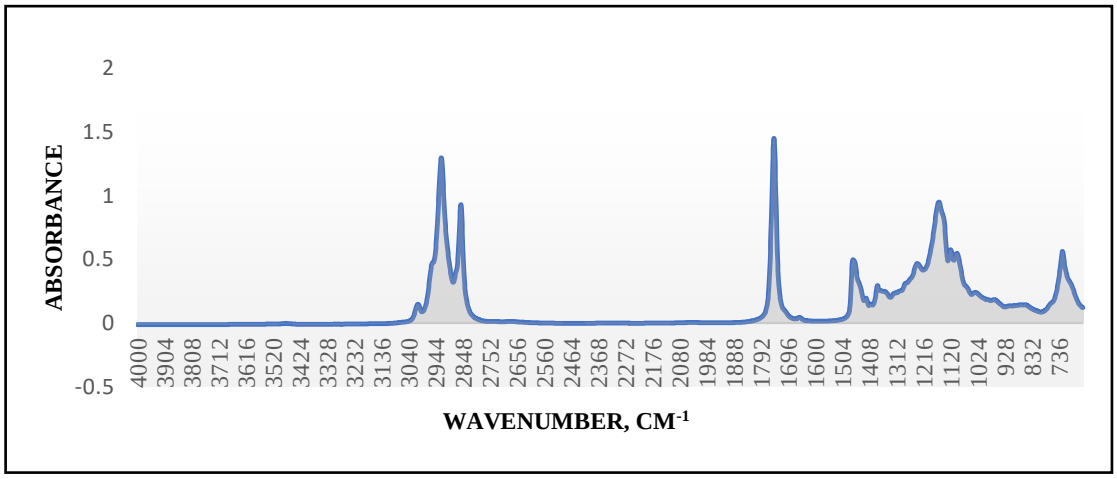


Figure 4.41. FTIR spectra of pure North region sample at the beginning of storage

OPLS-DA model developed with UV-vis spectra of North oils resulted in total separation with respect to storage times. Samples belonging to different storage periods are grouped in different quarters of the score plot (Figure 4.42). Another model developed for South oils, on the other hand, differentiates the initial and 6th months samples totally (Figure 4.43). However, the samples from 12th and 15th months are clustered together. North and South oils are also combined together in the same data set to investigate the changes in the olive oils with respect to storage time using UV-vis spectral data (Figure 4.44).

Differentiation with respect to storage time is successful using UV-vis spectra of combined two regions. Samples before storage and after 6 months of storage are separately located in the upper half of the score plot while the oils stored for 12 and 15 months are very closely placed to each other in the lower half. Therefore, initial and 6 months' samples could be differentiated from other two storage periods with respect to second principal component. Although the samples in the same storage period are clustered together oils from North are placed on the one side of cluster and South samples on the other end.

OPLS-DA models developed for both North and South Region oils using FTIR spectral data also have the same type of trend with UV-vis models (Figure 4.45 and 4.46). Score plots show separation of initial and 6th months samples, and other two storage periods are clustered together separately from the rest. Combining the data from two regions also results the same conclusion (Figure 4.47).

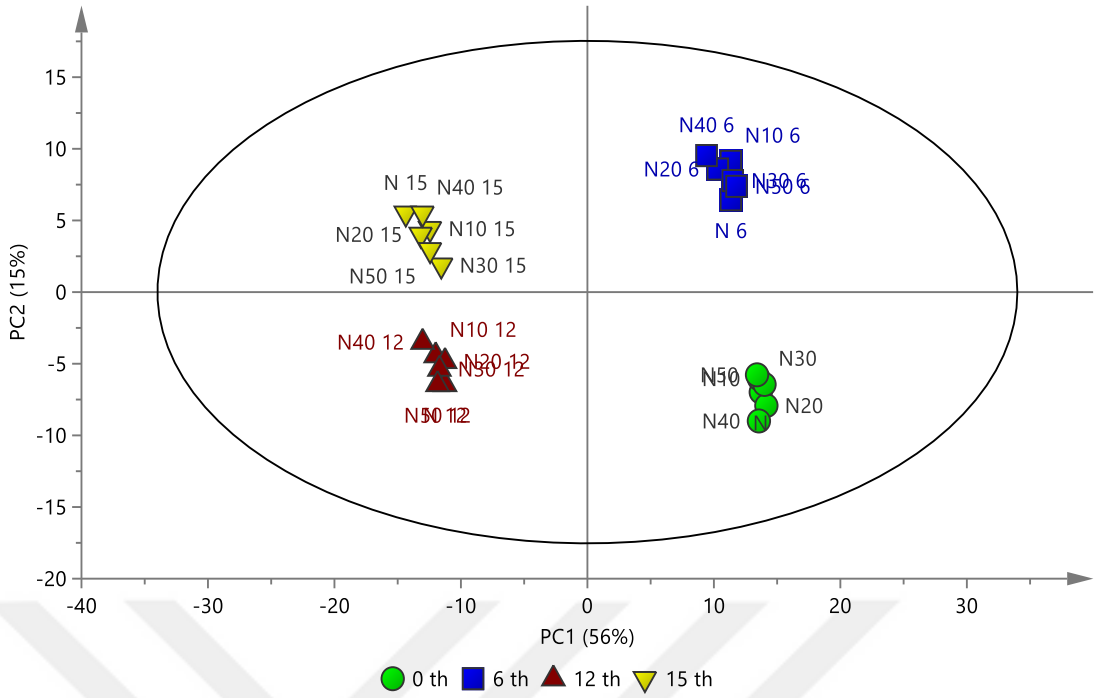


Figure 4.42. OPLS-DA score plots of second derivative of UV-Vis data of olive oils from North region adulterated with old olive oil with respect to storage time (N=North, First two numbers indicate adulteration levels and last number is the storage time)

FTIR data from two combined regions were also used in creating OPLS-DA model with respect to adulteration level (Figure 4.48). According to score plot, fresh, 10% and 20% oils are clustered together indicating no separation at these levels. Some of the samples of 30% could not be separated from fresh ones with respect to first principal component. However, better differentiation is obtained for 40% and especially 50% adulterated samples.

