MINOR COMPONENTS OF OLIVE OILS AS INDICATORS FOR THE AUTHENTICITY OF VIRGIN OLIVE OILS

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ABSTRACT

MINOR COMPONENTS OF OLIVE OILS AS INDICATORS FOR THE AUTHENTICITY OF VIRGIN OLIVE OILS

Adulteration of olive oil is a major problem of olive oil industry and may result in health problems as well as unfair earnings. Especially after the update in EU regulations about the labelling of olive oils, a need is arisen to detect the mixtures of old and fresh olive oils. Improvements in detection methods could fall behind of the inventiveness of the fraudsters. Detecting and preventing adulteration could be a challenging task; therefore, new methods and solutions are always in demand to solve this problem. First purpose of this theses is to characterize Aegean region olive oils with respect to their quality parameters such as fatty acid alkyl esters, diacylglycerols, and pigment compositions and to investigate differentiation power of these parameters on harvest year and geographical origin in comparison with spectroscopic methods. It is also aimed to predict these quality parameters by the fast and environmentally friendly ultraviolet-visible (UV-vis) and mid-infrared (mid-IR) spectroscopic techniques in combination with multivariate statistical methods. Finally, the applicability of spectroscopic methods (UV-vis, mid-IR, fluorescent) to detect adulteration of fresh olive oil with old olive oil is investigated. Olive oils were successfully differentiated with respect to geographical location by spectroscopic methods, fatty acid alkyl esters and pigments. In general, prediction of investigated chemical parameters was achieved robustly with mid-IR spectral data except pigments which were estimated better with UV-vis spectral data. Fluorescence and mid-IR + UV-vis spectroscopies were successful in detecting old olive oils in fresh olive oils.

ÖZET

SIZMA ZEYTİNYAĞLARIN OTANTİSİTESİ İÇİN İNDİKATÖR OLARAK MİNÖR BİLEŞİKLER

Zeytinyağı endüstrisi için bu ürünün tağşişi hem sağlık problemlerine hem de haksız kazanca sebebiyet verebilen önemli bir problemdir. Özellikle Avrupa Birliğinin zeytinyağının etiketleme kurallarını güncellemesinden sonra, eski ve taze zeytinyağlarının karışımını saptama ihtiyacı oluşmuştur. Tağşiş saptama yöntemlerindeki gelişmeler dolandırıcıların yaratıcılığının gerisinde kalabilmektedir. Tağşişi tespit ve engelleme zorlu bir konu olabilmektedir; dolayısıyla yeni yöntemlere ve çözümlere her zaman ihtiyaç duyulmaktadır. Bu tezin ilk amacı, kalite parametrelerinden yağ asidi alkil esterleri, diaçilgliserolleri ve pigment içeriklerine göre Ege Bölgesi zeytinyağlarını karakterize etmek ve bu parametrelerin coğrafi konum ve hasat yılı üzerindeki farklılaşma gücünü spektroskopik yöntemlerle karşılaştırarak araştırmaktır. Bununla birlikte bu kalite parametrelerinin hızlı ve çevre dostu ultraviyole-görünür (UV-vis) ve orta-kızılötesi (orta-IR) spektroskopik tekniklerinin çok değişkenli istatistiksel yöntemler ile kombinasyon halinde tahminidir. Son olarak, spekstroskopik metotların (ultraviyole-görünür, orta-kızılötesi ve floresan) taze zeytinyağlarının eski zeytinyağları ile tağşişinin saptanmasında uygulanabilirliği araştırılmıştır. Zeytinyağları, spektroskopik yöntemlerle, yağ asidi alkil esterleri ve pigmentler ile coğrafi konuma göre başarılı bir şekilde ayırt edilmiştir. Genel olarak, UV-vis spektral verilerle daha iyi tahmin edilen pigmentler dışında, araştırılan kimyasal parametrelerin tahmini orta-IR spektral verileriyle sağlam bir şekilde elde edilmiştir. Floresan ve orta-IR + UV-vis spektroskopileri, taze zeytinyağlarında eski zeytinyağlarının tespitinde başarılı olmuştur.

Dedicated to my father Salih UNCU

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CHAPTER 1

INTRODUCTION

Redrafted, modified, and extended from:

Uncu, Oguz, Banu Ozen, and Figen Tokatli. 2019. “Use of FTIR and UV–Visible spectroscopy in determination of chemical characteristics of olive oils.” *Talanta* 201: 65–73. https://doi.org/10.1016/j.talanta.2019.03.116.

Uncu, Oguz, and Banu Ozen. 2019. "A comparative study of mid-infrared, UV–Visible and fluorescence spectroscopy in combination with chemometrics for the detection of adulteration of fresh olive oils with old olive oils." *Food Control* 105: 209-218. https://doi.org/10.1016/j.foodcont.2019.06.013.

Uncu, Oguz, Banu Ozen, and Figen Tokatli. 2020. “Authentication of Turkish Olive Oils by using detailed pigment profile and spectroscopic techniques.” *Journal of the Science of Food and Agriculture* 100 (5): 2153–65. https://doi.org/10.1002/jsfa.10239.

Uncu, Oguz, and Banu Ozen. 2021. "Fatty acid alkyl ester and wax compositions of olive oils as varietal authentication indicators." *Journal of Food Measurement and Characterization* (in press). https://doi.org/10.1007/s11694-021-01184-2.

Olive oil is a high profit food product due to its proven health benefits and its unique sensory characteristics. These positive characteristics are mainly associated with the unique chemistry of olive oil which is mainly composed of monounsaturated fatty acids (mainly oleic acid) and minor components (phenolic compounds, α-tocopherol and carotenoids) (Li and Wang 2018). These chemical characteristics of virgin olive oils are well preserved during its production, which is based on mechanical extraction without

the use of any chemical solvent (Uncu and Ozen 2015). A rise in the price of this product due to increasing demand, makes olive oil quite prone to adulteration. Unfortunately, it is a very common practice to mix good quality olive oils with other vegetable oils as well as low quality olive oils such as pomace or deodorized olive oils in the market to obtain extra profit. Quality problems comprising fraudulent representation and mislabeling of olive oils cause consumers to lose confidence to this product (Jolayemi et al. 2017).

Fraudsters continuously update their adulteration techniques as a response to new adulteration detection methods. In addition, olive oils have been started to be produced outside of traditional growth area of olives and olive oils coming from untraditional olive growth areas might have significant compositional differences compared to the limits of regulations based on European production area, even without any adulteration (Aparicio et al. 2013; Bajoub et al. 2018). Therefore, new chemical parameters have been continuously introduced as quality indicators for olive oil.

Minor compounds could be effective indicators of the authenticity and quality of the olive oils since they are hard to mimic in complex matrices (Uncu and Ozen 2020). Color pigments (carotenoids, chlorophyll and derivatives), diacylglycerols (DAGs), and fatty acid alkyl esters (FAAEs) were proposed as potential quality and adulteration detection parameters (European Commission 2013). Ability of these chemical measures were tested to differentiate olive oils with respect to olive variety/geographical growth location in this study using chemometric methods.

Some of these constituents in olive oil are present in the highest level immediately after the extraction and there could be dramatic changes in their quantity during the storage mostly due to oxidative processes. As a result, “best before” date is critical for the quality of olive oil (Tena, Aparicio, and García-González 2018). An update in European Union regulation was done about olive oil labelling requirements in 2012 (Commission Implementing Regulation (EU) 2012). According to this regulation, harvest year can be placed on the label only if 100% of the product was obtained from the olives harvested in the same year. Therefore, mixing of the olive oils from the previous harvest with freshly extracted olive oils is regarded as an adulteration if the label indicates harvest year and a need arises to determine this type of mixing to prevent unfair profits and to protect the consumers. However, detection methods which aim to differentiate old oils in fresh oils have not been thoroughly studied in the literature.

In general, spectroscopic methods provide rapid analysis of the adulterated samples; and they require treatment of the data with multivariate statistical analysis tools.

There are many examples of successful applications of these methods in determination of adulteration of different oils in literature (He et al. 2021; Lohumi et al. 2015).

As another part of this thesis, spectroscopic methods were also used in detection of old olive oils in fresh olive oils by evaluating the data with chemometric techniques.

In the light of these, this thesis has three main aims which will be covered under Chapters 4 to 6 as listed below.

- In Chapter 4, it was aimed to determine the chemical characteristics and authenticity of olive oils from Aegean Region of Turkey. For this purpose, basic quality parameters, fatty acid profile, DAGs, FAEEs, FAAEs, waxes and detailed pigment contents of Turkish olive oils were studied and their ability as authentication tools have been investigated and also compared with spectroscopic methods.
- In Chapter 5, it was aimed to predict FAAE, wax, DAG and color pigment contents of olive oils by using rapid and non-destructive spectroscopic techniques (FTIR and UV–vis) individually and in combination.
- Lastly, in Chapter 6, it was aimed to detect and quantify adulteration of fresh olive oils with old olive oils from the previous harvest year by using fluorescence, Fourier transform infrared (FT-IR), and ultraviolet–visible (UV–vis) spectroscopic techniques in combination with chemometrics.

This thesis was based on the publications derived from the present Ph.D. study. At the beginning of each chapter, bibliographic information of the publications is given. In order to keep the integrity of the thesis structure, these publications were redrafted, modified, and extended.

CHAPTER 2

LITERATURE REVIEW

Redrafted, modified, and extended from:

Uncu, Oguz, and Banu Ozen. 2019. "Authentication of Olive Oil with Mid-Infrared Spectroscopy." in *Authentication and Detection of Adulteration of Olive Oil*, edited by Michael G. Kontominas, 127-152. New York: Nova Science Publishers.

Uncu, Oguz, and Banu Ozen. 2020. "Importance of some minor compounds in olive oil authenticity and quality." *Trends in Food Science and Technology* 100: 164–76. https://doi.org/10.1016/j.tifs.2020.04.013.

2.1. Minor Compounds in Olive Oil Authenticity and Quality

Well-established health effects and desirable sensory properties of olive oil are the major driving forces for the high economical value of this product. Major components of olive oil are triacylglycerols and this oil also contains various minor components such as chlorophylls, carotenoids, phenolic compounds, and squalene (Yan et al. 2018).

Minor components of virgin olive oil which does not need to go through refining steps are highly preserved during mechanical extraction (Olmo-García et al. 2019). Minor compounds are not only significant for physicochemical characteristics of the product, but they are also correlated with taste and nutritional value (Olmo-García et al. 2019). In addition, they are important markers for olive oil quality, purity and authenticity (Olmo-García et al. 2018; Tena et al. 2015). Therefore, the concentration and type of minor compounds are of great importance for both the consumers and the manufacturers (Olmo-

García et al. 2018). The quality and quantity of these metabolites are affected by olive variety, growth conditions of olives, extraction and refining procedures of oil as well as storage conditions (Dais and Hatzakis 2013).

Besides their health-promoting effects, minor components (volatiles, phenolic compounds, terpenoids, sterols, etc.) are also found to be more successful descriptors of olive oil compared to major metabolites due to the fact that it is hard to mimic minor compounds during preparation of illegal formulations (Dais and Hatzakis 2013). Importance of minor compound composition has become even more significant since olive fruits have been started to be cultivated outside the Mediterranean zones. Even for the same olive type, differences in olive growth locations are also leading to compositional differences between oils obtained from relatively new areas and the products from traditional olive producer countries (Aparicio et al. 2013). As a result, olive oils from new cultivation areas could be out of the limits set by official regulatory agencies mainly based on Mediterranean countries (Uncu, Ozen, and Tokatli 2019). In addition, some traditional but minor cultivars, even grown in the Mediterranean region could still have chemical compounds out of the described limits (García-González, Aparicio, and Aparicio-Ruiz 2018). Thus, the data of the minor compounds of olive oils have become more valuable for statistical evaluation as a significant part of authentication studies (Dais and Hatzakis 2013).

As a solution to these emerging problems, new chemical parameters mainly exploiting minor compounds of olive oil have been put into action as quality and/or authenticity indicators (Dais and Hatzakis 2013). If the official and recently proposed methods are examined, it could be seen that the methods that determine quality and adulteration in general are intertwined with each other. Fatty acid alkyl esters (FAAEs), diacylglycerols (DAGs), natural color pigments, particularly pyropheophytins (PPPs) as the degradation product of chlorophylls and phenolic compounds are regarded as some of the potential quality and authenticity indicators of olive oil (European Commission 2013).

Some well-known minor compounds such as sterols, stigmastadienes, aliphatic hydrocarbons and phenolic compounds along with major compounds (triacylglycerols, fatty acid contents) which have official limits in regulations were evaluated in detail in the literature (Aparicio, Conte, and Fiebig 2013; Arvanitoyannis and Vlachos 2007; Ben-Ayed, Kamoun-Grati, and Rebai 2013; Boskou 2008; García-González, Aparicio, and Aparicio-Ruiz 2018; Montealegre, Alegre, and García-Ruiz 2010). In this part, several

minor compounds (FAAEs, color compounds with their derivatives (e.g. PPPs), DAGs with derivatives (e.g. monochloropropanediol esters (MCPDEs) and glycidyl esters (GEs)) that have been studied in recent years will be examined in terms of the authenticity and quality of olive oil.

2.1.1. Authentication Studies

The olive oil industry has several significant problems such as seasonal price fluctuations caused by variations in production capacity, waste disposal management and authentication issues. Among these problems, adulteration is a major concern and it has not only economic consequences but also health implications besides creating a negative publicity for the product (Lai, Kemsley, and Wilson 1994). An authentic food product is defined as "any food product which has the labeling that represents its actual content in accordance with regulations of responsible authorities in the defined territory" (Aparicio et al. 2013; Lees 1998). Authenticity problems of olive oil could be grouped under four main headings as indicated in the literature (Aparicio et al. 2013):

- adulteration of high-quality olive oils with different seed oils, and lower quality olive oils such as pomace oil or olive oil from previous season,
- inexact labelling and traceability problems related to geographical origin of olive oils,
- inexact labelling related to cultivation of olives (organic or conventional farming), and
- cultivar related problems such as false labelling of mixture of different olive oil cultivars as monovarietal olive oil

There are various regulations dealing with different food authentication issues. Two successive regulations EEC 2081/92 (Council Regulation (EEC) 1992a) and 2082/92 (Council Regulation (EEC) 1992b) which were replaced with EC 510/2006 (Council Regulation (EC) 2006b) and 509/2006 (Council Regulation (EC) 2006a), respectively were put into action to protect geographical identity and designation of origin of food products (Luykx and van Ruth 2008). In EC regulation 510/2006, two slightly different concepts were described as "Protected Designation of Origin (PDO)" and

"Protected Geographical Indication (PGI)". According to this regulation, PDO means that "qualities or characteristics of a defined foodstuff are attributed to a particular geographical environment in which production, processing and preparation steps occurred in that specified region" while PGI indicates that "attributed characteristic or quality of a food product is due to any steps of the production and/or processing and/or preparation taking place in the defined geographical region" (Council Regulation (EC) 2006b). According to EC 509/2006, "Traditional Specialty Guaranteed (TSG)" regulation is related with labelling of 'any foodstuff that possesses a traditional specific character which may be related to either its composition (physical, chemical, microbiological or organoleptic features) or production method" (Council Regulation (EC) 2006a). Moreover, olive oil chemical and organoleptic characteristics and their measurement methods were defined in accordance with International Olive Council (IOC) to ensure olive oil authenticity in EU 1348/2013 (Commission Implementing Regulation (EU) 2013), a revised version of EEC 2568/91 (Commission Regulation (EEC) 1991). In addition, regulation EU 432/2012 (Commission Regulation (EU) 2012) prepared through the recommendation of European Food Safety Agencies (European Food Safety Authority (EFSA) 2011) states that a positive health claim which renders the product a candidate for a higher price on the market can be placed on an olive oil label under certain conditions (5 mg of hydroxytyrosol and its derivatives per 20 g of olive oil).

Despite the progresses in analytical methods, developments may still not be enough to find absolute solutions to some of the major problems (European Commission 2013). One of these cases is addition of soft-deodorized virgin olive oil to extra virgin olive oil and this type of mixing could not be detected by standard methods (Kulling et al. 2019). Some proposed solutions for these problems include the determination of PPPs and alkyl esters (Aparicio-Ruiz, Romero, et al. 2017).

Another problem is related with freshness of olive oils. To obtain an extra profit, fraudsters add old olive oils from previous harvest year into the fresh olive oil. This is an emerging adulteration case and there is an update in European Union regulation (Commission Implementing Regulation (EU) 2012) about olive oil labelling requirements indicating the freshness of olive oil. According to the regulation, harvest year could be placed on the label only if 100% of the olive oil is from the olives harvested in the same year. However, there is not any official method in the literature to determine this type of adulteration. It has been proposed that new quality parameters such as FAAEs, pigments

(PPPs, carotenoids, etc.) and DAGs have potential for olive oil quality and authenticity (European Commission 2013).

Production of fake extra virgin olive oil mixtures is another type of fraud. A recent report on deliberately mislabeling the mixture of olive oil made with refined olive oil as extra virgin olive oil was the case occurred in 2018 which was detected by compulsory controls (Kulling et al. 2019). Another case was also reported in 2019 by Europol in which chlorophyll, β-carotene and soya oil were added to sunflower oil to prepare a fake olive oil. The last two adulteration examples were detected easily by existing regulations based on methods using chromatographic techniques (Kulling et al. 2019). In order to solve emerging issues in olive oil, official methods have been updated regularly as a result of new scientific findings about the quality and authenticity of olive oils. Examples of several relatively new regulations about minor components of olive oil are provided in Table 2.1.