As a result, UV-vis and FTIR spectral data, in general, provide similar results and separate initial, 6th and 12th months oils; however, differentiation after this period is not very clear. This could be due to levelling of the changes in the chemical properties of olive oil after 12 months or the shorter duration between 12 and 15 months. This conclusion also could be supported by another OPLS-DA model which includes all chemical properties measured and both region olive oils. According to score plot of this model, samples before storage and after 6 months of storage are separately placed while 12th and 15th months oils are very close to each other (Figure 4.49). Therefore, this is also

a confirmation of the result that while there is a progressive change in the quality parameters and chemical composition of olive oils in the first 12 months of storage this change slows down after 12 months. FTIR spectral data in combination with chemometric analysis could also detect old olive adulteration of fresh olive oils around 40% and higher levels regardless of geographic origin and storage time.

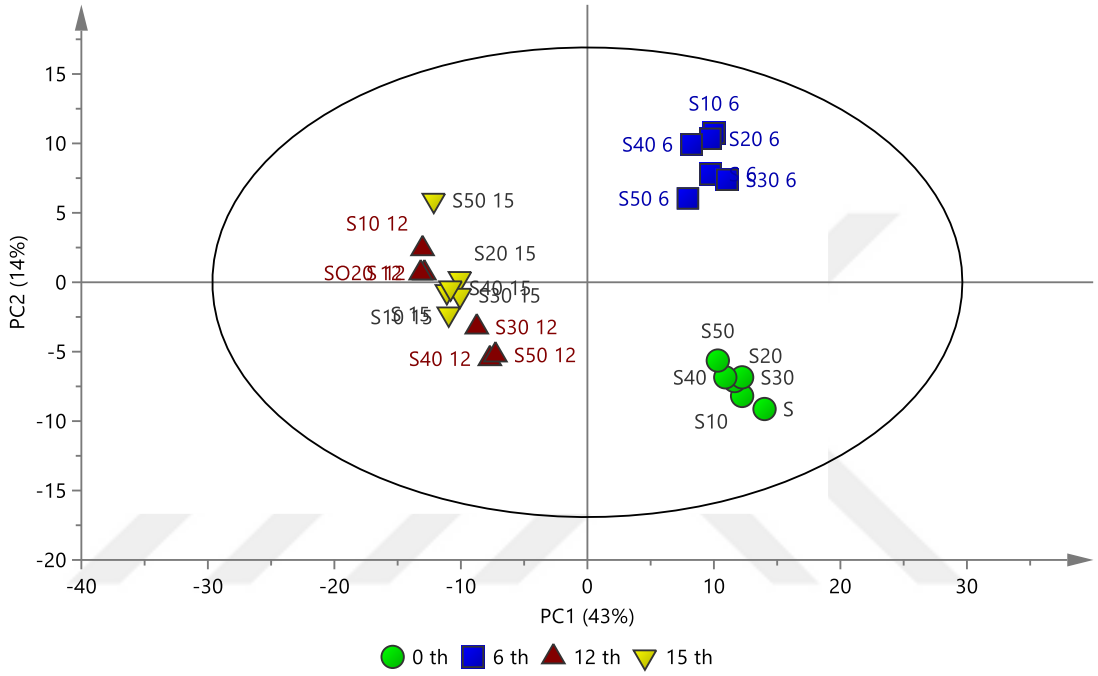


Figure 4.43. OPLS-DA score plots of second derivative of UV-Vis data of olive oils from South region adulterated with old olive oil with respect to storage time (S= South, First two numbers indicate adulteration levels and last number is the storage time)

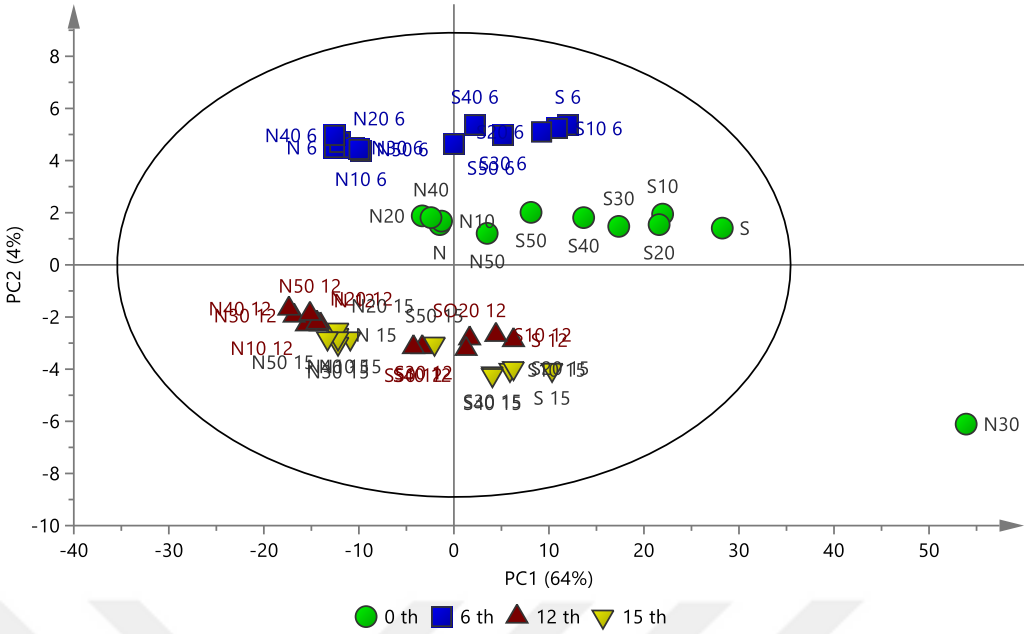


Figure 4.44. OPLS-DA score plots of second derivative of UV-VIS spectra of olive oils from both regions adulterated with old olive oil with respect to storage time (N=North, S= South, First two numbers indicate adulteration levels and last number is the storage time)

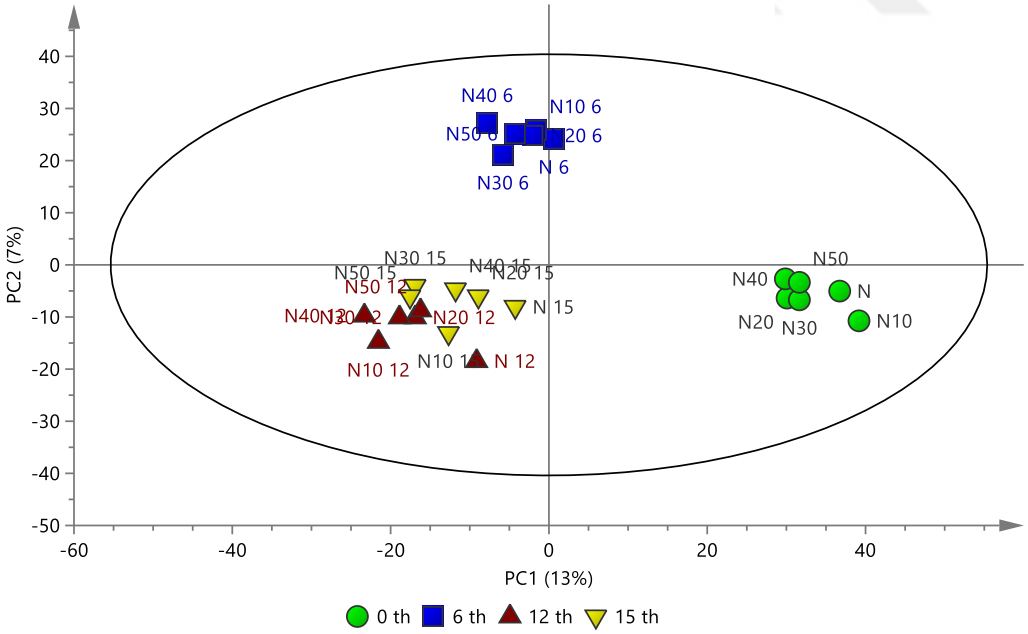


Figure 4.45. OPLS-DA score plots of second derivative of FTIR data of olive oils from North region adulterated with old olive oil with respect to storage time (N=North, First two numbers indicate adulteration levels and last number is the storage time)

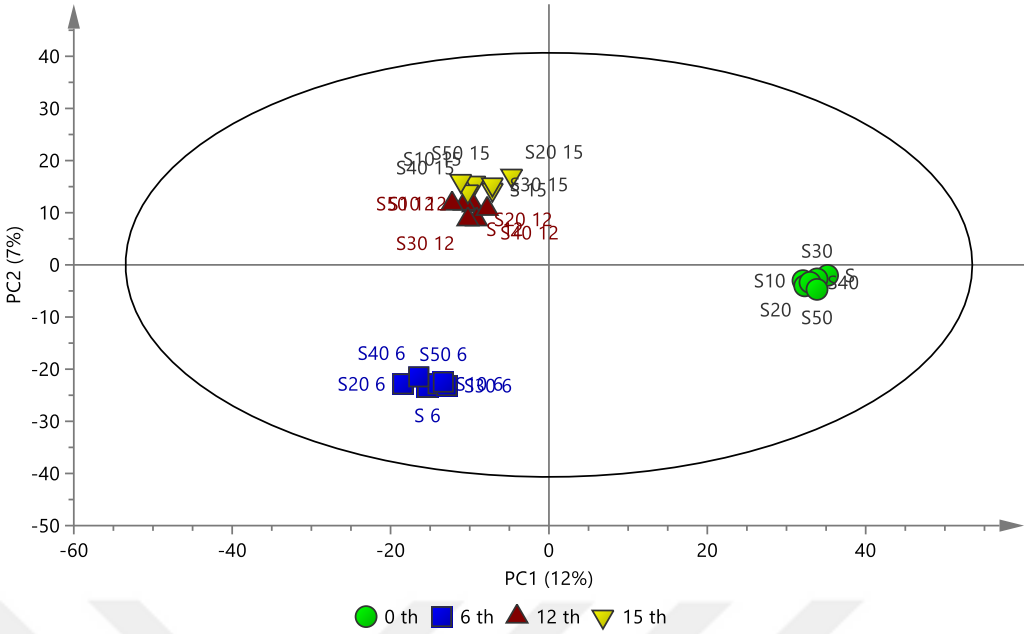


Figure 4.46. OPLS-DA score plots of second derivative of FTIR data of olive oils from South region adulterated with old olive oil with respect to storage time (S= South, First two numbers indicate adulteration levels and last number is the storage time)

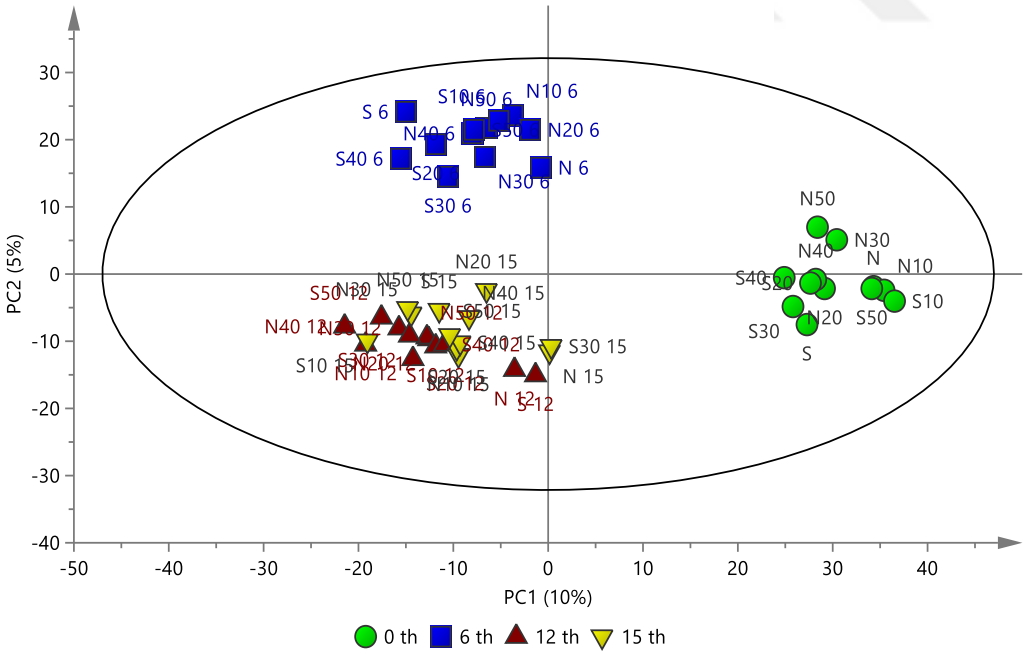


Figure 4.47. OPLS-DA score plots of second derivative of FTIR data of olive oils from both regions adulterated with old olive oil with respect to storage time (N=North, S= South, First two numbers indicate adulteration levels and last number is the storage time)