Table 2.1. Official regulations about reviewed parameters of olive oil quality and authenticity

Parameters	Legislations
Fatty acid ethyl esters (FAEEs)	Quality criteria defined in IOC (2019) and EU (2016) regulations which state that olive oil could be graded as extra virgin only if it contains ethyl esters less than or equal to 35 mg/kg.
Diacylglycerols (DAGs)	Quality and freshness indicator only found in Australian (Standards Australia 2011) and Californian (California Department of Food and Agriculture 2014) standards to grade olive oil as extra virgin under certain conditions. Both standards define threshold value for 1,2-DAGs as 35% as the ratio between 1,2- to total 1,2- and 1,3- DAGs.
Pyropheophytins (PPPs)	Used in freshness evaluation by both Australian (Standards Australia 2011) and Californian (California Department of Food and Agriculture 2014) standards. According to both standards olive oils are graded as extra virgin when they contain less than or equal to 17% of PPPs.

All these regulations related to adulteration limits and/or detection methods for olive oil are based on wet chemistry analytical techniques. In general, the analytical methods for authentication studies can be divided into two main categories: a) "targeted analysis"; based on identification of specific compounds from the fractionation of olive oil components, and b) "profiling or non-targeted" analyses which aim to identify molecular structures based on pre-defined metabolic pathways (Aparicio et al. 2013). Targeted approaches which focus on many individual components of olive oil have been used for many years, and new application areas have been brought into practice such as the introduction of limits for fatty acid alkyl and ethyl esters (Jabeur et al. 2015), and stigmastadiene analyses (Crews, Pye, and Macarthur 2014) to detect adulteration in olive oil, and pyropheophytin *a* and 1,2-diacylglycerol content determination as olive oil quality parameters (Guillaume, Gertz, and Ravetti 2014) and methodological developments are still in progress. Although these applications might have high precision power regarding the determination of the targeted analyte, they still possess some drawbacks such as long analysis time, high operation cost, and hazardous waste production. As non-targeted analysis approaches, spectroscopic techniques such as mid-infrared (mid-IR), UV-Vis, and fluorescence spectroscopy, provide speed, low cost and environmentally friendly applications for determination of authenticity, overall quality and chemical composition of olive oils.

2.1.1.1. Fatty Acid Alkyl Esters

Fatty acid alkyl esters (FAAEs) are produced by enzymatic reaction of free fatty acids with low molecular weight alcohols, mainly methanol and ethanol under acidic conditions yielding methyl (FAME) and ethyl esters (FAEE), respectively (Bajoub et al. 2018; Pérez-Camino et al. 2002; 2008). Critical levels of FAAEs (sum of FAME and FAEE) for olive oil have been defined first by a Commission Regulation (EU) No 61/2011 (Commission Regulation (EU) 2011) as a quality parameter since the formation of these compounds indicates fermentation (mainly ethanol formation) as well as degradation processes (mainly methanol formation) occurred during storage (Purcaro, Barp, and Conte 2015). In addition, it is not possible to remove FAAEs without leaving

by-products such as stigmastadiene in high temperature treatment (Purcaro, Barp, and Conte 2015). All of these make FAAEs as suitable markers for olive oil quality as well as sensorial assessment (Biedermann et al. 2008). Moreover, storage and processing conditions of olive fruit are also other factors for FAAEs formation (Caponio et al. 2018; Jabeur et al. 2015; Squeo et al. 2017). It was observed that oil that was produced from olives stored in closed plastic bags rather than in perforated plastic containers have higher concentrations of FAAEs due to fermentation activity in the closed plastic bags (Jabeur et al. 2015).

Former regulation has been amended by substituting FAAE (sum of FAME and FAEE) with only FAEE by EU Commission Implementing Regulation 1348/2013 (Commission Implementing Regulation (EU) 2013). Reason for this substitution is that FAEE presence depends on level of its substrate, ethanol, which is produced chemically as a result of fermentative processes. On the other hand, amount of FAMEs depends on methanol content, and unlike ethanol, methanol is physiologically formed during pectin degradation of cell wall as olive fruit ripens (García-Vico et al. 2018). The concentrations of FAEEs depend first on the availability of substrates (ethanol and free fatty acids), and then storage time and temperature, agricultural practices (health status of olive fruits) as well as manufacturing conditions (Bajoub et al. 2018; Conte et al. 2020; García-Vico et al. 2018). In two separate studies, ethanol content of olives being precursor of ethyl ester formation in olive oil was investigated with respect to two different parameters as maturation stage (Beltrán et al. 2015) and harvest method (Beltrán et al. 2016). It was observed that ethanol content of olive fruit increased during the ripening process (Beltrán et al. 2015). Furthermore, ground-picked olives were more susceptible to sensory defects with increasing level of ethanol content compared to tree-picked fruits (Beltrán et al. 2016). In another study, FAAE levels of olive oils were investigated during storage (Conte et al. 2014). The results indicated that high quality olive oils with initially low content of free ethanol and FAAEs did not show any increment of ethyl esters during storage in contrast to lower quality ones. Since these findings confirmed the necessity of an update based on omission of FAME from the regulation and lowering the limit for FAEEs, modifications in regulation were done (Conte et al. 2014). As a result, only the amount of FAEEs have been used as a threshold value for virgin olive oil in determination of the quality in terms of category after this change. According to the latest EU (2016) and IOC (2019) regulations, olive oil could be graded as extra virgin only if it contains FAEEs≤35 mg/kg. As an alternative method, GC Electron Ionization Mass Spectroscopy

(GC-EI-MS) method has been also used in determination of FAAEs of olive oils as a fast way without sample preparation. It was observed that this method was at least as successful as official EU method in discrimination of extra virgin and lower quality olive oils (Boggia et al. 2014). Moreover, very recently GC-IMS has been used promisingly in quantification of ethanol content in olive oils without sample pretreatment and found as being faster than the method based on GC-FID/MS (del Mar Contreras, Aparicio, and Arce 2020). In addition, spectroscopic methods have been applied to the prediction of FAAE content due to their environmentally friendly and easy to use characteristics compared to wet chemical methods. Fourier transform infrared (FTIR) spectroscopy was used in quantification of FAAEs and ratio of ethyl and methyl esters value successfully (Valli et al. 2013). The same type of application was also performed with near infrared (NIR) spectroscopy (Cayuela 2017; Garrido-Varo et al. 2017). In addition, FTIR and UV-visible spectroscopy separately and in combined form were applied to predict FAAE and FAEE content of olive oils (Uncu, Ozen, and Tokatli 2019). FTIR spectroscopy also achieved discrimination of extra virgin from non-extra virgin olive oils based on FAEEs content (Squeo et al. 2019). Dielectric spectroscopy as time domain reflectometry (TDR) was another method used in screening of FAMEs, FAEEs, and FAAEs in olive oils (Berardinelli et al. 2013). In a review paper, determination of various quality parameters of olive oils including FAAEs by different rapid and innovative instrumental approaches were discussed (Valli et al. 2016).

In addition to their quality determining characteristics, these parameters have been also used in detection of mildly refined olive oil which is one of the most recent and common way of adulteration of extra-virgin olive oil. It has been very hard to detect this type of mixing with any other chemical test (Jabeur et al. 2015). FAAE has been firstly proposed as a useful marker to detect soft deodorized olive oils (Pérez-Camino et al. 2008) since this compound is not affected by mild refining conditions significantly. Recent studies are focusing on FAEE contents of olive oils rather than FAAE due to the update in the legislations mentioned in the previous paragraph. Later on, the weak side of this approach as an authentication tool was also discussed in different studies (Aparicio-Ruiz, Romero, et al. 2017; García-Vico et al. 2018; Gómez-Coca et al. 2016). In one of these investigations, it was proven that FAEE content of olive oil could be related with factors other than the quality and health of olives used in olive extraction as opposed to prior knowledge and this could be explained by two main factors (Gómez-Coca et al. 2016). One of these factors is ethanol (precursor of FAEE) formation which had been

previously thought to be produced by only fermentation. However, it was found out that healthy fruits could also be the sources of ethanol during maturation which contribute to aroma development (Beltrán et al. 2015). Other factor is related to technological aspects such as addition of water during the extraction process and this could change ethanol concentration as well as FAEE formation (Gómez-Coca et al. 2016). As a result, extra virgin olive oil could be out of the limits in a few months' time if FAEE content would be measured (Gómez-Coca et al. 2016). Therefore, in a recent study, it was proposed that strict regulations should take into account of the presence of ethanol basal levels in the oils which were found quite high in many cultivars. As a result, it becomes an important point to differentiate physiologically formed and fermentative ethanol contents in the olive fruits (García-Vico et al. 2018). In the light of these findings, the latest EU regulation about FAEE might need an update for including the initial ethanol content. In some cases, deodorized low quality (especially rancid) oils might not have very high FAEE content and if this oil is used as an adulterant current critical levels in legislation might not be enough to detect the adulteration. Hence, it could be concluded that FAEEs are suitable adulteration markers for the oils possessing significantly high content of FAEEs compared to virgin olive oils (Conte et al. 2020). Another important factor making FAEEs insufficient in detection of adulteration is masking effect of the certain processing conditions of the soft deodorization on the oils. It was observed that deodorization at 100℃ for 60 min is the optimum condition to remove volatiles responsible for sensory defects without significant losses of quality parameters such as total phenols, PPPs and FAEEs and the critical limits of regulations are still met using these parameters (Aparicio-Ruiz, Romero, et al. 2017). Therefore, monitoring FAEEs could only be useful in detecting highly degraded oils with initial concentration already higher than the threshold values of the regulations prior to process. Otherwise, mixture of soft deodorized olive oil and extra virgin olive oil could not be detected up to 50% with current standard methods (Aparicio-Ruiz, Romero, et al. 2017).

Another attention-grabbing point is the relationship between FAEEs content and sensory defects. First comprehensive effort to reveal a relationship between the FAAEs concentration of olive oils and their sensory classification was conducted by Gómez-Coca, Moreda, and Pérez-Camino (2012) and a connection between the FAAEs and fermentative organoleptic defects was determined (Gómez-Coca, Moreda, and Pérez-Camino 2012). In another study, FAEEs are also correlated with the fermentation processes responsible for organoleptic defects and it was concluded that their relations

could be used to determine olive oils that have undergone mild refining processes (Di Serio et al. 2017). In a recent study, correlation between sensory characteristics and various chemical parameters of Brazilian olive oils were investigated (Zago et al. 2019). A positive correlation was obtained between concentration of FAEE and vinegary defect. Therefore, FAEE amount could be useful not only for authentication but also for quality control of olive oils in terms of sensory characteristics. Other examples of recent applications of alkyl esters in olive oil authentication are listed in Table 2.2.

Table 2.2. Examples of studies from the literature for the determination of olive oil authenticity and quality using fatty acid alkyl esters (FAAEs).

Aim	Main Findings	Reference
Checking authenticity	FAAEs could be used to detect adulteration of olive oil with mild deodorized low-quality olive oil up to 30%.	Jabeur et al. (2015)
Shelf life prediction	FAEEs could be used to predict shelf-life of olive oil along with main chemical, physicochemical, and sensory characteristics under standard shelf-life conditions.	Di Serio et al. (2018)
Detection of adulteration	FAEEs were found successful in the detection of extra virgin olive oil fraud with 2% refined pomace olive oils.	Jabeur et al. (2017)
Effect of processing parameters	FAAEs increase from the decanter to the vertical centrifuge during production. Use of water decreases the formation of FAEE and FAME.	Alcalá et al. (2017)
Shelf life determination	After 6 months of storage, FAEEs content of extra virgin olive oil could be off limit although the other quality related parameters (peroxide index, K 232, K 270 and ΔK) were not.	Grompone et al. (2016)
Evaluation of quality	Good correlation was established between FAAEs and free acidity. Moreover, FAAEs as well as many other parameters as free acidity, waxes, stigmastadienes, extinction coefficients, and peroxide values were all negatively correlated to the sensorial characteristics.	Di Loreto et al. (2014)
Characterization in terms of PDO	Olive oils from Sicilian region were below the critical limit of FAAEs except some aged ones.	Costa et al. (2017)
Characterization in terms of variety and growing area	Both variety and growing environment of olives have significant effect on qualitative indexes such as free fatty acid, peroxide value, specific extinction coefficient values, waxes, fatty acids, FAAEs content and sterols of olive oils.	Piscopo et al. (2016)

2.1.1.2. Diacylglycerols (DAGs) and Derivatives

Diacylglycerols (DAGs) have been considered as another quality parameter especially by some relatively new olive growing areas, USA (particularly California state) and Australia. DAGs are found in virgin olive oil in minor amounts ranging from 1% to 3% and they are generally produced before or during olive oil extraction process. 1,2-DAGs are the intermediate products that form as a result of the incomplete biosynthesis of triacylglycerols (TAGs) while 1,3-DAGs are the products of enzymatic or chemical hydrolysis of TAGs (Pérez-Camino, Moreda, and Cert 2001). Health status of the olive fruits is one of the major factors determining the amount, type and ratio (1,2- to 1,3-) of DAGs. Olive oils extracted from poor quality olive fruits showed a significant raise of 1,3-DAGs while the product obtained from healthy olive fruits contains almost exclusively 1,2-DAGs (Garcia, Martins, and Cabrita 2013). In addition, storage conditions and time as well as extraction process (high temperature and water dilution during extraction), presence of macromolecules, and metals had also major effect on DAG ratio of olive oils (Circi et al. 2018; Vlahov, Giuliani, and Del Re 2010). During storage, the concentration of 1,2-DAGs gradually decreased by isomerization resulting in the formation of more stable 1,3-DAGs. Thus, ratio of these isomeric forms was found to be reliable markers for the freshness (age) and the quality of virgin olive oils (Bajoub et al. 2018). According to both Californian and Australian standards, olive oils are graded as extra virgin if it contains 1,2 DAGs ≥35% in terms of C32+C34+C36 and this value actually is the ratio between 1,2-DAGs and total DAGs content known as *D* value. The methods used in the determination of DAGs are based on gas chromatography (GC), high performance size exclusion chromatography and high-performance liquid chromatography (HPLC) all of which requires tedious derivatization steps before injection of the sample (Vlahov, Giuliani, and Del Re 2010). GC-FID has been used most commonly to determine fractionated isomeric DAGs in olive oil (Gertz and Fiebig 2006a). GC-EI-MS is another technique applied to characterize and quantify DAGs without any requirement for a standard which was reported as a problem for the previous method (Zhu et al. 2013). Thin layer chromatography (TLC) coupled with visible (Vis) spectrophotometry was also used as a simple method to quantify DAGs in edible oils (Li et al. 2018). As a relatively new approach some spectroscopic methods were also used in

DAGs determination. Recently, DAG content of olive oils were predicted from Fourier transform near infrared (FT-NIR) spectroscopic data (Azizian et al. 2018; Willenberg, Matthäus, and Gertz 2019). In addition, a very recent study investigated the use of FTIR and UV-vis spectroscopic methods jointly and separately to estimate DAGs composition of olive oils (Uncu, Ozen, and Tokatli 2019). Furthermore, NMR spectroscopy in the forms of ^{1}H, ^{13}C and ^{1}P NMR has been preferred in determination of acylglycerols of olive oil because of its ease of sample handling and rich data generation (several metabolites in single spectrum) as an alternative to wet chemical methods (Dais and Spyros 2007; Hatzakis et al. 2011; Vlahov, Giuliani, and Del Re 2010).

Three isomeric classes of DAGs (1,2-, 2,3-, and 1,3-) of extra virgin olive oils stored in different temperatures of 15 ℃ and 30 ℃ and time up to 12 months were evaluated in order to observe the effect of these parameters on DAGs content in a study (Cossignani et al. 2007). The results indicated that significant differences existed in the amount of different DAG classes as well as the ratios between the classes. The samples inspected just after extraction possessed the highest contents in terms of percentage for 1,2-DAGs and the lowest for 1,3- and 2,3-DAGs. On the other hand, the samples kept at 30 ℃ had the highest content of 1,3 DAGs due to isomerization reaction favored mainly by temperature. Therefore, it was concluded that storage temperature was the most important factor on the DAGs content, and their isomerization provided information regarding the storage conditions as well as the preservation status of olive oils. In addition to the aforementioned parameters, other possible storage factors for the isomerization of DAGs in fresh olive oils were examined for 24 months (Caponio et al. 2013). The results showed that storage time was the significant factor in increasing amounts of 1,3-DAGs due to isomerization causing higher 1,3/1,2 ratio for oils. Besides, it was found that degree of isomerization was also affected by the initial hydrolysis level of the olive oil. However, storage conditions such as the bottle glass color, the light, and the air had no effect on isomerization of DAGs except the speed of the reaction. Therefore, it was confirmed that the DAGs ratio could be used as a freshness index for extra virgin olive oil since concentrations of these compounds were not affected by either oil variety or storage conditions (glass color, light, and air) (Caponio et al. 2013). In a similar study (Ayyad et al. 2015), effects of different conditions of storage at 20°C in darkness and in light, at 4-6°C in light and at 20°C in light with argon in the headspace were observed for 14 months. The results confirmed that not only the storage time but also temperature had effects on isomerization of DAGs. Inert gas was not that efficient in the protection of olive oils from

isomerization under storage in the light. In another study (Salvo et al. 2017), ^{1}H NMR spectroscopy was also used in monitoring of olive oil aging with respect to DAG content. The olive oils were stored in the dark and at room temperature for one year. It was already known that the isomerization rate was affected by the free fatty acidity, additionally it was proven that the presence of specific macromolecules (lipases) had effect on DAG content as well (Salvo et al. 2017).

The studies mentioned so far focused on the investigation of the change in olive oil DAGs content with different parameters during storage. However, kinetic studies were also performed to correlate the age of olive oil with DAGs concentration (Dais and Spyros 2007). Kinetics of DAG formation and isomerization in virgin olive oil were formulized in terms of the *D* value and the free fatty acid values by using ^{31}P NMR spectroscopy (Spyros, Philippidis, and Dais 2004). Robust prediction models were obtained between actual and theoretical storage time up to 10-12 months (Spyros, Philippidis, and Dais 2004). In another study, a more comprehensive mathematical expression was established for the determination of shelf life of olive oils with respect to many parameters such as alkyl esters, volatiles and 1,2-DAGs etc. (Di Serio et al. 2018). In a recent study, artificial intelligence derived system as adaptive neuro-fuzzy inference predicted the oxidative stability of virgin olive oil during storage as a function of time, temperature, DAGs as well as other well studied parameters (Arabameri et al. 2019). According to this study, minor constituents including DAGs were found as the most important factors influencing the preservation status and freshness of olive oils during storage. Furthermore, it was concluded that the changes in DAGs content could be a good indicator for olive oil oxidative stability. While the direct effect of DAGs concentration on olive oil organoleptic characteristics during storage was not observed, they are essential in determination of aging. As a result of aging, degradation of various health promoting components of olive oil such as tocopherol and phenolic compounds were also observed which further decrease the nutritional and organoleptic characteristics by increasing rancidity (Dais and Spyros 2007). Therefore, it becomes an important point to know the storage history of olive oil to be sure about its actual quality. Relation between DAG concentration and storage time could also mean that these compounds can be used in detection of adulteration of fresh olive oils with old oils.

In addition to their applicability in quality determination, DAGs are used as a tool in authenticity determination of olive oils. It is known that fresh extra virgin olive oil samples do not contain high amounts of total DAGs (1–3% mainly 1,2-DAGs) compared

to lower quality olive oils such as refined olive oils (4–5% mainly 1,2-DAGs) and pomace olive oils (15-20% mainly 1,2-DAGs). Moreover, the isomerization from 1,2-DAGs to 1,3-DAGs results an immediate equilibrium state in refined olive oils (Dais and Spyros 2007). In this respect, adulteration of virgin olive oil with deodorized oils was inspected with a study in which 1,2- and 1,3-DAG isomers in olive were determined with solid phase extraction followed by GC analysis (Pérez-Camino, Moreda, and Cert 2001). The results indicated that the relationship between acidity and total DAGs were not an efficient indicator for the genuineness of olive oils. While the 1,3-/1,2-DAGs ratio was found useful in authentication of virgin olive oils as well as in determining the oil aging and evaluating the storage conditions (Pérez-Camino, Moreda, and Cert 2001). Therefore, the studies on olive oil authenticity have been focused on the ratio of DAGs as *D* value rather than total content of these compounds. However, the increase of 1,3-DAG could be also due to the long storage of olive oil. Therefore, any change in *D* value may not necessarily be a sign of adulteration (Dais and Hatzakis 2013). Aforementioned studies deal with only DAGs and their derivatives. However, NMR metabolic profiling which quantifies DAGs as well as many other parameters at the same time and NMR fingerprinting were also proposed as an efficient tool in adulteration detection of olive oil. In the literature, there are various studies which used NMR spectroscopy to identify DAGs content as well as other important authenticity parameters for the determination of olive oil adulteration as shown in detail in Table 2.3. In general, DAGs were regarded as quality parameters to grade olive oil. However, the methodological approach based on investigation of many physicochemical parameters together as in the previous examples was also valid for the classification studies of olive oil with respect PDO and variety in terms of their DAGs contents. There are several examples of the use of DAGs content in classification and/or differentiation as well as adulteration and quality determination of olive oils (Table 2.3)

Table 2.3. Examples of studies about the use of diacylglycerols in determination of authenticity and quality of olive oils.

Aim	Main Findings	Reference
Quality determination	The *D* value and total DAGs have potential to determine quality of virgin olive oils, commercial olive oils, refined olive oils, and pomace oils by using ^{31}P NMR	Fronimaki et al. (2002)
Quality determination	All types of DAGs content of olive oil could be detected with ^{19}F NMR and then could be used to monitor the quality of olive oil as well as ordinary edible oils	Zhou et al. (2015)
Adulteration detection of olive oils with hazelnut oil	^{31}P NMR was used for the quantification of minor compounds as phenolic compounds, DAGs, sterols, and free fatty acids. Detection limit was found at 5% for refined hazelnut oils in refined olive oils	Agiomyrgianaki, Petrakis, and Dais (2010)
Adulteration detection of olive oils with seed oils	The detection of olive oil adulteration with various refined seed oils was accomplished by using the combination of ^{1}H NMR and ^{31}P NMR. Adulteration could be determined as low as 5% by using *D* value of fresh olive oils.	Vigli et al. (2003)
Adulteration detection of olive oil with lampante and refined olive oil	High field ^{31}P NMR was used to detect the targeted adulterations at varying levels by determining 1,2-DAGs, 1,3-DAGs, total DAGs, *D* value, sterols and acidity. 5% was the limit of detection for both adulterant type (refined and lampante)	Fragaki et al. (2005)
Adulteration detection of olive oil with refined olive oil and seed oil	^{19}F NMR and ^{1}H NMR were compared to detect lower grade oil in olive oil with respect to DAGs content and ^{19}F NMR was suitable for detection of refined olive oil while ^{1}H NMR was suitable for seed oil for the same type of application	Jiang et al. (2018)
Cultivar characterization	Geographical classification of Turkish and Slovenian extra virgin olive oils by ^{1}H NMR spectra was performed in terms of aldehydes, phenolic compounds, terpenes and DAGs as major discriminants	Özdemir, Dağ, Makuc, et al. (2018)
Geographical characterization	Fatty acids, phenolics, DAGs, total free sterols, free acidity, and iodine number were used to determine geographical identity of olive oil samples up with 87% success rate by means of ^{1}H and ^{31}P NMR spectroscopy	Petrakis et al. (2008)
Effect of storage conditions	1,2-DAGs were found very promising to control overall olive oil quality and freshness as well as easily indicate any problems during the storage of the olive oil	Guillaume, Gertz, and Ravetti (2014)

More recently, authentication studies have been investigating monochloropropanediol esters (MCPDE) as (2- and 3-MCPD) and glycidyl esters (GEs) presence in olive oils as well as in other vegetable oils (Kamikata et al. 2019; Yan et al. 2018). MCPDEs and GEs are the minor compounds derived from DAGs and MAGs, respectively through refining processes (Yan et al. 2018). These compounds are formed during the deodorization step of the refining process, and they are also known as heat-induced contaminants. They could be used as an indicator of extra virgin olive oil adulterated with refined oils since these compounds were not expected to be present in the extra virgin olive oil produced without any chemical treatment from healthy olive fruits (Kamikata et al. 2019). Besides temperature, pressure, water activity and other processing parameters also speed up the formation of 3-MCPD esters (Weißhaar 2008; Yan et al. 2018). In a recent study, it was found that these processing derived contaminants could be used to detect lower grade oils in olive oil in varying limits of detection as 2% when using 3-MCPD esters, 5% for 2-MCPD esters, and 13–14% for GEs (Yan et al. 2018). Especially, quantification of MCPDEs were found to be promising with lower limit of detection compared to GEs. In another study, potential of these compounds as an adulteration detection tool was also emphasized (Kamikata et al. 2019). Determination of these compounds are important not only for adulteration studies but also for the health concerns. It was reported that after consumption of highly contaminated foods with these derivatives gastrointestinal tract can easily convert these compounds to their free forms which are known to have toxicological effects on human (Nguyen and Fromberg 2020).

2.1.1.3. Color pigments and derivatives

The color of a virgin olive oil is attributed to the lipophilic chlorophyll and carotenoid pigments present in the olive fruit (Montealegre, Alegre, and García-Ruiz 2010). Green olives having high chlorophyll content give greenish color to the oils whereas mature olives yield yellowish oils due to their higher carotenoid content. As a result, combination as well as proportions of these pigments determine the ultimate color of the olive oils (Lazzerini, Cifelli, and Domenici 2016). Olive oils contain comparably

rich variety of carotenoids (*β*-carotene, lutein, violaxanthin, neoxanthin and other xanthophylls) and chlorophyll derivatives (chlorophyll *a* and *b*, pheophytin *a* and *b*, and other minor derivatives) (Lazzerini and Domenici 2017). The level of these pigments in olive oil could go up to an almost 100 ppm. The major pigments were reported as pheophytin *a* (up to 25 ppm), followed by *β*-carotene (up to 15 ppm) and lutein (up to 10 ppm) (Lazzerini, Cifelli, and Domenici 2016); however, amounts may differ depending on various factors. The main factors affecting the concentration of each pigment found in olive oils are highly correlated with the physiochemical characteristics of olive fruits and they rely on botanical as well as geographical origin, environmental conditions (climate and/or irrigation), and also extraction process (mainly malaxation). In addition, the storage conditions of olive oil are also important factors in pigment type and concentration (Gandul-Rojas, Roca, and Gallardo-Guerrero 2016; Lazzerini, Cifelli, and Domenici 2017; Lazzerini and Domenici 2017; Lazzerini, Cifelli, and Domenici 2016).

In the literature, the pigments have been identified mostly by chromatographic techniques and most successfully by HPLC coupled with diode array (DAD), UV-Vis as well as other types of detectors (Lazzerini, Cifelli, and Domenici 2016; Mínguez-Mosquera, Gandul-Rojas, and Gallardo-Guerrero 1992; Seppanen, Rahmani, and Csallany 2003). In addition, total pigment contents of olive oils have been evaluated in terms of chlorophylls at 470 nm and carotenoids at 670 nm after dilution with proper solvent by UV-vis spectrophotometer (Cerretani et al. 2008; Mínguez-Mosquera et al. 1991; Reboredo-Rodríguez et al. 2016).

In the recent years, other spectroscopic techniques are also becoming alternatives to the HPLC, and UV-vis spectroscopic methods used in quantification of individual (Domenici et al. 2014) and total pigments of olive oil (Cayuela et al. 2014), respectively. Direct analysis of olive oils with UV-Vis-NIR spectroscopy was found promising compared to timely and waste producing reference analysis of total chlorophylls and carotenoids (Cayuela et al. 2014). Absorption spectra in the near UV-vis region were mathematically treated by Ayuso, Haro, and Escolar (2004) to reveal its potential uses in color characterization. Then, suitability of near-UV-vis region for the determination of major pigments of olive oils as two carotenoids (lutein and *β*-carotene) and two chlorophylls (pheophytin *a* and *b*) was proposed in another study (Domenici et al. 2014). This finding was also confirmed with an investigation in which pigment contents of Mediterranean olive oils obtained from UV-vis spectroscopy and HPLC-DAD measurements were compared with similar success (Lazzerini, Cifelli, and Domenici

2017). Moreover, a very recent study (Borello and Domenici 2019) compared two different approaches for determining olive oil pigments using the near UV-Vis spectroscopy. First method was the standard method (Mínguez-Mosquera et al. 1991) based on absorption spectra at single wavelengths (470 and 670 nm) while mathematical deconvolution of the absorption spectra developed in a previous study (Domenici et al. 2014) was the other approach used in the same type of application. The results indicated that overall approach used in standard method was not as effective as newly proposed method in determination of total carotenoids' and chlorophylls' derivatives in olive oils due to the fact that standard method underestimates the contents of both carotenoids and the chlorophyll derivatives compared to whole spectrum (Borello and Domenici 2019). In another study, use of UV-vis spectroscopy in the whole range of 200-800 nm was found promising in prediction of detailed pigment profile of olive oils compared to FTIR spectroscopy since pigment profile is highly correlated with UV-vis absorption profile (Uncu, Ozen, and Tokatli 2019). Fluorescence spectroscopy was also used in determination of major pigments (chlorophylls *a* and *b* and pheophytins *a* and *b*) of olive oils (Galeano Díaz et al. 2003). The recent attempt has been exploiting ultra-fast high-performance liquid chromatography with fluorescence excitation–emission detection in quantification of these pigments directly without previous sample treatment (Lozano et al. 2013).

Measurement of some pigment compounds has been proposed as a way of determining the quality and adulteration of olive oils (Tena et al. 2015). They are regarded as quality tools due to their relationship with freshness, nutritional and antioxidant properties of olive oils (Lazzerini, Cifelli, and Domenici 2017). Natural color pigments have also been used in authentication of olive oils (Lazzerini, Cifelli, and Domenici 2016). According to one of the studies using chlorophyll and carotenoid pigments of virgin olive oils as authenticity and quality index, total chlorophylls to total carotenoids ratio should be around 1 and also the ratio of minor carotenoids to lutein should be around 0.5 to indicate the authenticity of olive oils (Gandul-Rojas, Cepero, and Mínguez-Mosquera 2000). Moreover, it was concluded that these thresholds were valid for olive oils in general regardless of fruit variety. In addition, certain pigments such as the percentages of lutein, violaxanthin, and total pigment contents could be used as discriminatory tools for monovarietal virgin olive oils (Gandul-Rojas, Cepero, and Mínguez-Mosquera 2000). Some pigment fractions such as chlorophylls/carotenoids, minor carotenoids/lutein, and percentages of violaxanthin and lutein as well as total

pigment content were found to be stable during one year of storage irrespective of the variety and degree of ripeness of the olive fruit (Roca et al. 2003). It was determined that degradation of chlorophylls as pheophytinization started from malaxation and increased during storage (Aparicio-Ruiz, Aparicio, and García-González 2014). The chlorophylls *a* and *b* being naturally present in the olive fruit are irreversibly converted into more stable pigments (pheophytins *a* and *b*, orderly) as the central Mg^{+2} ion of the porphyrin ring is replaced by two hydrogen atoms, and further to pyropheophytins (PPPs) which are the ultimate products of degradation of chlorophyll by the removal of the carboxy-methyl group from the pheophytins (Garcia, Martins, and Cabrita 2013; Giuliani, Cerretani, and Cichelli 2011). Formation of chlorophyll *a* derivative (pheophytin *a* and pyropheophytin *a* (PPP *a*)) in small amounts were identified as an indication of oil storage (Roca et al. 2003). This finding was also confirmed in another study in which increasing amounts of PPP *a* as a new compound was observed during the storage (Gallardo-Guerrero et al. 2005) whereas none or trace amounts existed in fresh olive oils (Anniva et al. 2006). It was also indicated that temperature was a significant factor favoring the formation of PPPs. Thus, the content and proportion of PPP *a* in terms of ratio between pheophytin *a* (the precursor pigment) to PPP *a* could indicate the storage conditions of the olive oils (Gallardo-Guerrero et al. 2005). The effect of thermal abuse and lengthy storage on PPP formation was also determined in a different study (Anniva et al. 2006). Thermal degradation kinetics of carotenoids as well as chlorophylls were analyzed in detail in several studies (Aparicio-Ruiz and Gandul-Rojas 2012; Aparicio-Ruiz, Mínguez-Mosquera, and Gandul-Rojas 2010; 2011). Decoloration kinetics of chlorophylls and carotenoids in virgin olive oil triggered by autoxidation were examined under varying time and temperature. The results indicated that chlorophylls were more resistant to heat treatment due to requirement of higher activation energy compared to carotenoids. Additionally, it was concluded that obtained kinetic models could be used to construct a mathematical model to predict the decoloration of chlorophyll and carotenoids pigments in olive oil in terms of time and temperature (Aparicio-Ruiz and Gandul-Rojas 2014). In addition, chemical changes in thermoxidized virgin olive oil with respect to various parameters including pigments were monitored by fluorescence spectroscopy (Tena, Aparicio, and García-González 2012). Photooxidation reaction of pigments especially chlorophyll was followed effectively through UV-visible spectroscopy in combination with artificial neutral networks (Torrecilla et al. 2015). In another study, effect of light exposure on functional compounds of olive oil such as vitamin E and chlorophyll was

evaluated successfully by fluorescence spectroscopy (Díaz et al. 2019). These studies were based on investigating the effects of various storage conditions and time on the quality of olive oils similar to a study of Guillaume, Gertz, and Ravetti (2014). Effects of different factors such as environment, cultivar, storage conditions as well as time on several physico-chemical parameters including PPP were determined. The results showed that PPP *a* and 1,2-DAGs were good indicators for overall olive oil quality and freshness as well as storage history (Guillaume, Gertz, and Ravetti 2014). Recently, shelf-life prediction was also investigated by using induction time, 1,2-DAGs, PPPs, and free fatty acids of olive oils (Guillaume and Ravetti 2016).

The method for determination of the degradation products of the chlorophyll *a* (pheophytin *a*, *a'* and PPP) in olive oil was officially described by the German Society for Fat Science (Gertz and Fiebig 2006b). The method was based on HPLC analysis with UV detector measurement after solid phase extraction of the olive oil samples and it was then adopted by the International Standards Organization (International Organization for Standardization (ISO) 2009b) as a quality measurement method (Li, Woodman, and Wang 2015). PPPs content, ultimate degradation product of chlorophyll *a*, was calculated as ratio of PPP *a* to PPP *a* + pheophytin *a* + *a'* in terms of percentage with a limit up to 17% to grade an olive oil as extra virgin in official regulations. After official recognition of the PPPs content by some official bodies (Table 2.1), rapid determination of pigment composition become more important. An alternative method based on HPLC analysis with fluorescence detection which is comparably less in cost and time was proposed for the same purposes (Li, Woodman, and Wang 2015). In addition, amount of PPP *a* formed in olive oil during storage was tried to be predicted with promising results using a mathematical expression (Aparicio-Ruiz, Roca, and Gandul-Rojas 2012). Prediction of extra virgin olive oil freshness correlated with PPPs content during storage was successfully accomplished using fluorescence spectroscopy (Aparicio-Ruiz, Tena, et al. 2017). As a result, effectiveness of PPPs in shelf-life determination was indicated. In addition, PPPs were recently proposed as adulteration determination criteria along with FAAEs, volatiles, and phenols for olive oils passing through deodorization process (Aparicio-Ruiz, Romero, et al. 2017).