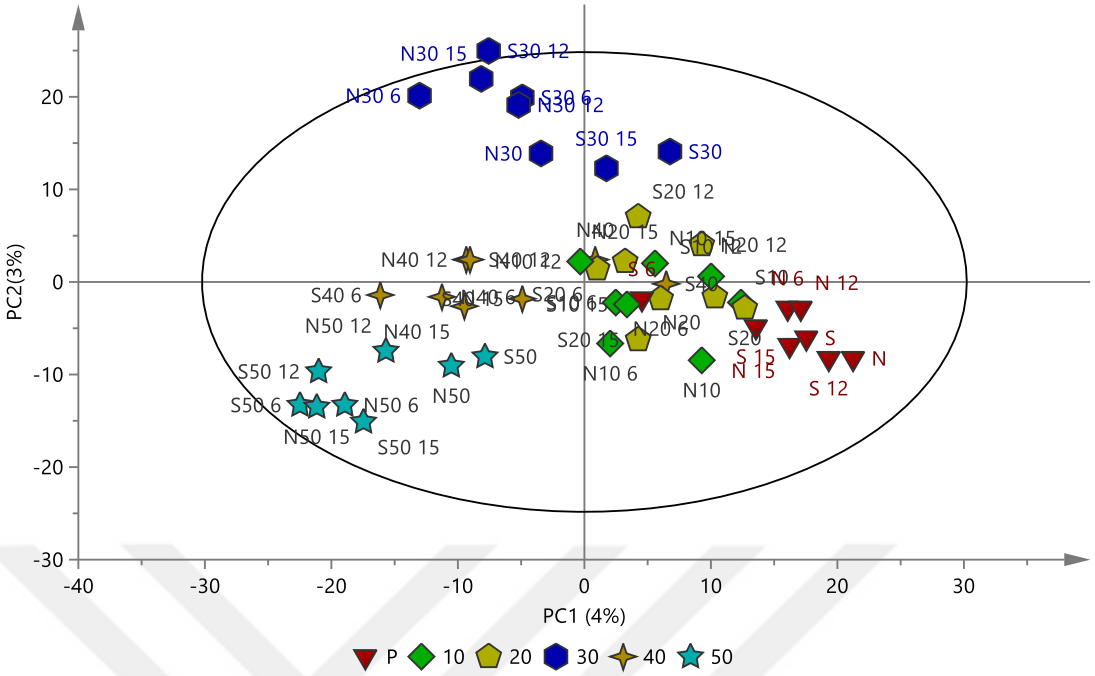


Figure 4.48. OPLS-DA score plots of second derivative of FTIR data of olive oils from both regions adulterated with old olive oil with respect to adulteration level (P= Pure, 10= 10% adulterated, 20= 20% adulterated, etc., N=North, S= South, First two numbers indicate adulteration levels and last number is the storage time)