Authentication of olive oils with respect to variety and geographical origin was also investigated in olive oil studies. Pigment content was useful in this type of application because genetic as well as environmental conditions have significant effects on pigment content (Montealegre, Alegre, and García-Ruiz 2010). In addition, it was found that

pigments could be correlated to other factors such as ripeness stage, geographic origin and cultivars (Lazzerini, Cifelli, and Domenici 2017). Varietal characterization and differentiation of olive oil was performed by determining the content of some chlorophyll and carotenoid compounds (Cichelli and Pertesana 2004). Discrimination based on harvest year was also accomplished by using main pigments of Italian olive oils (Lazzerini and Domenici 2017). Furthermore, instead of using only the pigment profile, there is a trend of combining total chlorophyll and carotenoid contents with other chemical parameters for geographical and/or varietal classification (Karabagias et al. 2013; Karabagias et al. 2019; Taamalli et al. 2010). It could be very hard to characterize an olive oil with a unique compositional marker by knowing that compositions of these markers are easily affected by the environmental conditions, the fruit ripening, and the extraction technology (Montealegre, Alegre, and García-Ruiz 2010). Therefore, bringing together different markers to obtain the discriminatory information as much as possible by using chemometric tools could provide better results (Montealegre, Alegre, and García-Ruiz 2010).

Pigment content of olive oil could also be susceptible to the alterations and frauds (Lazzerini, Cifelli, and Domenici 2016). Illegal addition of artificial pigments to olive oil to prevent any color loss due to refining is still a common adulteration method and European regulations do not allow the addition of colorants to any oils and/or fats from animal or vegetable origin (Roca et al. 2010). Therefore, if any artificial color is detected this situation is considered an adulteration. As a greenish colorant, copper complexes of chlorophyll known as E-141i, are obtained by solvent extraction from plant sources. The additive E-141i is produced by the addition of Cu^{+2} salts to the pigments in which the inner metal ion Mg^{+2} is replaced with the more stable Cu^{+2} causing the formation of copper–chlorophyll derivatives and it has been mostly used in the fraud of olive oils due to its stable color characteristics during the processing and storage (Gandul-Rojas, Roca, and Gallardo-Guerrero 2016; Lazzerini, Cifelli, and Domenici 2016; Roca et al. 2010). The adulteration studies about color pigments in olive oils showed that Cu–pyropheophytin *a* was the major component among copper–chlorophyll derivatives (Gandul-Rojas, Roca, and Gallardo-Guerrero 2016). Naturally, almost none of these derivatives exist in olive oils; therefore, detection of the presence of any of these compounds reveals the adulteration of the oil (Gandul-Rojas, Roca, and Gallardo-Guerrero 2016). Several techniques are available to determine Cu-chlorophyll derivatives in olive oil and the majority of these methods are based on HPLC analysis with different

detector systems (Fang et al. 2015; Roca et al. 2010). Capillary electrophoresis was also used for the same type of application (Del Giovine and Fabietti 2005). Recently, some alternative techniques such as Raman spectroscopy (Lian et al. 2015) and other spectrophotometric measurements (Wang, Hou, and Hsieh 2018) were also developed to determine these compounds in a fast way without harming the environment. Other examples of recent application of pigments usage in olive oil authenticity and/or quality determination are presented in Table 2.4.

Table 2.4. Recent studies in quality and authenticity determination of olive oils by using pigment content

Aim	Main Findings	Reference
Photo and thermal-oxidation determination	Excitation-emission matrices of olive oil samples correlated to polyphenols, and chlorophyll and derivatives. All could discriminate non-irradiated and irradiated as well as non-heated and heated samples	Manzano, Muñoz de la Peña, and Merás (2019)
Differentiation of virgin olive oils from a specific mill	Concentration of some key isoprenoids and color compounds (*β*- carotene, lutein, and pheophytin *a*) could achieve differentiation (88%) between olive oils from a specific mill and other mills	Mapelli-Brahm et al. (2018)
Quantification of binary and ternary mixtures of monovarietal olive oils	The artificial neural networks applied to visible spectroscopic data could be used to determine varietal quantifications based on the pigment profile of monovarietal extra virgin olive oils at 10% and 2.8% for the linear and non-linear models, respectively	Aroca-Santos et al. (2016)
Detection of possible fraud markers	Ultra-high-performance liquid chromatography tandem mass spectrometry coupled with two types of atmospheric pressure ionizations (chemical and photoionization) were found efficient in determining natural color pigments (carotenoids, chlorophylls and chlorophyll derivatives) as well as artificial ones (E141i) in olive oils. These methods were applicable in pigment profile identification as well as the detection of possible exogenous adulterants	Arrizabalaga-Larrañaga et al. (2019)
Cultivar differentiation	Five different Greek olive oil cultivars were successfully characterized and classified based on acidity, total chlorophylls and carotenoids, myristic, margaric, stearic, arachidic, and eicosenoic acids at a rate of 91.9% and 81.1% by using original and cross-validation methods, respectively	Karabagias et al. (2019)
Geographical differentiation	Geographical discrimination power of several chemical parameters (total phenol content, fatty acid and phenol profile, total carotene and chlorophyll content and oxidative stability) and mid-infrared spectroscopy on olive oils was investigated. It was found that combination of chemical parameters was better than mid-IR spectroscopy in classification of monovarietal olive oil obtained from geographically close regions of Turkey	Uncu and Ozen (2016)

(cont. on next page)

Table 2.4. (cont.) Recent studies in quality and authenticity determination of olive oils by using pigment content

Aim	Main Findings	Reference
Cultivar discrimination	Monovarietal extra virgin olive oils could be classified up to 94.4% according to their variety by using Raman spectroscopy highly correlated with both carotenoid and fatty acid composition of olive oils. Also, distinct Raman spectral bands could be used for the prediction of major fatty acids as well as lutein/*β*-carotene content ratio both known as quality parameters for olive oils	Portarena et al. (2019)
Geographical discrimination	Olive oil samples from PDO production areas of coastal Italy were analyzed in terms of their isotopic composition and carotenoid content with isotope ratio mass spectrometry and resonant Raman spectroscopy, respectively. The combination of isotopic and carotenoid data yielded a promising result with correct classification of 82% of olive oil samples with respect to geographical origin	Portarena, Baldacchini, and Brugnoli (2017)
Authentication	Detailed pigment profile including major and minor color compounds as well as their derivatives were successful in authentication of olive oils with respect to harvest year and geographical origin. On the other hand, UV-visible and FTIR spectroscopic techniques were reliable alternatives for the same purposes with the higher discriminatory power of FTIR alone and in combination	Uncu, Ozen, and Tokatli (2020)

2.2. Authentication of Olive Oil with Spectroscopic Methods

Although targeted type of approaches might have high precision power regarding the determination of the specific analyte(s), they still possess some drawbacks such as long analysis time, high operation cost, and hazardous waste production. As non-targeted analysis approaches, spectroscopic techniques such as mid-infrared (mid-IR), UV-Vis, and fluorescence spectroscopy, provide speed, low cost and environmentally friendly applications for determination of authenticity, overall quality and chemical composition of olive oils.

2.2.1. Mid-Infrared Spectroscopy and Chemometry

Mid-IR spectroscopy (4000 - 400 cm^{-1} in the electromagnetic spectrum) has been widely used in the qualitative and quantitative analysis of organic compounds such as food products in order to identify specific chemical structure of a food matrix called as fingerprint. This spectroscopic technique is based on the fact that bonds of certain atomic groups (diatomic or more complex molecules) have specific mode of vibrations (e.g., stretching and/or bending) in mid-IR wavelength range which lead to qualitative representation of molecular structure at characteristic frequencies. In addition, mid-IR spectroscopy has been used to quantify target molecular groups by a correlation explained with Lambert's-Beer law ($A = \varepsilon bc$) which indicates that intensity of the absorption bands (A) are proportional to the concentration of the functional groups (c) of molecules with molar absorptivity (ε) and pathlength (b) (Guillén and Cabo 1997; Karoui, Pierna, and Dufour 2008).

At earlier periods of mid-IR spectroscopy, the technique relied on monochromatic dispersion which was difficult to process and evaluate due to problems in sample preparation and data acquisitions (Guillén and Cabo 1997; Manning 1972). However, development of sampling techniques such as diffuse reflectance (DRIFT), photoacoustic (PAS) and attenuated total reflection (ATR) as well as replacement of dispersive mid-IR

technology with Fourier transform infrared (FTIR) spectroscopy, provided a wider application area for this spectroscopic technique. FTIR spectroscopy, based on an interferometer of mostly Michelson type, initially produces a signal called an interferogram. This signal is further converted into a frequency domain by a mathematical operation named as Fourier transform, leading to an increase in accuracy and speed of spectral acquisition (Downey 1998; Karoui, Pierna, and Dufour 2008). FTIR spectroscopy possesses superior characteristics over classical dispersive mid-IR spectroscopy and some of the prevailing features are simultaneous detection of frequencies rather than collection of individual wavelengths, higher signal to noise ratio, internal wavelength calibration ability, higher beam intensity, superior wavelength resolution and accuracy simultaneously, and reduction in the scan time without any effect on the resolution (Guillén and Cabo 1997; Rodriguez-Saona and Allendorf 2011).

The new technological developments both in data production and sampling techniques resulted in an increase also in the use of FTIR spectroscopy in food applications especially in olive oil studies. FTIR data could be evaluated in the same way as in classical chromatographic data which provide information interpretable both in a qualitative and quantitative manner (Szymańska et al. 2015). However, there is a major difference between chromatographic and spectroscopic techniques since the data generated by spectroscopic measurements are considerably more complex than the chromatographic ones due to simultaneous detection of all chemical information at molecular level (Ellis et al. 2012). In order to obtain meaningful interpretation from a complex data set, chemometric methods are commonly used in data analysis. Chemometry could be defined as the science used to extract useful chemical information from multidimensional data by reducing the dimension of the data set with multivariate statistical methods (Rodriguez-Saona and Allendorf 2011). Besides the complexity of the spectroscopic data, there are other factors such as light scattering, instrumental drift, base line shifts and slope variation which make the use of chemometric methods inevitable in order to extract desirable information from the raw data (Lohumi et al. 2015). Prior to the use of multivariate statistical analysis methods, pre-treatment techniques could be applied to the data to remove all interferences and variations. These pre-treatment techniques can be divided into a) signal correction methods (first or second order derivative, multiplicative scattering correction (MSC), standard normal variate (SNV) transformation, and orthogonal signal correction (OSC)), and b) signal enhancement methods (mean centering and variance scaling) (Moros, Garrigues, and Guardia 2010).

Multivariate statistical methods are applied to mid-IR and any other spectroscopic data in food studies in different manners as follows:

- Qualitative approach includes explanatory analysis based on unsupervised chemometric methods such as principal component analysis (PCA), parallel factor analysis (PARAFAC), independent component analysis (ICA), k-means, projection pursuit (PP), and hierarchical cluster analysis (HCA) to summarize and visualize the complex data. Classification methods are also used to develop suitable models having the ability of distinguishing samples according to their class memberships, based on supervised chemometric methods such as partial least squares-discriminant analysis (PLS-DA), linear discriminant analysis (LDA), quadratic discriminant analysis (QDA), support vector machines (SVM), k-nearest neighbors (k-NN), artificial neural network (ANN), and soft-independent modeling of class analogy (SIMCA) (Szymańska et al. 2015).
- Quantitative approach covers supervised methods such as partial least squares regression (PLSR), and other regression methods (e.g., multiple linear regression (MLR), principal component regression (PCR), artificial neural networks (ANNs), and SVM regression) which predict compositional parameters and/or properties of food materials by maximizing correlation between building blocks of the models (Borràs et al. 2015; Moros, Garrigues, and Guardia 2010).

The use of these statistical techniques requires a medium to large size of data sets. Number of the samples for data analysis should be representative of the investigated case and chemometric techniques produce more accurate results with increasing number of samples. In addition, a sufficient number of samples should be used for validation of the chemometric model. Furthermore, ranges of the parameters measured become quite important and have an effect on the prediction ability of the models especially for quantitative analysis.

The overall process and strategies of FTIR usage in olive oil studies are illustrated in Figure 2.1. Basically, spectroscopic data obtained from mid-IR spectroscopy are processed in three steps; 1) pre-processing of the raw data, 2) analysis of the calibration data set with suitable multivariate methods, and 3) checking the reliability of the calibration data set with another data set obtained independently as external validation and dependently as cross-validation (leave-one-out). External validation is based on splitting the raw data set into two independent sets as training or calibration (2/3 of data) and test or validation sets (1/3 of data) while cross-validation is performed by discarding

one observation at a time from the available observation data set and running the rest of the data to obtain a suitable model (Defernez and Kemsley 1997). As a last step, the correlation coefficient (R^2) is used to reveal goodness (expected to be close to 1 for a good fit) of the corresponding models (Bauer et al. 2008) together with several other statistical parameters. These statistical parameters are related to errors of generated data (calibration and prediction) sets such as bias and standard error of performance (SEP) which is closely correlated to root mean square error of prediction (RMSEP) for independent validation set, root mean square error of calibration (RMSEC), root mean square value of cross-validation (RMSECV) and predicted residual error sum of squares (PRESS) (Esbensen et al. 2002; Muik et al. 2004).

Acquired infrared spectra consists of information which can be evaluated both in a qualitative and quantitative manner. Various information that could be obtained from the spectra is described in Figure 2.2. The next part of this chapter will focus on the application of mid-IR spectroscopy for the determination of olive oil authenticity and prediction of quality parameters which are used for the authentication of olive oil.

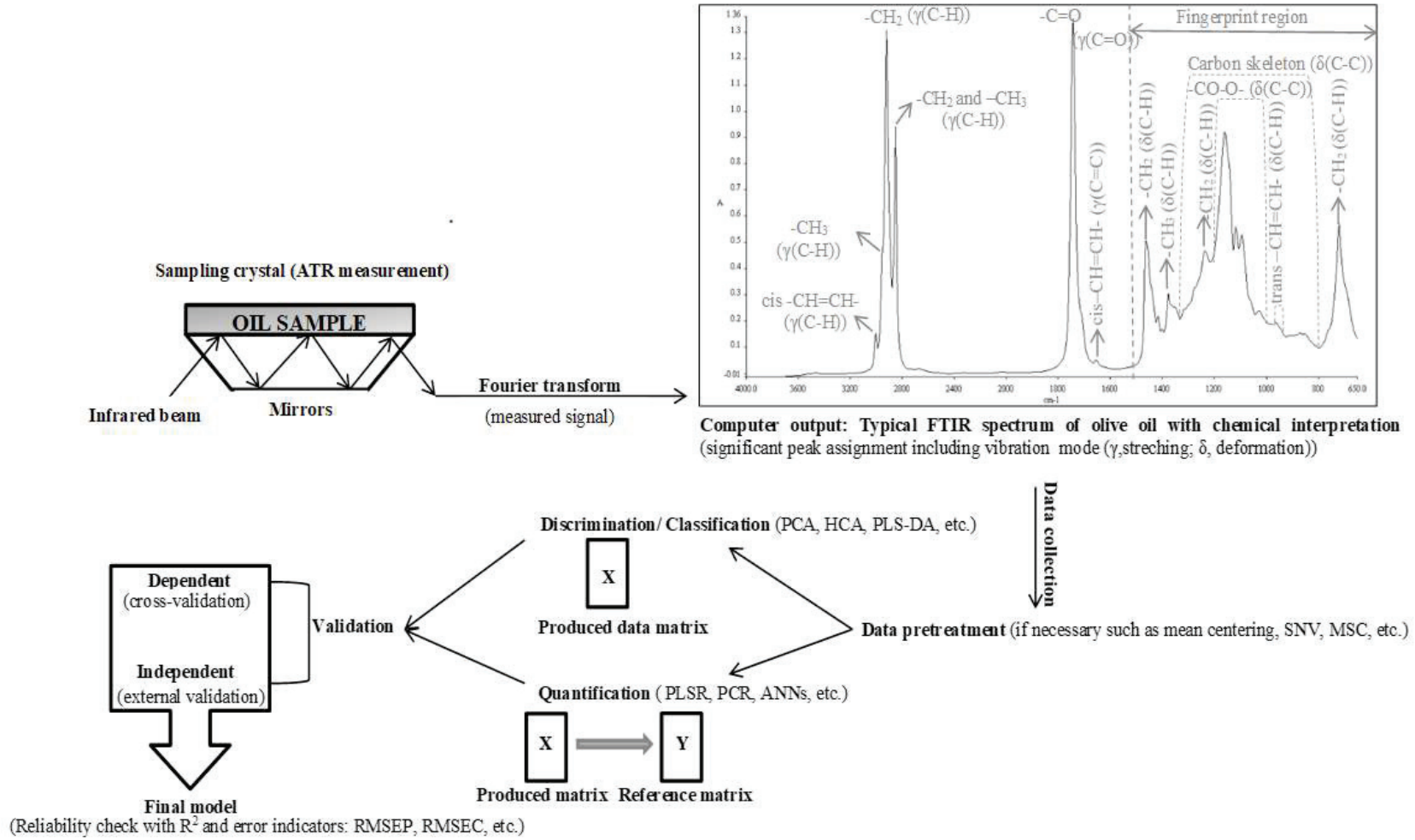

Figure 2.1 Chemometric evaluation of data obtained from mid-IR spectroscopy (peak assignments are obtained from Baeten et al. (2000)).

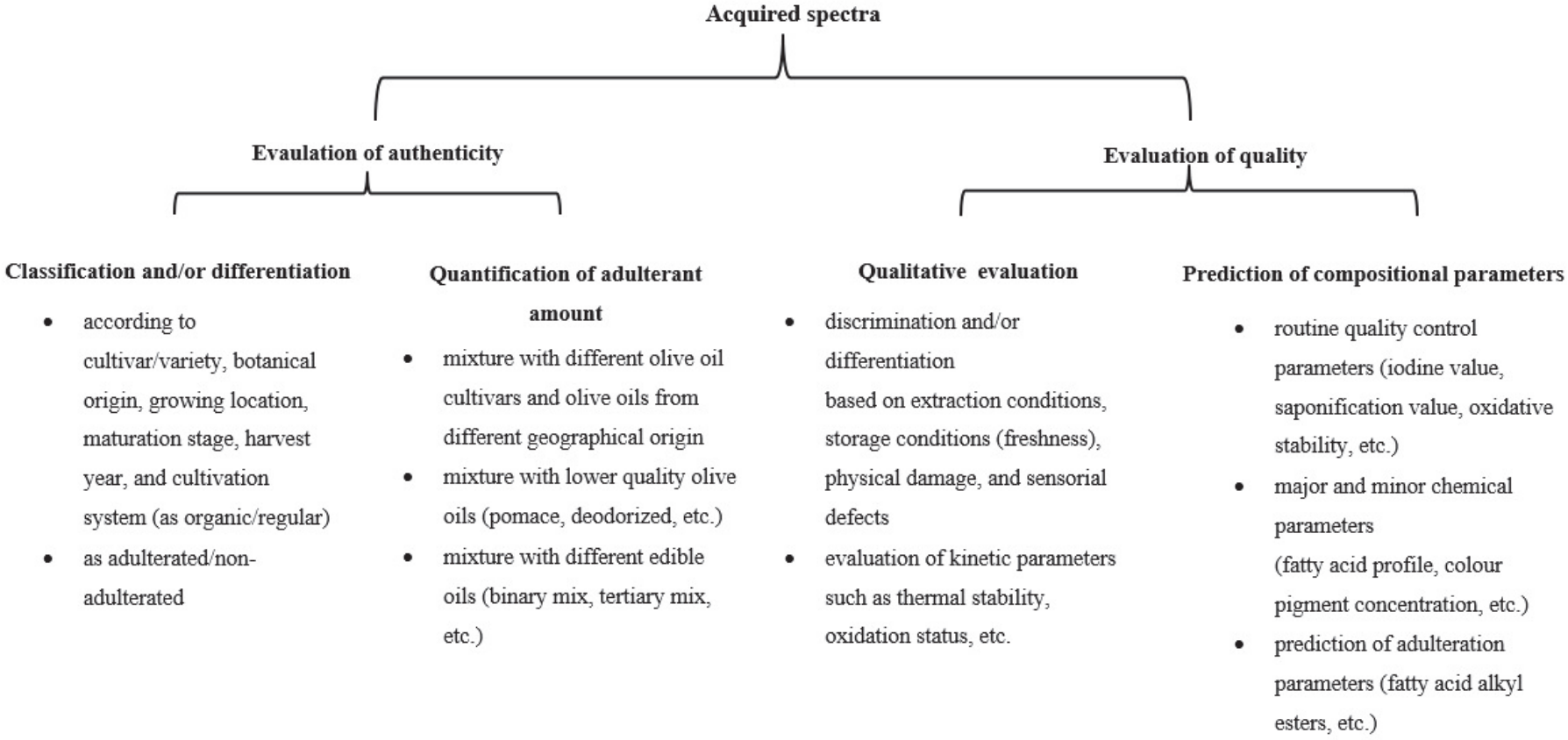

Figure 2.2 Scheme showing the applications of mid-IR spectroscopy in olive oil studies

2.2.1.1. Applications of Mid-Infrared Spectroscopy for Detection of Adulteration of Olive Oil

There are various studies in literature on the use of mid-IR spectroscopy for the detection of different categories of olive oil adulteration: detection of mixtures of olive oils from different genetic varieties (multi-varietal) and falsely labelled as "monovarietal" olive oil (Gurdeniz, Tokatli, and Ozen 2007), detection of mixtures with lower grade olive oils such as pomace, refined, and deodorized oils and sold as extra virgin olive oil (Yang and Irudayaraj 2001) and detection of mixtures with cheaper seed oils (soybean, corn, sunflower, hazelnut, etc.) and commercialized as pure olive oil (Gurdeniz and Ozen 2009; Obeidat, Khanfar, and Obeidat 2009; Lerma-García et al. 2010; Rohman and Che Man 2010; Rohman et al. 2011; Oussama et al. 2012; Rohman and Che Man 2012; Rohman, Che Man, and Yusof 2014; Sun et al. 2015; Vasconcelos et al. 2015). Mid-IR spectroscopy has been used for discriminating pure olive oils from different sources and adulterated vs. pure olive oils. Examples of such studies are given in Table 2.5. Data from this spectroscopic technique have been also used in combination with multivariate regression techniques such as partial least square (PLS) for the prediction of adulterant concentrations in olive oil and Table 2.6 provides the examples of these studies.

Table 2.5. Examples of mid-infrared spectroscopy applications in the classification and/or discrimination of olive oil

Aim	Sampling technique	Spectral range	Chemometric tools			Significant results	References
			pre-treatment and/or pre-processing of data*	Multivariate data modelling**	Validation technique		
Varietal/and or cultivar	ATR	2500-750 cm^{-1}	division and normalization of spectra	LDA	cross and external validation	88% correct classification	Concha-Herrera et al. (2009)
	ATR	4000-600 cm^{-1}	division and normalization of spectra	LDA	cross and external validation	All the samples from seven Tunisian cultivars were correctly classified	Abdallah et al., (2016)
Geographical	ATR	4000-600 cm^{-1}	MSC, SNV, derivative elaborations (Savitzky–Golay) algorithm, and wavelength optimization (Martens' Uncertainty Test)	CA, PCA, and PLS-DA	cross validation	Successful classification with wavelength optimization ($R^2 \geq 0.986$ and RMSEP≤ 0.049).	De Luca et al. (2011)
	ATR	1720-700 cm^{-1}	differentiation and smoothing of spectra	PCA	not available	Geographical discrimination of olive oils from some distinct regions (>96%).	Bendini et al. (2007)
	ATR	3000-700 cm^{-1}	first derivative	CART and SVM	external validation	Successful application of SVM and CART as a geographical classification tool	Caetano et al. (2007)
	ATR	4000-600 cm^{-1}	SNV, first and second derivatives, inclusion of only defined spectra (3000-2400 + 2250-700 cm^{-1})	PCA, FDA, and PLS-DA	cross and external validation	Improvement of geographical classification efficiency with inclusion of more harvest year	Hennessy, Downey, and O'Donnell (2009)
	ATR	4000-600 cm^{-1}	not available	PCA and PLS-DA	cross validation	Successful differentiation of virgin olive oils (in similar cultivar) from four Moroccan geographical areas (100%) with PLS-DA	Benlamaalam et al. (2015)

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Table 2.5. (cont.) Examples of mid-infrared spectroscopy applications in the classification and/or discrimination of olive oil

Aim	Sampling technique	Spectral range	Chemometric tools			Significant results	References
			pre-treatment and/or pre-processing of data*	Multivariate data modelling**	Validation technique		
Maturation stage	ATR	3000-500 cm^{-1}	Mean-centered and also standardized spectra with inclusion of only defined spectra (600-1800 cm^{-1} + 2750-3000 cm^{-1})	PCA, DA, and PLS	cross validation	DA showed 100% correct classification for calibration set with cross-validation (73.6%) of tolerable success according to maturation index and discrimination of different cultivars	Gouvinhas et al. (2015)
Botanical origin	Transmittance with NaCl cell windows	4000-450 cm^{-1}	extended MSC	PLS-DA, iPLS-DA, ECVA and iECVA	cross and external validation	Discrimination of butter from vegetable oils (corn, canola, sunflower, soya, olive) with all chemometric techniques.	Javidnia et al. (2013)
	ATR	4000-400 cm^{-1}	variable selection (MC-UVE, modified MC-UVE, CARS, SPA,CARS-SPA, and MC-UVE-SPA	PLS-DA	cross and external validation	MC-UVE with the highest classification rate (97.6%) for the discrimination of adulterated olive oil from peanut oil.	Li et al. (2016)

*MSC: Multiplicative Scattering Correction, SNV: Standard Normal Variate, MC-UVE: Monte Carlo Uninformative Variable Elimination, CARS: The Competitive Adaptive Reweighted Sampling Method, SPA: Successive Projection Algorithm.

**LDA: Linear Discriminant Analysis, CA: Cluster Analysis, PCA: Principal Component Analysis, PLS-DA: Partial Least Squares Discriminant Analysis, CART: Classification and Regression Trees, SVM: support vector machines, FDA: Factorial Discriminant Analysis, DA: Discriminant Analysis, iPLS-DA: Interval Partial Least Squares, ECVA: Extended Canonical Variates Analysis, iECVA: Interval Extended Canonical Variates Analysis.

Table 2.6. Examples of mid-infrared spectroscopy applications in quantitative determination of adulteration of olive oils

Aim	Adulterant (concentration)	Sampling technique	Spectral region	Chemometric tools			Significant results	References
				Pre-treatment and/or pre-processing of data	Multivariate data modelling*	Validation technique		
Detection of oils from different botanical origins	binary; cottonseed and rapeseed (2–20%, v/v), ternary; corn-sunflower oil (2–20%, v/v)	HATR	3620–2520 + 1875.5–675 cm^{-1}	scaling, mean-centering, wavelet compression, and orthogonal signal correction	PCA, SIMCA, PLS-DA and PLS regression	external validation	Successful prediction for all adulterants ($R^2 > 0.90$ and SEP ≈1) with detection limit of 5%. Differentiation of adulterated and non-adulterated samples at 10% detection limit regardless of the type of adulterant oil using PLS-DA models	Gurdeniz and Ozen (2009)
	binary: sunflower, corn, soybean, and hazelnut (0%, 5%, 10%, 30%, 50%, 75%, 100%, w/w)	KBr disks	4000-500 cm^{-1}	division of spectral range into 26 regions, normalization, stepwise algorithm	LDA and MLR	cross and external validation	Successful detection of adulterants with high values of R^2 (>0.90), low prediction errors (<2% for cross and <5% for external validation), and low limit of detection for all cases (<5%)	Lerma-García et al. (2010)
	binary: palm oil (1–50%, w/w)	KBr disks and ATR	1500–1000 cm^{-1}	first and second derivative	DA, PLS and PCR	cross and external validation	Perfect classification of pure and palm oil adulterated EVOO (%100) with DA and also differentiation of olive oil from other vegetable oils (sunflower, corn and canola)	Rohman and Che Man (2010)
	binary: canola oil (1–50 %, v/v)	HATR	3028–2985 + 1200–987 cm^{-1}	first and second derivative	DA, PLS and PCR	cross and external validation	Slightly better performance of PLS than PCR in prediction with a detection limit of 1 % v/v. Perfect discrimination of adulterated samples from pure EVOO with DA	Rohman, Che Man, and Yusof (2014)
	binary: peanut oil (0.5-5% v/v with 0.5% increments and 6-30% v/v with 2% increments)	ATR	17 bands & 1800-600 cm^{-1} + 3050-2750 cm^{-1}	smoothing, mean-centering and standard normal variates	PCA, LDA, PCR, and PLS	cross and external validation	Effective application of PLS to the discrete wavenumbers of 3007, 2922, 2853, 1754, 1160, and 1117 cm^{-1} compared to whole spectrum. Differentiation of adulteration level at 5% v/v or less with LDA	Vasconcelos et al. (2015)

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Table 2.6. (cont.) Examples of mid-infrared spectroscopy applications in quantitative determination of adulteration of olive oils

Aim	Adulterant (concentration)	Sampling technique	Spectral region	Chemometric tools			Significant results	References
				Pre-treatment and/or pre-processing of data	Multivariate data modelling*	Validation technique		
	binary: pure camellia, soybean, sunflower, and corn oils (1-90%, w/w).	ATR	4000-400 cm^{-1}	mean centering, absorbance normalization, absorbance normalization +mean centering and first derivative + smoothing and mean centering	PCA, locally linear embedding (LLE), supervised LLE (SLLE) in conjunction with the nearest centroid classification, and PLS	cross and external validation	Best classification and quantification using SLLE with first derivative followed by smoothing and mean centering. Detection of 1% adulterant level using PLS	Sun et al. (2015)
	binary: lard (1–50%, v/v)	Smart ATR	1500-1000 cm^{-1}	first and second derivative	PLS and DA	cross and external validation	Highest quantification level for lard in olive oil with PLS. Classification of pure vegetable oils (including olive oil) and those adulterated with lard perfectly (100%) except soybean oil with DA	Rohman et al. (2011)
	binary: soybean and sunflower (1–24%, w/w).	ATR	2453-481 cm^{-1}	mean centered and orthogonal signal corrected	PLS and PLS-DA	cross and external validation	Successful quantification of adulterants. Perfect classification of sunflower and soybean oil adulterated olive oil at 1% with PLS-DA	Oussama et al. (2012)
	binary: sunflower, soybean, sesame, corn, and olive-kernel oil (1–24%, w/w).	KBr disks	4000-400 cm^{-1}	not available	linear regression analysis	not available	Detection limits of 9% for corn oil and sesame seed oil, 6% for sunflower oil and soybean oil	Vlachos et al. (2006)
	binary: canola, hazelnut, pomace olive and high linoleic/oleic sunflower oil (5, 10, 20, 30, 40%, w/w)	ATR	4000–700 cm^{-1}	first derivative and mean centering	PLS	cross and external validation	Quantification of all types of adulteration with pre-treated IR spectra (R^2>0.9).	Maggio et al. (2010)

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Table 2.6. (cont.) Examples of mid-infrared spectroscopy applications in quantitative determination of adulteration of olive oils

Aim	Adulterant (concentration)	Sampling technique	Spectral region	Chemometric tools			Significant results	References
				Pre-treatment and/or pre-processing of data	Multivariate data modelling*	Validation technique		
	ternary: soybean oil + corn oil (0–100%, v/v)	HATR	4000-650 cm^{-1}	first and second derivative	PLS and PCR	cross and external validation	Better results with PLS compared to PCR with higher R^2 (0.999) and lower RMSEC (0.975), values	Rohman and Che Man (2011b)
	quaternary: grape seed oil + rice bran oil + walnut oil (0–100%, v/v)	HATR	1200–900 cm^{-1} + 2949–2885 cm^{-1}	mean centering, standard normal variate, first and second derivatives	PLS and PCR	cross and external validation	Successful prediction using PLS with low error values (RMSEP = 3.65%, v/v; RMSEC = 1.55%, v/v).	Rohman and Che Man (2011a)
Detection of different mono-varietal olive oils	binary: a distinct olive oil cultivar, (2-20%, v/v).	HATR	3120–2520 + 1875.5–675 cm^{-1}	not available	PCA and PLS	cross validation	Successful separation between pure and adulterated samples. Detection of mixture of monovarietal olive oils as low as 2% with high R^2, and low RMSE and SEP values by PLS	Gurdeniz Tokatli, and Ozen (2007)
Detection of low-quality olive oils	binary: pomace olive oil (0-100%)	ATR & PAS	4000-600 cm^{-1}	multiplicative scatter correction	PLS	external validation	PLS model for PAS with R^2 0.99, SEP 6.51% and for ATR with R^2 0.991, SEP 3.28%	Yang and Irudayaraj (2001)
	binary: canola, hazelnut, pomace, sunflower	ATR	3805.3-2840.9 + 1876.6-1105.1 cm^{-1} and 1876–912 cm^{-1}	first derivative and mean centering	PLS	cross and external validation	PLS model for pomace oil quantification with R^2 0.9733 and limit of quantification 0.003	Maggio et al. (2010)

* ATR: Attenuated Total Reflectance, PCA: Principal Component Analysis, SIMCA: Soft Independent Modeling of Class Analogy, PLS-DA: Partial Least Squares Discriminant Analysis, PLS regression: Partial Least Squares regression, LDA: Linear discriminant analysis, MLR: Multiple Linear Regression, DA: Discriminant Analysis, PCR: Principal Component Regression, HATR: Horizontal Attenuated Total Reflectance, PAS: Photoacoustic Spectroscopy.

Olive oil is a high value edible oil compared to many other oils from different botanical origins. Therefore, mixing of olive oil with different edible oils (corn, sunflower, canola, and etc.) is a common adulteration practice. Classification and discrimination of oils from different botanical origins is an important application area of mid-IR spectroscopy. Discrimination ability of FTIR spectroscopy for edible oils (corn, canola, sunflower, soya, and olive) and butter by different class modelling techniques such as PLS-DA, interval PLS-DA (iPLS-DA), extended canonical variates analysis (ECVA), and iECVA was investigated. It was observed that PLS-DA and iPLS-DA were not as successful as ECVA and especially iECVA which was able to discriminate oil samples perfectly (Javidnia et al. 2013). In mid-IR authentication studies, detection limit of adulterants and validity of generated statistical models determine the success of the method. Detection limit is quite important since fraudsters could make enormous gross profits on sales even with addition of small amounts of adulterants. FTIR spectroscopy was used to detect and quantify the adulteration of extra virgin olive oil mixed with different seed oils (corn, sunflower, rapeseed and cottonseed as a binary mixture, and corn–sunflower as a ternary mixture) (Gurdeniz and Ozen 2009). As a result of this study, successful prediction on adulterant level of 5% for both binary and ternary mixtures with tolerable error limits was obtained with PLS regression. There is limited number of adulteration studies in the literature dealing with ternary (Rohman and Che Man 2011b) and quaternary (Rohman and Che Man 2011a) mixtures. Moreover, detecting the presence of adulterants could be generally more important than identifying the adulterant type for the industry; therefore, the same study also investigated adulteration detection regardless of the type of adulterants and a detection limit of 10% was determined for this case (Gurdeniz and Ozen 2009). Another study (Lerma-García et al. 2010) revealed that different statistical approaches such as linear discriminant analysis (LDA) and MLR with suitable wavelength division and selection were able to successfully differentiate oils from different botanical origins such as extra virgin olive oil (EVOO), sunflower oil, corn oil, soybean oil and hazelnut oil, and also to detect binary mixtures of low cost oils with EVOO (<5%) in quantities as close to the findings of Gurdeniz and Ozen (2009). In addition, the presence of commonly used cheap adulterants such as palm oil (Rohman and Che Man 2010), canola oil (Rohman, Che Man, and Yusof 2014), peanut oil (Vasconcelos et al. 2015), camellia oil (Sun et al. 2015) and lard (Rohman et al. 2011) in olive oil was also detected and quantified by FTIR spectroscopy in recent studies. A study on the quantitative determination ability of FTIR spectroscopy on virgin coconut oil in

binary mixtures with olive oil and palm oil by PLS and PCR analyses was also performed (Rohman et al. 2010). The results indicated that frequency regions between 1,120–1,105 and 965–960 cm^{-1} were the most suitable spectral ranges to predict virgin coconut oil percentages in olive oil supported by higher R^2 and lower RMSEC values when compared to full spectral range (Rohman et al. 2010). Due to the multivariate nature of IR spectroscopy, many variables could be measured simultaneously which comprises informative variables, uninformative variables, and interferential variables. Therefore, there is a need of elimination of unnecessary variables (uninformative and interferential ones) by different variable selection methods such as Monte Carlo uninformative variable elimination (MC-UVE), the competitive adaptive reweighted sampling method (CARS), and successive projection algorithm (SPA). Application of these methods to discriminate adulterated olive oil from peanut oil (5-90% with 5% increment, w/w) samples was investigated and higher discriminating ability of modified MC-UVE than the other pre-process methods were shown (Li et al. 2016). Legal oil blends which are in demand due to economical and nutritional reasons are also available in the market. The rules for oil blends are regulated by legal authorities such as The European Union as highlighted by de la Mata et al. (2012). According to this legislation (Commission Regulation (EC) 2002a) presence of olive oil in an oil blend could be indicated with images or graphics only when it contains more than 50% (w/w) olive oil. In a related study, classification of oil blends containing olive oil higher or lower than 50% (w/w) by PLS-DA of FTIR data was possible as required by the regulation (de la Mata et al. 2012). Also, semi-quantification (only blends with olive oil content up to 50%) could be achieved relatively successfully by PLS regression.