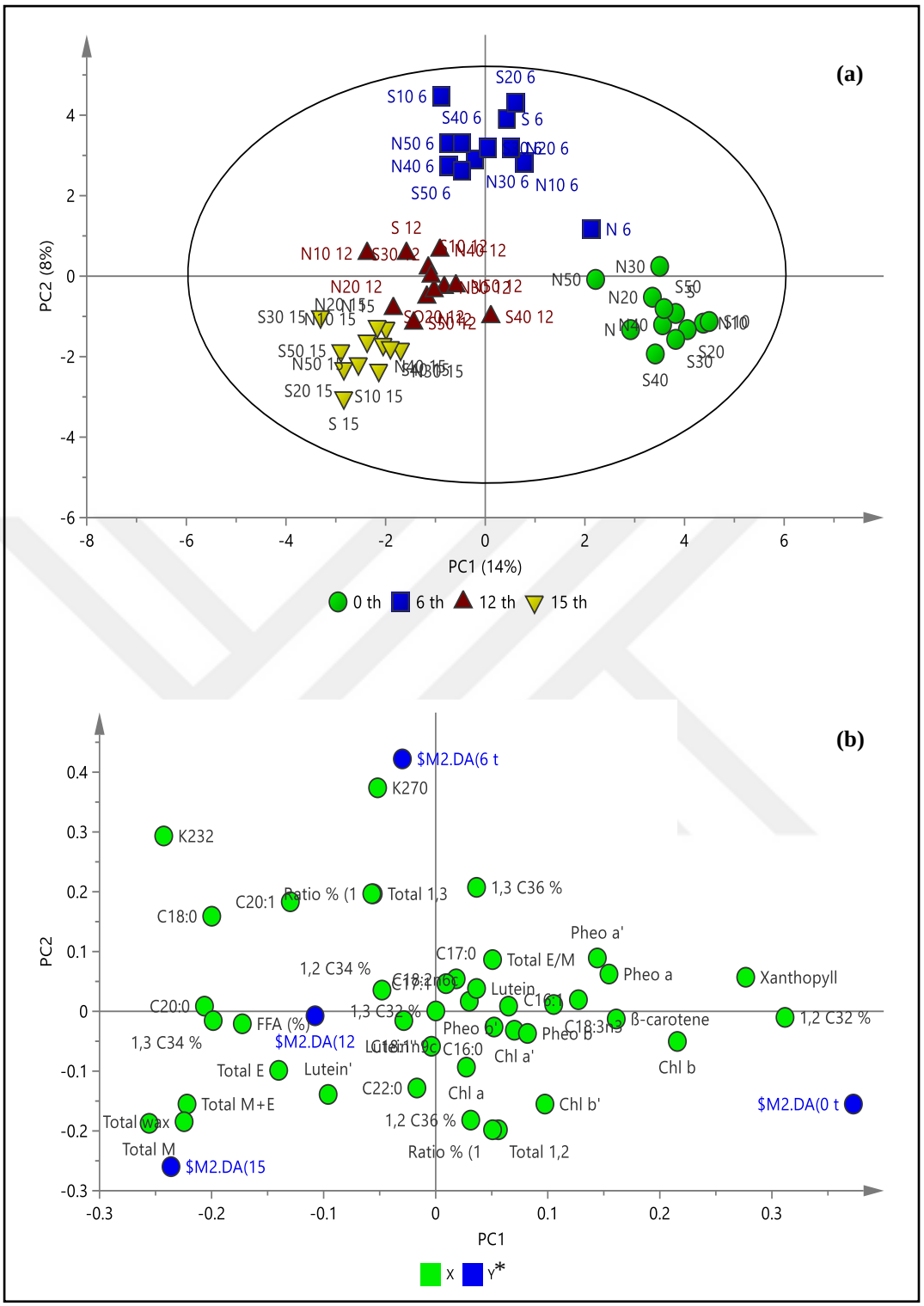


Figure 4.49. OPLS-DA (a) score and (b) loading plots of all chemical parameters of olive oils from both regions adulterated with old olive oil with respect to storage time (N=North, S= South, First two numbers indicate adulteration levels and last number is the storage time) *X: Variables Y:Classes

CHAPTER 5

CONCLUSIONS

Olive oils from two different locations were mixed with old olive and various chemical parameters including basic quality indices (K values and free fatty acidity), fatty acid profile, fatty acid alkyl and ethyl esters, diacylglycerols and pigment profile as well as UV-visible and FTIR spectral profiles were determined throughout 15 months of storage period in this study. It was aimed to determine which of these measurements are good indicators of quality and provide information regarding the adulteration of fresh olive oils with old olive oils.

Free fatty acid value is a good indicator of the quality during storage since it gradually increased throughout the storage both for adulterated and non-adulterated oils. However, K values decreased after 6 months due to the possible conversion of one form of oxidation product to the other; therefore, they can be misleading to monitor the quality in the long term. There are small changes in major fatty acid contents of olive oils regardless of adulteration level. However, more significant and gradual increase in the amounts of fatty acid alkyl esters of non-adulterated and adulterated oils were observed in the first 12 months of storage. Changes in diacylglycerol values of the oils were not also very notable. Chlorophyll and carotenoid content of all oils decreased considerably throughout the storage. These observations were clearly confirmed by multivariate statistical analysis of the data and statistical analysis provided more clear picture regarding the differences between storage times as well as the specific compounds causing the differentiation of oils. Multivariate analysis of both UV-visible and FTIR spectral data provided similar results and these results also agree with the conclusion obtained by evaluating all chemical parameters together and olive oil samples could be differentiated well with respect to their storage period. Fatty acid alkyl esters and pigments could provide separation of fresh oils from fresh oils containing 50% old olive oil regardless of geographic origin and storage time while FTIR spectroscopic data provided the same differentiation at 40% and higher levels.

More olive oil samples from different locations need to be evaluated to investigate this type of adulteration. In addition, other spectroscopic techniques such as NMR

spectroscopy could be also used for the same purpose along with other statistical analysis methods.

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APPENDIX A

EXPERIMENTAL DATA

Table A.1. Basic quality parameters of samples during 15-month storage

	North Aegean		**South Aegean**		**Old**	
Parameter*	2015/16 N =10		2015/16 N =11		2015/16 N =3	
	Mean	**Range**	**Mean**	**Range**	**Mean**	**Range**
FFA (%)	0.58	0.33-0.99	0.79	0.27-2.30	2.46	0.85-12.11
K232	2.12	1.81-2.82	2.20	2.15-2.78	2.40	2.26-3.14
K270	0.23	0.10-0.36	0.18	0.22-0.37	0.13	0.22-0.70

* FFA standard deviation: 0.03, K232 standard deviation: 0.07, K270 standard deviation: 0.03

Table A.2. Fatty Acid Profiles of samples during 15-month storage

	North Aegean		**South Aegean**		**Old**	
Parameter*	2015/16 N =10		2015/16 N =11		2015/16 N =3	
	Mean	**Range**	**Mean**	**Range**	**Mean**	**Range**
C16:0	15.08	12.23-15.85	13.26	12.78-15.62	13.91	12.09-15.09
C16:1	0.88	0.59-1.06	0.89	0.70-1.16	0.76	0.44-1.39
C17:0	0.16	0.14-0.19	0.07	0.05-0.17	0.19	0.11-0.18
C17:1	0.24	0.22-0.30	0.12	0.08-0.29	0.23	0.19-0.26
C18:0	2.70	2.45-3.77	2.66	2.27-3.41	2.70	2.48-3.53
C18:1n9c	68.31	67.65-74.32	71.85	65.92-72.47	66.24	64.09-70.75
C18:2n6c	11.28	7.02-13.19	9.61	8.04-12.96	14.42	9.47-17.67
C20:0	0.41	0.45-0.51	0.38	0.42-0.56	0.41	0.38-0.52
C18:3n3	0.64	0.61-0.75	0.80	0.68-0.97	0.73	0.59-1.03
C20:1	0.26	0.24-0.33	0.30	0.26-0.34	0.30	0.25-0.36
C22:0	0.04	0.00-0.20	0.06	0.00-0.16	0.11	0.09-0.15

*Standard deviations; C16:0: 0.02, C16:1: 0.00, C17:0: 0.01, C17:1: 0.00, C18:0: 0.01, C18:1n9c: 0.10, C18:2n6c: 0.04, C20:0: 0.02, C18:3n3: 0.07, C20:1: 0.01, C22:0: 0.01.