Another category of olive oil authenticity issue is related to geographical origin and cultivar/variety of olives used for oil production. Monovarietal olive oil demand is in the rise in the market due to superior sensorial and organoleptic properties of these oils coming from certain regions and these properties are protected by PDO labelling, PGI and TSG designations of European Union. As a result, monovarietal olive oils are generally marketed at higher prices which make them targets for mixing with other oils; therefore, there are various studies in the literature aiming at discriminating oils coming from different olive varieties and also geographical origin by mid-IR spectroscopy. FTIR discriminatory power on differentiation of different Spanish olive oil varieties was studied by Concha-Herrera et al. (2009).

2.2.2. UV-Vis and Fluorescence Spectroscopies

Both UV-Vis and fluorescence spectroscopies are commonly used in olive oil authentication since they have easy to use, environmentally friendly and informative characteristics. They could also provide qualitative and quantitative information about the analyzed samples (Valli et al. 2016). However, these techniques are not thoroughly studied in olive oil authenticity and quality as opposed to vibrational spectroscopy techniques. UV-Vis spectroscopy exploits quantitative information obtained from chromophores which is relying on Beer's law while the fluorescence intensity depends directly on concentration of fluorophore molecules (Gaigalas et al. 2001). Fluorescence characteristics of each molecule are defined by two types of spectra: excitation and emission. However, not all of the absorbing molecules have fluorescent characteristics, and fluorescent emitting and non-emitting properties of molecules contribute to higher selectivity of fluorescence as opposed to absorption spectra (Sikorska, Khmelinskii, and Sikorski 2012). The same chemometric techniques explained for FTIR analysis are also valid for both spectroscopic methods.

UV-Vis spectroscopy was used in identification of possible adulterants in olive oil as well as in discrimination of olive oils with respect to their geographical and/or botanical origin (Valli et al. 2016). It was also utilized in prediction (Torrecilla et al. 2010b) and classification (Torrecilla et al. 2013) of lower quality oils in virgin olive oil. Data fusion was also applied to UV-Vis spectra to enhance its classification and discrimination power. In the literature, UV-Vis spectral data were combined with NIR spectroscopy to determine adulteration made with sunflower oil (Downey, McIntyre, and Davies 2002) and to predict basic quality and purity parameters such as free fatty acids, peroxide value, phenolic compounds, oxidative stability, total chlorophyll content and fatty acid profile (Mailer 2004). In addition, it was applied to geographical classification of olive oils (Casale et al. 2010; Downey, McIntyre, and Davies 2003). 230-270 nm band shows high absorption in the presence of conjugated dienes and trienes of unsaturated fatty acids and also 300-400 nm band correlates with polyphenol contents in UV-Vis spectra of olive oil (Mignani et al. 2012).

Fluorescence spectroscopy have some advantages due to its high sensitivity, selectivity and simplicity of use (Gaigalas et al. 2001; Sikorska, Khmelinskii, and

Sikorski 2012). Fluorescence spectra of the olive oils were also promisingly used in discrimination of quality grades, in adulteration detection, in authentication with respect to geographical origin, in quantification of fluorescent components, in monitoring thermal and photo-oxidation, as well as in assessing the quality changes during storage (Sikorska, Khmelinskii, and Sikorski 2012; Valli et al. 2016). This technique owes its capabilities to fluorescence properties of olive oil components such as vitamins (excitation:290-297 nm and emission: 320-324 nm), chlorophylls (excitation:405-458 nm and emission:648-673 nm), and phenolic compounds (excitation:270 and emission:310-457 nm) (Sikorska, Khmelinskii, and Sikorski 2012).

CHAPTER 3

MATERIALS AND METHODS

Redrafted, modified, and extended from:

Uncu, Oguz, Banu Ozen, and Figen Tokatli. 2019. “Use of FTIR and UV–Visible spectroscopy in determination of chemical characteristics of olive oils.” *Talanta* 201: 65–73. https://doi.org/10.1016/j.talanta.2019.03.116.

Uncu, Oguz, and Banu Ozen. 2019. "A comparative study of mid-infrared, UV–Visible and fluorescence spectroscopy in combination with chemometrics for the detection of adulteration of fresh olive oils with old olive oils." *Food Control* 105: 209-218. https://doi.org/10.1016/j.foodcont.2019.06.013.

Uncu, Oguz, Banu Ozen, and Figen Tokatli. 2020. “Authentication of Turkish Olive Oils by using detailed pigment profile and spectroscopic techniques.” *Journal of the Science of Food and Agriculture* 100 (5): 2153–65. https://doi.org/10.1002/jsfa.10239.

Uncu, Oguz, and Banu Ozen. 2020. “Importance of some minor compounds in olive oil authenticity and quality.” *Trends in Food Science and Technology* 100: 164–76. https://doi.org/10.1016/j.tifs.2020.04.013.

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3.1. Materials

Three different olive oil sets were used for three different investigations as explained in detail in the following parts

3.1.1. Olive Oil Samples Used in Characterization and Authentication

Ninety-one olive oil samples extracted with two-phase decanters from various parts of the Aegean Region of Turkey were collected from trusted sources for two consecutive harvest years in 2015-16 and 2016-17. The olive oil samples, belonging to 38 different places, as shown in Figure 3.1, were scattered in three main cultivation area of the Aegean Region as North (N=29 samples), South (S=36 samples) and Middle (M=26 samples). In the first harvest year, 19, 25, and 10 samples and in the second harvest year 10, 11, 16 samples were analyzed from North, South and Middle, respectively as shown in detail in Table 3.1. The North and South Aegean Regions are the designated areas for PDO labeling of olive oils on national scale, whereas the Middle Aegean Region could be a candidate for this type of labeling due to the unique characteristics of olive oils from this region. Ayvalik/Edremit is the olive variety cultivated in the northern part of the Aegean Region, whereas Memecik variety is the predominant variety of the South Aegean Region. Erkence is the unique variety of the Middle Aegean Region. All the olive oils obtained from North and South regions for two successive harvest years were graded as extra virgin according to the European regulations, whereas 70% of Middle region olive oils were in a lower grade due to varietal characteristics of Erkence olives. The maturity index of the commercial oil samples was in the range of 6–7 (purple to black). The samples were kept in the dark at refrigeration temperature (4 °C) before analysis, and the headspace of the samples was flushed with inert gas (nitrogen) before storage. Samples were analyzed shortly after they were received.

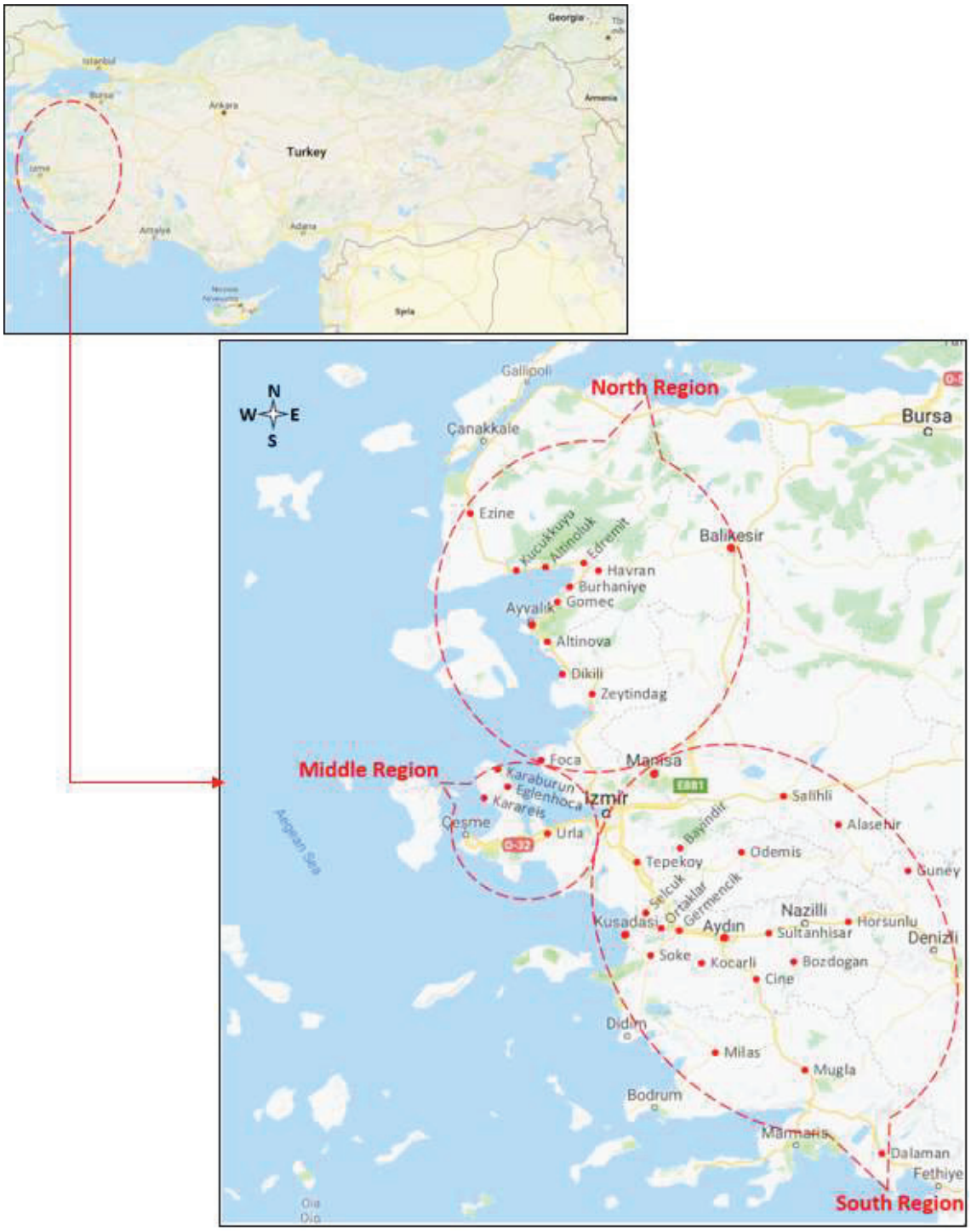


Figure 3.1 Map showing the approximate locations of olive oil samples (red spotted) obtained from various places of the Aegean Region of Turkey (Source: Google Map (2019))

Table 3.1. Code and location of olive oil samples obtained in 2015 and 2016 harvest year

Middle (M) region				North (N) region				South (S) region			
2015 Harvest year		2016 Harvest year		2015 Harvest year		2016 Harvest year		2015 Harvest year		2016 Harvest year	
Code	Location	Code	Location	Code	Location	Code	Location	Code	Location	Code	Location
M1-1**	Karareis	M2-1	Urla	N1-1	Ezine	N2-1*	Edremit	S1-1	Mugla	S2-1*	Salihli
M1-2	Eglenhoca	M2-2	Urla	N1-2	Altinoluk	N2-2*	Edremit	S1-2	Alasehir	S2-2	Alasehir
M1-3	Eglenhoca	M2-3	Urla	N1-3**	Edremit	N2-3*	Ezine	S1-3	Alasehir	S2-3*	Manisa
M1-6	Akseki	M2-4	Urla	N1-4	Edremit	N2-4*	Kucukkuyu	S1-4	Alasehir	S2-4*	Milas
M1-7	Eglenhoca	M2-5	Eglenhoca	N1-5	Burhaniye	N2-5*	Zeytindag	S1-5	Alasehir	S2-5*	Ortaklar
M1-8	Eglenhoca	M2-6	Eglenhoca	N1-6	Burhaniye	N2-6*	Kucukkuyu	S1-6**	Alasehir	S2-6*	Germencik
M1-9	Urla	M2-7	Eglenhoca	N1-7	Altinoluk	N2-7*	Ayvalik	S1-7	Mugla	S2-7*	Kocarli
M1-10	Urla	M2-8	Eglenhoca	N1-8	Edremit	N2-8*	Ayvalik	S1-8	Alasehir	S2-8*	Selcuk
M1-11	Karaburun	M2-9	Eglenhoca	N1-9	Zeytindag	N2-9*	Balikesir	S1-9	Alasehir	S2-9*	Memecik
M1-12	Karaburun	M2-10	Eglenhoca	N1-10	Altinova	N2-10*	Balikesir	S1-10	Kocarli	S2-10*	Mugla
		M2-11	Eglenhoca	N1-11	Ezine			S1-11	Selcuk	S2-11*	Guney
		M2-12	Eglenhoca	N1-12	Ayvalik			S1-12	Germencik		
		M2-13	Eglenhoca	N1-13	Gomec			S1-13	Tepekoy		
		M2-14	Eglenhoca	N1-14	Foca			S1-14	Horsunlu		
		M2-15	Urla	N1-15	Havran			S1-15	Dalaman		
		M2-16	Urla	N1-16	Altinoluk			S1-16	Ortaklar		
				N1-17	Burhaniye			S1-17	Bayindir		
				N1-18	Kucukkuyu			S1-18	Soke		
				N1-19**	Dikili			S1-19**	Aydin		
								S1-20	Bozdogan		
								S1-21	Kusadasi		
								S1-22	Odemis		
								S1-23	Selcuk		
								S1-24	Sultanhisar		
								S1-25	Cine		

* Fresh and ** old olive oil samples used in adulteration study

3.1.2. Olive Oil Samples Used in Prediction Studies

Two outlier samples (S1-17 and S1-5) were omitted from the data set. As a result, total of 89 samples from two consecutive harvest years (52 samples from 2015 and 37 samples from 2016) were used for the prediction of chemical characteristics of olive oils from spectral data.

3.1.3. Olive Oil Samples Used in Adulteration Studies

Fresh olive oil samples obtained in 2016 harvest year were analyzed immediately after the production whereas olive oils from 2015 harvest year were used as old olive oil samples after one year of storage. Olive oils were from the different parts of Aegean Region (14 different locations for fresh olive oils and 5 different locations for old olive oils) (Table 3.1). Twenty different fresh and 5 different old oils were used in the analyses and 4 fresh, and 5 old olive oils were mixed with each other in cross combinations and the rest of the fresh samples (16 samples) were independently used. As a result, 100 adulterated samples in five different concentrations from 10% to 50% level with 10% increments (20 samples for each level) were prepared with a total volume of 10 mL by mixing samples with a vortex.

3.1.4. Chemical Reagents

All reagents used in the analyses were analytical grade and obtained from Sigma-Aldrich (Germany) and Merck (Germany) unless otherwise stated.

3.2. Wet Chemical Methods

All wet chemical methods are grouped under four subtopics as in the following.

3.2.1. Determination of Free Fatty Acid Content, K Values and Fatty Acid Profile

Basic quality parameters, free fatty acid (FFA) and specific extinction coefficients (K232 and K270) and fatty acid profile of the olive oil samples were determined according to European Official Methods of Analysis (Commission Regulation (EEC) 1991).

FFA value was determined by first dissolving 20 g of olive oil sample in 150 mL diethyl ether-ethanol solution (1:1) and then titrating this solution with a standardized 0.1 mol L^{-1} solution of potassium hydroxide until a change in indicator color (phenolphthalein). Results were expressed in terms of % oleic acid.

Absorbance values of 0.25 g of the olive oil samples diluted to 25 mL with cyclohexane were measured at 232 and 270 nm with a spectrophotometer (Shimadzu UV-2450 Spectrophotometer, Japan) using the pure cyclohexane as the blank.

Fatty acid profile of the methyl esterified olive oil samples was determined by a GC with flame-ionization detector (FID) (Agilent 6890, Agilent Technologies, USA) possessing an auto-sampler (Agilent 7863) with a split/splitless inlet. As a capillary column, HP-88 with dimensions of 100 m × 0.25 mm ID × 0.2 mm (Agilent, USA) was used. Experimental conditions were as follows; 1 μL eluent was injected with a split ratio 1/50, helium was used as a carrier gas at constant 2 mL m^{-1} flow, injection and detector temperatures were set to 250 ℃ and 280 ℃, respectively. Temperature program of oven was kept at 120 ℃ for 10 min and then increased to 220 ℃ with a rate of 3 ℃ m^{-1} and maintained at the same temperature for 5 min. The sample chromatogram peaks were compared with the retention times of fatty acid methyl ester (FAME) 37 components mix standards (Supelco-CRM47885). The results including major individual fatty acids, total

saturated fatty acids (SFA), total monounsaturated fatty acids (MUFA), and total polyunsaturated fatty acids (PUFA) were given as the relative percentage of FAME. Three replicates of each measurement were recorded and then averaged. This method was used to determine the basic quality parameters of olive oil samples used in authentication and characterization as well as in prediction and adulteration studies.

3.2.2. Determination of Fatty Acid Alkyl Ester and Wax Contents

Fatty acid alkyl esters (FAAEs) as sum of ethyl (FAEEs) and methyl esters (FAMEs) are defined as a family of natural neutral lipids present in olive oils (Jabeur et al. 2015). FAME and FAEE and wax contents of olive oil samples were determined according to a method by International Olive Council (2010). This method is based on fractionation of olive oil with addition of suitable internal standards then direct analysis of the eluent by capillary gas chromatography (GC). Briefly, 15 g of silica gel suspended in n-hexane was placed into a glass column and was percolated with n-hexane to remove any impurities. Then, about 0.5 g of the olive oil sample was placed into a flask with addition of internal standards as dodecyl arachidate solution (Sigma-Aldrich-A8671) for waxes and methyl heptadecanoate solution (Sigma-Aldrich- 51633) for alkyl esters together by mixing with sudan 1 indicator dye. Then, prepared sample was transferred to the chromatography column with the aid of n-hexane. Sample was percolated further with n-hexane/ethyl ether mixture (99:1) continuously until the sudan 1 color reached to the bottom of the column. Resultant fractions were evaporated in a rotary evaporator (Heidolph Laborota-4000, Germany) at 20 °C. Fraction containing the methyl and ethyl esters and waxes was collected and diluted with 2 mL n-heptane. Diluted sample was filtered into a deep brown vial and then injected into GC.

GC analyses were conducted with Agilent 7890A GC-FID (USA). An HP-5 (30 m × 0.32 mm ID, 0.25 μm film, Agilent, USA) column was used in analyses. The analytical conditions were as follows; on column inlet temperature was set to 70 °C and injection volume was 1 μL carried with hydrogen. The oven temperature was programmed as 80 °C (1 min), 20 °C/min to 140 °C (0 min), 5 °C/min to 335 °C (20 min). Detector temperature was 350 °C. Obtained peaks were further identified with GC-MS

(Agilent 6890 N / 5973 N Network GC / MSD System, USA) at the same conditions. The results were expressed in terms of mg/kg. The target compounds were determined as sum of ethyl of C16:0, C18:0, C18:1 and C18:2 in official method (Figure 3.2). This method was used in both authentication as well as prediction studies.

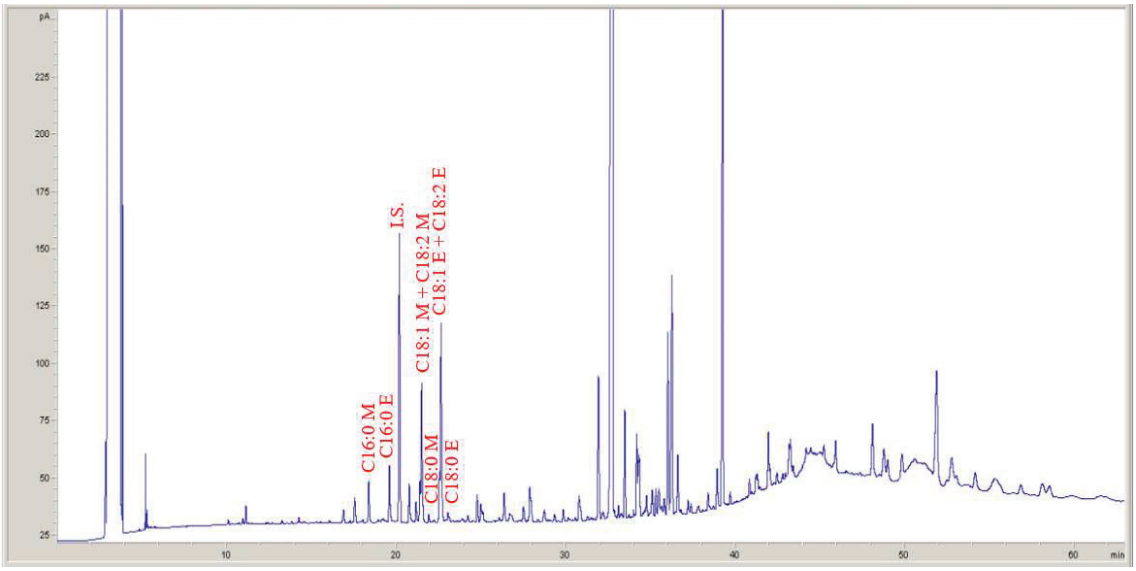


Figure 3.2. Sample GC chromatogram of alkyl esters of an olive oil according to International Olive Council method (International Olive Council (IOC) 2010)

3.2.3. Determination of Diacylglycerol Content

A miniaturized column chromatography on a silica gel column was used to separate the isomeric DAGs as 1,2- and 1,3-isomers of C32-, C34- and C36- according to International Organization for Standardization method (International Organization for Standardization (ISO) 2009a). Firstly, olive oil sample was weighted and dissolved in 1 mL toluene. Then, it was transferred on to the prepared column with wetted silica gel while purging the flask with solvent mixture (isooctane/diisopropyl ether). Column was washed with 2x3.5 mL portions of the solvent mixture. DAGs were eluted with diethyl ether two times and eluate was collected in a pointed flask. Solvent was removed from the eluate with a rotary evaporator (Heidolph Laborota-4000, Germany) at 20 °C. Then,

silylation reagent as 50 µl 1-methylimidazole (Sigma-Aldrich-M50834) in 1 ml of *N*-Methyl-*N*-(trimethyl-silyl) heptafluorobutyramide (MSHFBA) (Supelco-69484) was added to the reaction vial containing the DAGs, and mixture was sealed and allowed to react for 20 min. at room temperature. After silylation, 1 mL acetone was added into the mixture and 2 µL of the solution was used for the GC analysis. DAG isomers were identified with a GC by comparing the retention times of silylated reference standards composed of dipalmitin (Sigma-Aldrich-D2636) and distearin (Sigma-Aldrich-D9019).

GC analysis was carried with Agilent 7890A GC-FID (USA). The column was capillary GC column as Rtx-5MS (60 m × 0.25 mm ID, 0.1 µm film, Restek, USA). Injection volume was 2 µL having 1:20 split ratio carried with hydrogen. The oven temperature was programmed to 240 °C (1 min) followed by 10 °C/min to 320 °C (16 min). Both injector and detector temperatures were set to 340 °C. The results were expressed in terms of percentage. A typical DAG profile for an olive oil sample obtained with GC-FID analysis are shown in Figure 3.3. Data obtained from this analysis was used in both authentication as well as prediction studies.

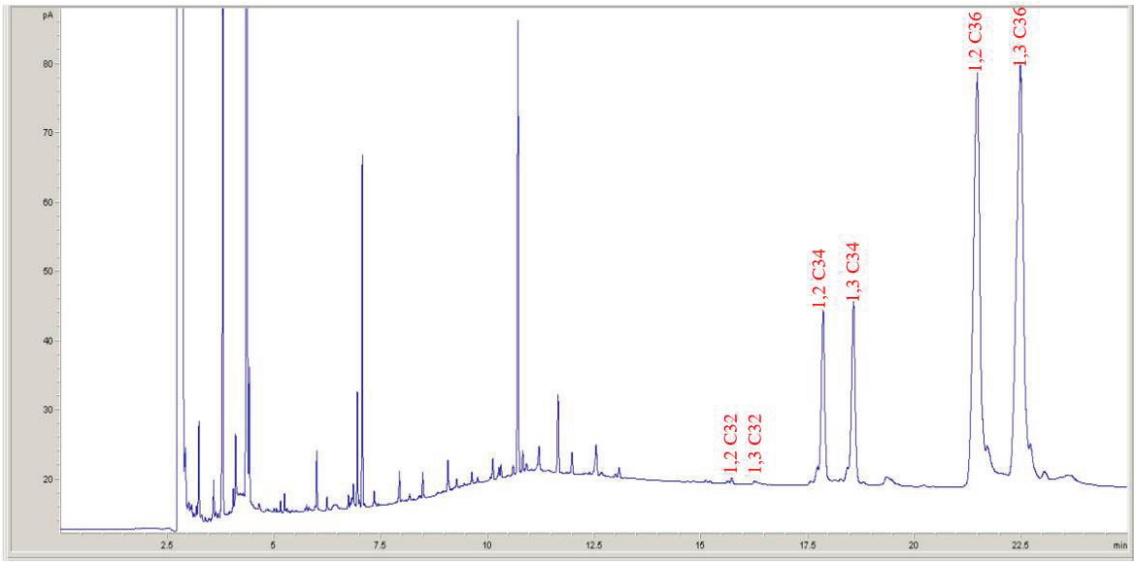


Figure 3.3. Typical GC chromatogram of an olive oil showing individual DAG peaks obtained by analysis according to International Organization for Standardization method (2009a)

3.2.4. Quantification of Individual Chlorophylls and Carotenoids

The method adapted from Mateos and García-Mesa (2006) was used to determine the pigment profiles of olive oils. Samples were extracted by the solid-phase extraction (SPE) using octadecyl (C18) disposable extraction columns (Agilent, USA). SPE column was conditioned first with methanol and then with hexane. One g of oil dissolved in 4 mL of n-hexane was injected to column and then washed with n-hexane. Firstly, hexanic phase containing *β*-carotene was collected and evaluated with UV-vis spectroscopy (Shimadzu UV-2450 Spectrophotometer, Japan). Then, the remaining pigments were eluted with 5 mL acetone. The acetone phase was taken to dryness and collected in 0.3 mL of acetone for HPLC (Agilent 1200 HPLC, USA) analysis. The sample dissolved in acetone injected into HPLC-DAD system. Separation was performed on a column packed with Waters Spherisorb S5ODS2 (25 cm × 4.6 mm ID, 5 μm particle size, Supelco, Germany) protected with a guard cartridge (3.2-4.6 mm ID, Supelco, Germany) packed with the same material as the column.

The pigments were eluted at a rate of 1 mL/min. The eluents were water + ion pair reagent as mobile phase (A) and acetone-methanol as mobile phase (B) (Mínguez-Mosquera, Gandul-Rojas, and Gallardo-Guerrero 1992). The gradient scheme for eluents indicated at Mateos and García-Mesa (Mateos and García-Mesa 2006)(2006) were as follows; initial composition as 75% (A) and 25% (B) and then (A) was decreased to 50% while (B) was increased to 50% in 10 min simultaneously and both maintained for 2.5 min. Then, (A) was further decreased to 20% in 1.5 min., (B) was increased to 80% at the same time and both maintained for 2 min. After that, (A) was lowered to 0% in 5 min while (B) was raised to 100% and both were kept constant for 14 min. After that, concentrations were turned back to the initial conditions in 5 min. The pigments were identified simultaneously at varying wavelengths by comparing the retention times of external standards. Pheophytins *a* and *b* standards were prepared with acid treatment of chlorophyll *a* and *b* solutions, respectively (Sievers and Hynninen 1977). The rest of the standards were obtained commercially for chlorophyll *a* (Sigma-Aldrich-C5753), chlorophyll *b* (Sigma-Aldrich-C5878), and lutein (Supelco-07168). 5-point calibration curves at distinct wavelengths were obtained for each standard as follows: 410 nm for pheophytin *a* and its derivative, 430 nm for chlorophyll *a* and its derivative, 435 nm for

pheophytin *b* and its derivative, 446 nm for lutein and its derivatives and other xanthophylls (as total xanthophylls), and 466 nm for chlorophyll *b* and derivative (Appendix A). The results were expressed in terms of mg/kg. A sample HPLC chromatogram of olive oil pigments were shown in Figure 3.4. This method was used determining the pigment contents of the samples in both authentication and prediction studies.

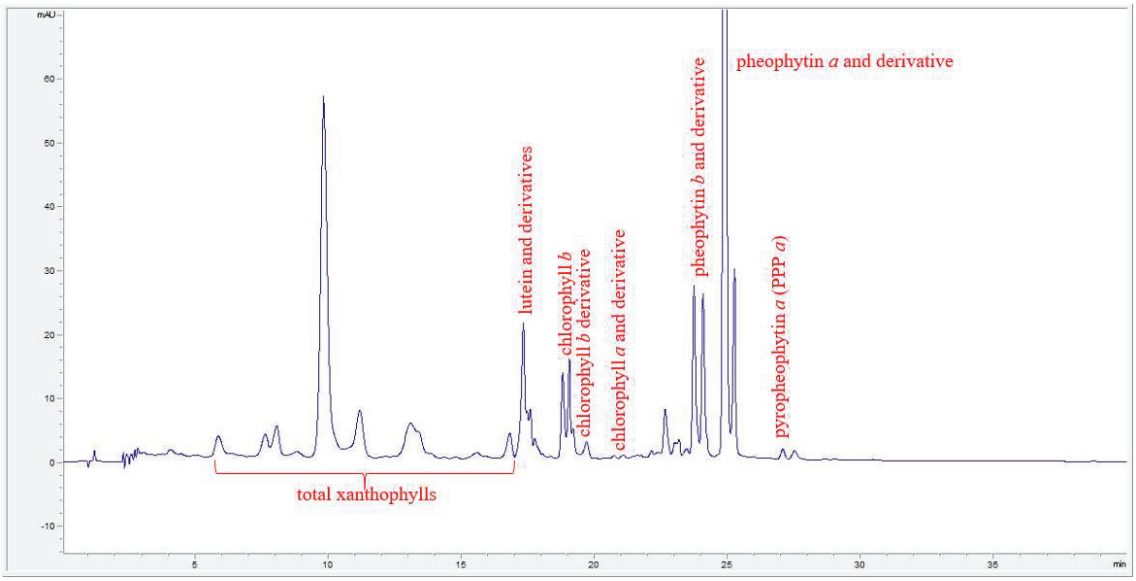


Figure 3.4. Pigment chromatogram of an olive oil sample obtained with HPLC analysis described in the literature (Mateos and García-Mesa 2006)

3.3 Spectroscopic Methods

Various spectroscopic methods were also used in the analysis of each olive oil as explained in the following sections.

3.3.1. FT-IR Analysis

Mid-infrared spectra between 4000-650 cm^{-1} of the olive oil samples were recorded by using Perkin Elmer Spectrum 100 FT-IR spectrometer (Perkin Elmer Inc., USA) equipped with a deuterated tri-glycine sulphate detector (DTGS). As a sampling technique horizontal attenuated total reflectance (HATR) accessory with ZnSe crystal was used. Scan speed, resolution, and number of scans for each spectrum were adjusted as 1 cm s^{-1}, 4 cm^{-1}, and 64 respectively. The spectrum for each sample was taken twice. After each analysis, the sampling crystal was cleaned with hexane, ethanol and deionized water. This method was applied to all olive oils.

3.3.2. UV-Vis Spectroscopy

UV-visible spectrophotometer (Shimadzu UV-2450 Spectrophotometer, Japan) was used to obtain the spectra of olive oil samples between 200-800 nm. Absorbance was measured with fast scan speed in a macro type polystyrene cuvette (12.5 x 12.5 x 45 mm) having 10 mm light path by using air as the blank. Sampling interval and slit width were set to 2.0 nm and 5.0 nm, respectively. Duplicated spectra were obtained for each olive oil sample. This method was applied to all olive oil data sets.

3.3.3. Fluorescence Spectroscopy

Fluorescence spectra of the olive oil samples were acquired with the LS-55 fluorescence spectrometer (Perkin Elmer Inc., USA) equipped with a pulsed xenon lamp. The slit width was adjusted to 5 nm for both excitation and emission. Data interval for

scan and integration time was set to 0.5 nm and 0.2 s, respectively. These parameters were selected to obtain the best resolution with optimal signal-to-noise ratio.

For each excitation wavelength (320, 330, 340 and 350 nm) fluorescence emission spectra were recorded twice for each sample between 300-800 nm simultaneously by using a quartz cell. By using trial and error method, an excitation wavelength at 350 nm was selected in the construction of both classification and prediction models.

3.4 Multivariate Statistical Analysis

In order to handle the large data clusters obtained from the spectroscopic measurements and wet chemical analysis, multivariate statistical tools were utilized in both classification and prediction studies. SIMCA 14.0 software (Umetrics, Sweden) was used for all the data analyses. Different multivariate approaches were used in each part of the study. Hence, this section was divided into three parts in order to clearly show each statistical strategy, and Table 3.2 provides a summary of overall investigation.

3.4.1. Adulteration Study

The whole spectra from FTIR (4000-650 cm^{-1}), UV-vis (200-800 nm), and fluorescence (300-800 nm) spectroscopy measurements were used in the analyses. In addition, low level data fusion was applied to FTIR and UV-vis spectroscopic data to obtain a single matrix and this combined form was also used in both classification and prediction models. Low level data fusion is a basic combination method relied on concatenating data sets obtained from different instruments into a large single matrix and could be used in generating classification or prediction models. Rows and colons of the matrix correspond to samples and signals (variables), respectively (Borràs et al. 2015).