Table A.3. Pigment Contents of samples during 15-month storage

Parameter*	North Aegean 2015/16 N =10		South Aegean 2015/16 N =11		Old 2015/16 N =3	
	Mean	**Range**	**Mean**	**Range**	**Mean**	**Range**
[1]Pheoohytin a	2.68	1.66-4.73	5.47	1.99-7.69	2.16	0.93-2.89
[2]Pheophytin a'	0.56	0.33-0.97	1.05	0.36-1.59	0.46	0.18-0.99
[3]Chlorophyll a	0.07	0.04-0.14	0.21	0.09-0.30	0.13	0.09-0.22
[4]Chlorophyll a'	0.37	0.03-0.05	0.05	0.04-0.06	0.03	0.03-0.04
[5]Pheophytin b	0.25	0.15-0.43	0.66	0.33-0.86	0.38	0.30-0.69
[6]Pheophytin b'	0.19	0.13-0.28	0.55	0.28-0.68	0.27	0.23-0.58
[7]Xanthophyll	0.85	0.52-1.93	1.11	0.62-1.82	0.53	0.36-0.69
[8]Lutein	1.52	1.02-1.86	2.67	1.39-4.19	1.27	0.80-1.68
[9]Lutein'	0.21	0.16-0.36	0.52	0.32-1.01	0.23	0.19-0.32
[10]Lutein"	0.13	0.09-0.23	0.25	0.19-0.38	0.11	0.07-0.14
[11]Chlorophyll b	0.17	0.07-0.21	0.44	0.16-0.98	0.17	0.05-0.27
[12]Chlorophyll b'	0.03	0.02-0.04	0.09	0.05-0.18	0.03	0.01-0.05
[13]β-carotene	3.20	1.62-5.13	3.31	1.96-4.51	1.82	1.69-2.35

Standard deviations: 0.52[1], 0.11[2], 0.01[3], 0.01[4], 0.02[5], 0.02[6], 0.10[7], 0.24[8], 0.08[9], 0.03[10], 0.05[11], 0.02[12], 0.82[13]

Table A.4. Fatty Acid Alkyl Esters (FAAEs) of samples during 15-month storage

Parameter*	North Aegean		South Aegean		Old	
	2015/16 N =10		2015/16 N =11		2015/16 N =3	
	Mean	**Range**	**Mean**	**Range**	**Mean**	**Range**
FAAE	39.74	20.97-60.14	40.97	28.82-62.03	72.12	59.63-85.54
FAME	25.32	13.94-35.82	22.46	15.38-33.57	35.06	28.26-42.59
FAEE	15.51	7.02-26.81	18.10	10.12-28.46	37.06	31.37-42.95
FAEE/FAME	0.64	0.34-1.00	0.81	0.58-1.03	1.06	1.01-1.11
Waxes	27.71	15.13-35.82	18.38	2.38-40.21	31.50	26.99-46.55

* FAAE standard deviation:2.39, FAME standard deviation:1.57 , FAEE standard deviation:0.83, FAAE/FAME standard deviation:0.01, Waxes standard deviation:0.53.

Table A.5. Diacylglycerols content (DAGs) of samples during 15-month storage

Parameter*	North Aegean		South Aegean		Old	
	2015/16 N =10		2015/16 N =11		2015/16 N =3	
	Mean	**Range**	**Mean**	**Range**	**Mean**	**Range**
1,2 C32%	0.13	0.00-0.47	0.14	0.00-0.44	0.16	0.00-0.47
1,3 C32%	0.00	0.00-0.00	0.00	0.00-0.00	0.05	0.00-0.15
1,2 C34%	14.98	14.23-15.37	13.41	12.48-14.21	14.88	14.40-15.17
1,3 C34%	6.75	6.40-7.30	6.27	5.70-7.11	6.74	6.26-7.37
1,2 C36%	54.88	51.89-56.32	56.45	54.37-59.06	54.15	51.95-55.42
1,3 C36%	23.26	22.05-25.52	23.75	20.82-25.52	24.03	22.64-26.28
Total 1,2%	69.99	67.26-71.39	70.00	67.98-72.80	69.18	66.35-70.95
Total 1,3%	30.00	28.61-32.74	30.02	27.20-32.02	30.82	29.05-33.65
Total 1,3%/1,2%	2.34	2.05-2.49	2.34	2.12-2.68	2.26	1.97-2.44

* 1,2 C32% standard deviation:0.03, 1,3 C32% standard deviation:0.06, 1,2 C34% standard deviation:0.03, 1,3 C34% standard deviation:0.68, 1,2 C36% standard deviation:1.31, 1,3 C36% standard deviation:0.52, Total 1,2% standard deviation:1.25, Total 1,3% standard deviation: 1.25,Total 1,3/1,2 standard deviation:0.02.

Table A.6. Specific extinction coefficients, Free fatty acidity and Fatty acid profiles of (% total fatty acids) of Old oil sample during 15-month storage

	*Specific extictions		*Free fatty acidity (%)	*Fatty acids (%)										
Time (Month)	**K232**	**K270**	**FFA**	**C16:0**	**C16:1**	**C17:0**	**C17:1**	**C18:0**	**C18:1n9c**	**C18:2 n6c**	**C18:3 n3**	**C20:0**	**C20:1**	**C22:0**
0	2.66	0.23	1.04	13.58	0.80	0.13	0.18	2.67	70.72	10.37	0.72	0.42	0.28	0.14
6	2.90	0.30	1.19	12.94	0.75	0.12	0.20	2.82	71.23	10.25	0.70	0.48	0.33	0.18
12	2.82	0.30	1.26	12.70	0.71	0.11	0.19	2.74	71.66	10.22	0.70	0.51	0.33	0.14
15	2.78	0.30	1.24	13.00	0.74	0.13	0.19	2.74	71.79	9.91	0.61	0.47	0.32	0.12

* FFA sd: 0.03, K232 sd: 0.07, K270 sd: 0.03, C16:0 sd: 0.02, C16:1 sd: 0.00, C17:0 sd: 0.01, C17:1 sd: 0.00, C18:0 sd: 0.01, C18:1n9c sd: 0.10, C18:2n6c sd: 0.04, C20:0 sd: 0.02, C18:3n3 sd: 0.07, C20:1 sd: 0.01, C22:0 sd: 0.01.

Table A.7. Fatty acid alkyl esters and pigments of Old olive oil sample during 15-month storage

	*Fatty Acid Alkyl Esters (mg/kg)					*Pigments (mg/kg)									
Time (Month)	**Total M**	**Total E**	**Total M+E**	**Total E/M**	**Total wax**	**[1]Pheo a**	**[2]Pheo a'**	**[3]Chl a**	**[4]Chl a'**	**[5]Pheo b**	**[6]Pheo b'**	**[7]Chl b**	**[8]Chl b'**	**[9]Lutein**	**[10]Lutein'**	**[11]Lutein''**
0	30.69	32.17	62.86	1.05	26.99	2.89	0.99	0.24	0.04	0.69	0.58	0.27	0.05	1.68	0.32	0.15
6	28.26	31.37	59.63	1.11	33.83	2.23	0.46	0.09	0.03	0.31	0.24	0.14	0.02	1.46	0.21	0.11
12	39.44	41.95	81.39	1.06	29.16	2.10	0.46	0.13	0.03	0.38	0.30	0.20	0.04	1.09	0.19	0.10
15	42.59	42.95	85.54	1.01	46.55	0.93	0.18	0.14	0.03	0.37	0.23	0.05	0.02	0.80	0.25	0.07

* FAAE sd: 2.39, FAME sd: 1.57 , FAEE sd: 0.83, FAAE/FAME sd: 0.01, Wax sd: 0.53, Pigments sd; 0.52^{1}, 0.11^{2}, 0.01^{3}, 0.01^{4}, 0.02^{5}, 0.02^{6}, 0.10^{7}, 0.24^{8}, 0.08^{9}, 0.03^{10}, 0.05^{11}.

Table A.8. Pigments and Diacylglycerols of Old oil sample during 15-month storage

	Pigments (mg/kg)			Diacylglycerols (%)*								
Time (Month)	**[11]Lutein''**	**[12]Xanthopyll**	**[13]β-carotene**	**1,2 C32**	**1,3 C32**	**1,2 C34**	**1,3 C34**	**1,2 C36**	**1,3 C36**	**Total 1,2**	**Total 1,3**	**Total (1,3/1,2)**
0	0.15	0.69	2.35	0.47	0.15	15.06	6.26	55.42	22.64	70.95	29.05	0.41
6	0.11	0.64	1.73	-	-	-	-	-	-	-	-	-
12	0.10	0.41	1.70	0.00	0.00	15.17	6.58	55.08	23.17	70.25	29.75	0.42
15	0.07	0.36	1.90	0.00	0.00	14.40	7.37	51.95	26.28	66.35	33.65	0.51

Pigments sd; 0.02[12], 0.82[13]. 1,2 C32% sd: 0.03, 1,3 C32% sd: 0.06, 1,2 C34% sd: 0.03, 1,3 C34% sd: 0.68, 1,2 C36% sd: 1.31, 1,3 C36% sd: 0.52, Total 1,2% sd: 1.25, Total 1,3% sd: 1.25,Total 1,3/1,2 sd: 0.02.

*The content of DAGs of old olive oil sample was determined throughout the storage at 0, 12 and 15 months.

APPENDIX B

STANDARD CALIBRATION CURVES FOR PIGMENTS

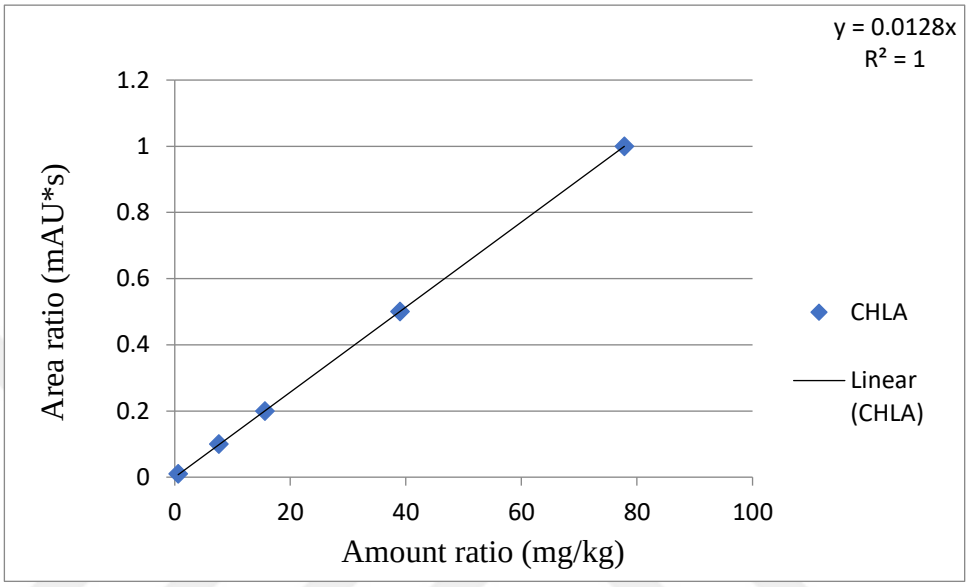

Figure B.1. Standard calibration curve for chlorophyll a

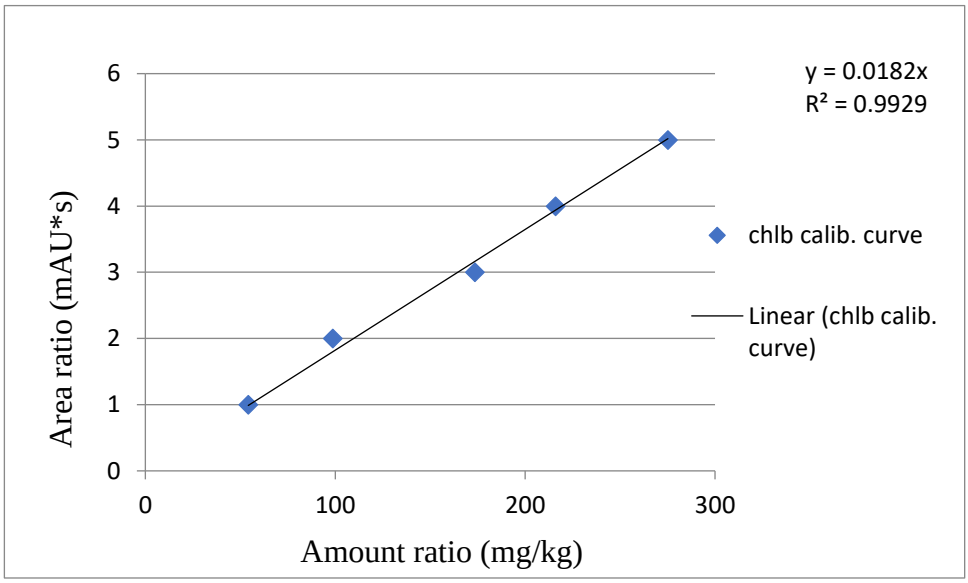

Figure B.2. Standard calibration curve for chlorophyll b

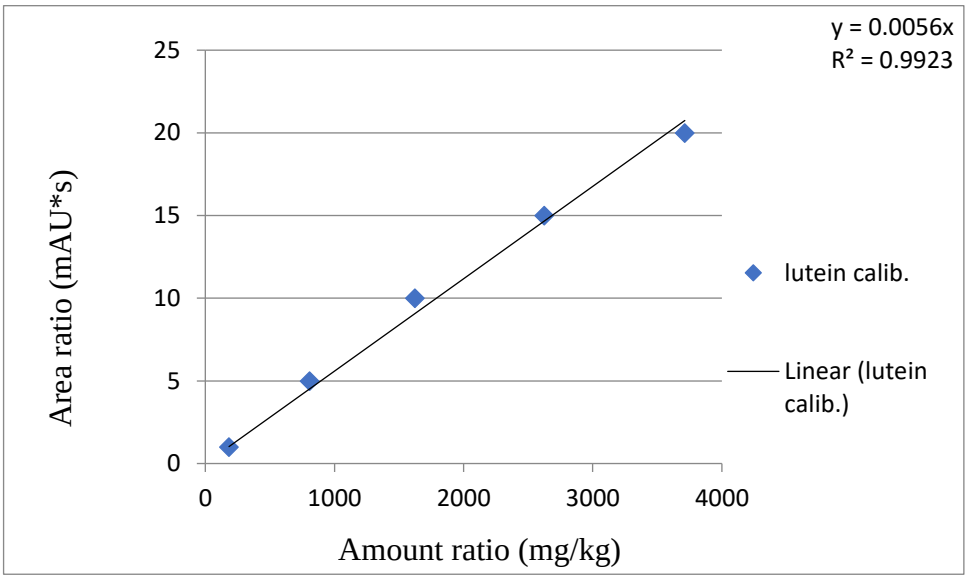

Figure B.3. Standard calibration curve for lutein

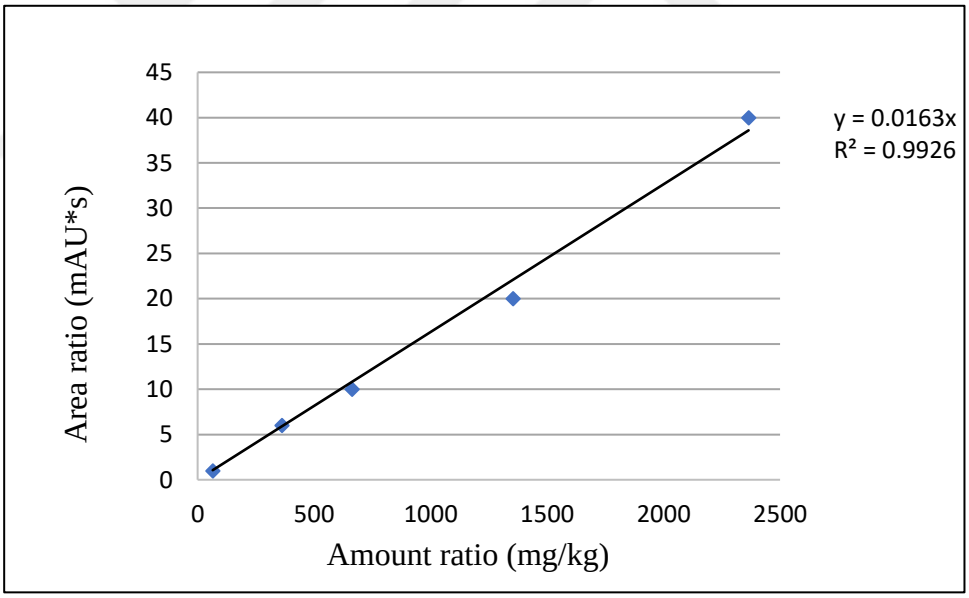

Figure B.4. Standard calibration curve for pheophytin a and its prime

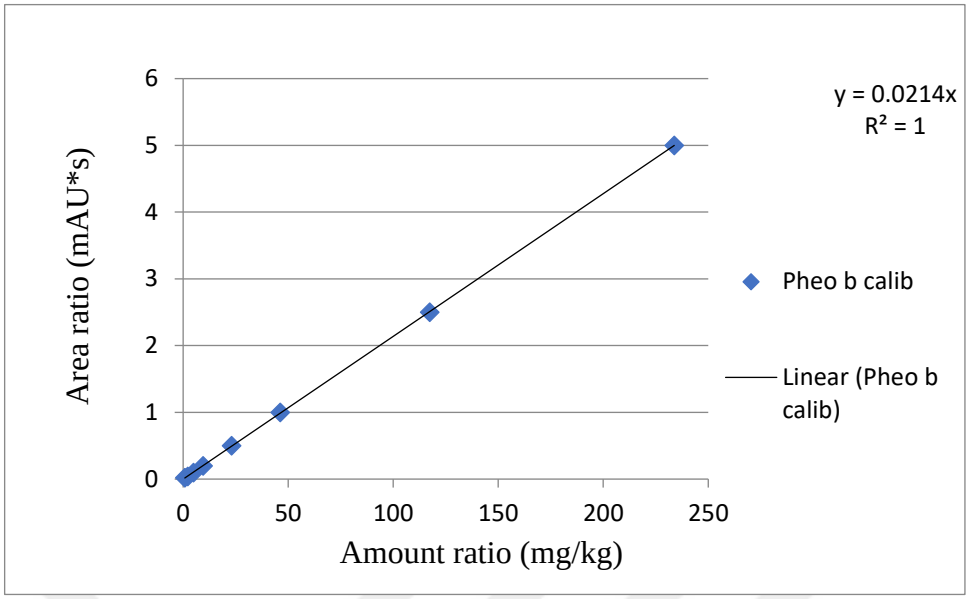

Figure B.5. Standard calibration curve for pheophytin b