Prior to the model development, replicated spectroscopic data were averaged and then appropriate pre-processing techniques were used to remove the undesirable instrumental and experimental variations (Engel et al. 2013). Pre-processing techniques could be divided into two main categories as signal enhancement and signal correction methods (Moros, Garrigues, and Guardia 2010). Mean-centering and unit variance scaling were applied as a signal enhancement strategy in the construction of all models. Advanced signal correction algorithms such as first derivative (FD), second derivative (SD), Savitzky-Golay (S-G), wavelet denoising techniques (WDTs), multiplicative scatter correction (MSC), and orthogonal signal correction (OSC) were used individually and in appropriate combinations (S-G:MSC, FD:S-G:MSC, and WDTs:OSC) for the development of the specific models. FD and SD of the spectroscopic data were calculated from moving quadratic sub-models with 15 data point long and the distance between each data point is set to 1 excluding the edge effects. As a wavelet function Daubechies-10 was chosen, and confidence interval was selected as 99.5%. Selection of the suitable pre-processing technique was accomplished with the trial and error method. For this purpose, different pre-processing techniques were applied and the best performing one was selected with respect to their classification and prediction efficiencies in terms of the statistical parameters provided in the next section (Engel et al. 2013).

For the classification and quantification, pre-treated data set of each spectroscopic technique was randomly divided into calibration and validation sets comprising 2/3 and 1/3 number of the data set, respectively. The calibration data set was used to generate the corresponding model. An optimal model with respect to the latent variables (LVs) was chosen by internal validation (cross validation) which was applied as leave-one-out cross validation (LOO-CV) to avoid over and/or under fitting of the model (Riedl, Esslinger, and Fauhl-Hassek 2015). The optimal number of LVs obtained from 7-fold cross validation revealed the model complexity, and the percentage of correct classification for the optimized number of LVs provided the classification accuracy (Engel et al. 2013).

In classification studies, orthogonal partial least square-discriminant analysis (OPLS-DA) was used to visualize the separation of adulterated and fresh olive oil samples by using pre-treated data. In OPLS-DA analysis, a dummy Y matrix (variable vector) consisting of class 1 and class 2 (adulterated and non-adulterated (fresh) samples, respectively) was correlated with X matrix (spectral data) (Sen and Tokatli 2016). The results of the OPLS-DA analysis are given in the form of a misclassification table. Both cross and external validation techniques were used to determine correct classification and

misclassification (known as rejection or error) rate (Riedl, Esslinger, and Fauhl-Hassek 2015). The correct classification rate (%CC) was determined when an examined oil sample from a defined olive oil class (as adulterated or non-adulterated) have a prediction value between 0.5 and 1.5; otherwise, it was considered as a misclassification (Hirri et al. 2016). In addition, other performance parameters such as number of LVs, regression coefficient for calibration (R^2_{cal}) and Q^2 (regression coefficient for cross-validation (R^2_{cv})) were determined for each classification model constructed with different spectroscopic data. These values were evaluated by automatic fitting function available in the SIMCA software.

Prediction for the quantification of the varying levels of adulteration (0–50% v/v) were conducted with PLS regression analysis. Basically, PLS regression was used to correlate spectroscopic absorbance of each adulterated and non-adulterated sample (X block) with the percentages of adulterant and non-adulterant olive oil (Y block) (Gurdeniz and Ozen 2009). The prediction ability of the generated PLS models were investigated with several performance parameters such as R^2_{cal}, R^2_{cv}, and regression coefficient for prediction (R^2_{pred}) Error values as RMSEP/C/CV were also used in the performance evaluation. R^2 values should be close to 1 while error values should be small and close to each other in order to minimize error as low as possible by sustaining balance between generated error values in terms of magnitude and to obtain a robust prediction model (Uncu and Ozen 2015). Additional parameters such as RPD for external validation and slope of the calibration models were also used to evaluate the model. The RPD value stands for the ratio of standard deviation of predicted values to RMSEP values revealing the predictive ability of the corresponding model (Riedl, Esslinger, and Fauhl-Hassek 2015). The RPD values were calculated according to formula provided in the literature (Ozturk, Yucesoy, and Ozen 2012). In RPD evaluation, values lower than 2.0 are considered to be insufficient for prediction while values between 2.0-2.5 are used for approximate quantitative predictions. Values between 2.5-3.0 and values higher than 3.0, on the other hand, indicate good and excellent predictions, respectively (Tamaki and Mazza 2011).

3.4.2. Characterization and Authentication

A set of basic quality parameters (free fatty acid and K values) including 91 rows (samples) and 3 columns (parameters) was obtained with titrimetric analyses, and a fatty acid profile matrix with 91 rows (samples) and 11 columns (individual fatty acids), and a DAGs matrix possessing 91 rows and 9 columns (individual DAGs including ratio), FAAEs and wax contents with their components in terms of 91 rows and 16 columns were determined with GC analysis. Finally, a pigment matrix having 91 rows (samples) and 13 columns (pigments) were generated with the results from HPLC analysis. In the spectral part, the whole spectra of FTIR (4000-650 cm^{-1}) and UV-visible (200-800 nm) measurements were used to create data matrices with dimensions of 91×3351 and 91×301, respectively. In addition, combination of FTIR+UV-visible spectra (650-4 000 cm^{-1}+12 500-50 000 cm^{-1}) in low-level fused form of 91×3652 dimensions were also used in the analysis.

Prior to construction of discrimination models, raw pigment data were standardized and regularized simply by applying unit variance scaling and mean-centering without any further pre-processing techniques. Whereas, spectroscopic data as FTIR and fused form were additionally pre-treated with second-order derivative (SD) to minimize baseline effect and random-noise contributions (Moros, Garrigues, and Guardia 2010). The SD data were treated with moving quadratic sub-models with 15 data point long including distance between them as 1 while excluding the edge effects. In addition to these spectral pre-processing techniques, SNV transformation was applied to UV-visible spectra to enhance the classification power by eliminating major effects of light scattering from the spectra (Moros, Garrigues, and Guardia 2010).

For the discrimination purposes, pre-treated data set of each matrix (chromatographic and spectral) was randomly divided into calibration and validation sets comprising 2/3 and 1/3 number of the samples, respectively. Orthogonal partial least square-discriminant analysis (OPLS-DA) was used to visualize the separation of olive oil samples according to geographical origin and harvest year by using the pre-treated data. In geographical discrimination, a calibration data set of total of 60 samples were divided into three classes as 17 Middle (class M), 19 North (class N), and 24 South (class S) samples while 31 samples (9 M, 10 N, and 12 S) were used as a validation set. In

differentiation of harvest year, a calibration data set (60 samples) belonging to two consecutive years (36 samples for the first harvest year (class 1) and 24 samples for the second harvest year (class 2)) and a validation set (31 samples) from the same harvest years (18 and 13 samples for class 1 and 2, respectively) were used.

Classification performance of the generated models were checked with several parameters as number of LVs and regression coefficients for both calibration (R^2_{cal}) and validation (R^2_{cv}) models as well as correct classification rate (CC%) for the same models. Cross-validation was also performed for the OPLS-DA models by applying 7-fold LVs built-in function of SIMCA software to avoid overfitting. As a last parameter, variable importance for the projection (VIP) values for geographical origin and harvest year, generated with SIMCA software, were used to determine the most influential variables of pigment, wavelength and/or wavenumber in chromatographic and spectral analysis, respectively. Variables having VIP values greater or close to 1 were considered as important variable in classification (Uncu and Ozen 2015).

3.4.3. Prediction Study

Partial least squares (PLS) regression was used to construct the prediction models of the chemical parameters from FTIR and UV-vis spectra. Moreover, data fusion approach was also used to enhance the prediction ability of the PLS models by combining FTIR and converted UV-vis spectra (650-4 000 cm^{-1} + 12 500-50 000 cm^{-1}) in a low-level fusion. In low-level fusion, all the data from different sources were simply concatenated into a single matrix (Borràs et al. 2015).

Prior to construction of calibration models by PLS regression, spectroscopic data were pre-processed to increase the prediction ability of the models by eliminating spectral variation. Mean-centering and UV-scaling were used in all of the model construction to enhance spectral signal. As pre-processing methods, first- or second-order derivative, MSC, and SNV transformation were used in specific model construction (Moros, Garrigues, and Guardia 2010). The first- and second-order derivative of the spectroscopic data were calculated from moving quadratic sub-models with 15 data point long and the distance between each data point is set to 1 excluding the edge effects.

After obtaining pre-processed calibration model by splitting 2/3 of the raw data (59 samples), reliability of the proposed models was checked with randomly selected external validation data set (1/3) (30 samples) as well as cross-validation. Performance of constructed models were checked by several performance parameters. R^2 was used to reveal robustness of the corresponding models as R^2_{cal}, R^2_{cv}, and R^2_{pred} for external validation (Ozturk, Yucesoy, and Ozen 2012). Parameters related with error such as root mean square error of prediction (RMSEP), root mean square error of calibration (RMSEC), root mean square value of cross-validation (RMSECV) were also evaluated. As another useful parameter, number of latent variables (LVs) were also used in the model performance assessment. To obtain a robust model without overfitting, it was expected to use as few numbers of LVs as possible with high value of R^2 and low value of RMSEC/RMSEP (Özdemir, Dağ, Özinanç, et al. 2018).

In addition to these parameters, residual predictive deviation (RPD) and slope of the models were calculated. The RPD value for external validation models was defined as the ratio of the standard deviation of the external validation variables to RMSEP and high value indicates a better model (Sinelli et al. 2008). All the statistical parameters except RPD values were calculated with SIMCA software while the RPD values were calculated according to Ozturk, Yucesoy, and Ozen (2012). Summary of this section is provided in the Table 3.2.

Table 3.2. Summary of overall approaches used during the studies

Investigation	Number of olive oil samples	Chemical analyses	Spectroscopic analyses	Statistical approaches
Adulteration of fresh oil with old oil	20 fresh and 100 adulterated	FFA, K values, fatty acid profile of fresh and old oils	FTIR, UV-Vis, FTIR+UV-Vis, Fluorescence for all samples	PLS and OPLS-DA
Characterization and authentication	91 samples from North, South and Middle parts of Aegean Region	FFA, K values, fatty acid profile, fatty acid alkyl esters and waxes, chlorophyll and carotenoids, diacylglycerols of all samples	FTIR, UV-Vis, FTIR+UV-Vis for all samples	OPLS-DA
Prediction	89 samples	Fatty acid alkyl esters and waxes, chlorophyll and carotenoids, diacylglycerols of all samples	FTIR, UV-Vis, FTIR+UV-Vis for all samples	PLS

CHAPTER 4

RESULTS AND DISCUSSION

CHARACTERIZATION AND AUTHENTICATION OF OLIVE OILS

Redrafted, modified, and extended from:

Uncu, Oguz, Banu Ozen, and Figen Tokatli. 2020. "Authentication of Turkish Olive Oils by using detailed pigment profile and spectroscopic techniques." *Journal of the Science of Food and Agriculture* 100 (5): 2153–65. https://doi.org/10.1002/jsfa.10239.

Uncu, Oguz, and Banu Ozen. 2021. "Fatty acid alkyl ester and wax compositions of olive oils as varietal authentication indicators." *Journal of Food Measurement and Characterization* (in press). https://doi.org/10.1007/s11694-021-01184-2.

4.1. Chemical Characterization and Authentication of Olive Oils from Aegean Region

Aegean Region is one of the most important olive oil producing areas in Turkey. Several important quality and purity parameters as free fatty acid (FFA) value, K values, fatty acid profile, fatty acid alkyl esters (FAAEs) and fatty acid ethyl esters (FAEEs) and

waxes were measured to investigate the chemical characteristics of olive oils from this region and to evaluate their importance in authenticity and quality determination. Moreover, chemical parameters recently proposed as quality indicators, chlorophyll and carotenoid profiles and DAG content were determined to study their effect on olive oil authentication. Ultraviolet-Visible (UV-VIS), and Fourier Transform Infrared (FTIR) spectroscopic profiles of the samples were also evaluated. Samples were obtained from North (N), Middle (M) and South (S) parts of Aegean Region for two consecutive harvest year. The origins of these oils are listed in the previous section (Table 3.1). Number of samples for 2015-16 and 2016-17 harvest year is 54 and 37, respectively. All data were analyzed with multivariate statistical methods.

4.1.1 Basic Quality Parameters

As basic quality parameters, FFA and K values were determined for the studied olive oil samples. It could be seen from Table 4.1 that M region samples were in lower quality in terms of all measured parameters when compared with the other two regions (N and S). N and S region samples had extra virgin oil grade in average.

These parameters are strict quality parameters for grading olive oils according to European Legislations. In this part of the study, it was aimed to investigate the differences in quality characteristics of the oil samples with respect to their geographical locations (varietal origins) and harvest year. Therefore, OPLS-DA classification models were constructed with the quality data set (FFA and K values) as shown in Figure 4.1 and Figure 4.2 and statistical parameters of these models could be found in Table 4.2 and Table 4.3. Geographical differentiation model was built with 2 predictive components and these LVs explained 31% of the total variance. 42% of the total variance of harvest year model was explained by 1 predictive and 1 orthogonal components l.

As far as the varietal origins are concerned it could be seen that N and S samples were not generally separated from each other while most of M region samples were grouped distantly from the others with respect to LV1 in the score plot (Figure 4.1). Moreover, Table 4.2 shows the details about the correct classification rates in calibration and external validation sets for the geographical origin model. It is clear that M region

samples were apart from the other two regions due to their lower quality characteristics. In detail, only three samples were misclassified for both external and calibration data sets of M region whereas other regions (N and S) were mostly placed together (Table 4.1). This could be explained by the fact that N and S samples were similar to each other in terms of their basic quality parameters having smaller ranges as it can be seen from Table 4.1.

Table 4.1. Basic quality parameters of olive oil samples with respect to their geographical location and harvest year

	North Aegean				South Aegean				Middle Aegean			
	2015/16 (n=19)		2016/17 (n =10)		2015/16 (n =25)		2016/17 (n=11)		2015/16 (n =10)		2016/17 (n =16)	
Parameters[±]	Average	Range	Average	Range	Average	Range	Average	Range	Average	Range	Average	Range
[1]FFA	0.58	0.33-0.99	0.63	0.30-0.76	0.79	0.20-1.42	0.71	0.27-1.30	2.46	0.64-4.82	3.79	0.85-12.11
[2]K232	2.12	1.81-2.82	2.33	1.87-2.57	2.20	1.87-2.57	2.41	2.15-2.78	2.40	1.99-2.55	2.73	2.26-3.14
[3]K270	0.22	0.10-0.36	0.20	0.15-0.44	0.18	0.07-0.35	0.20	0.22-0.37	0.13	0.07-0.19	0.40	0.22-0.70

[±]Standard deviations: 0.03[1], 0.08[2], 0.04[3]

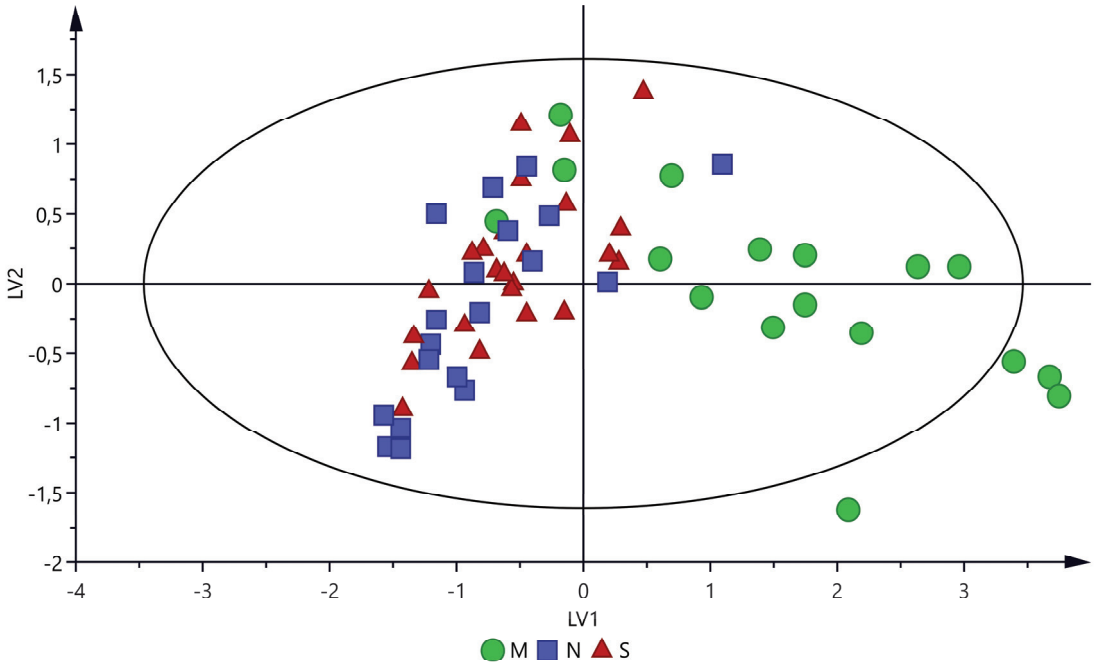


Figure 4.1. OPLS-DA score plot constructed with basic quality parameters showing their effects on geographical location

Table 4.2. Statistical parameters of OPLS-DA calibration and validation models of olive oils with respect to geographical location

Model	Number of samples	Basic quality parameters Pre-treatment: none, LVs: 2+0, R^2_{cal}: 0.31, R^2_{cv}: 0.28			
		M	N	S	%CC
Calibration					
M	17	14	0	3	82
N	19	1	9	9	47
S	24	0	5	19	79
Total	60	15	14	31	70
Validation					
M	9	6	1	2	67
N	10	1	3	6	30
S	12	1	5	6	50
Total	31	8	9	14	48

It was also aimed to investigate harvest year effect on the same quality parameters. OPLS-DA score plot (Figure 4.2) was constructed to observe the clustering with respect to harvest year. The first harvest year samples were mostly grouped in the left side according to LV1 whereas the second harvest year samples were located at the opposite side with some misclassification between each group (Figure 4.2). According to

misclassification table, first harvest year samples were classified with 94% success for both calibration and external validation sets, while the second harvest year samples were correctly classified at a lower rate. The basic quality parameters of the first harvest year samples were similar while the second harvest year samples had wider ranges of the measured variables compared to the previous harvest year (Table 4.1).

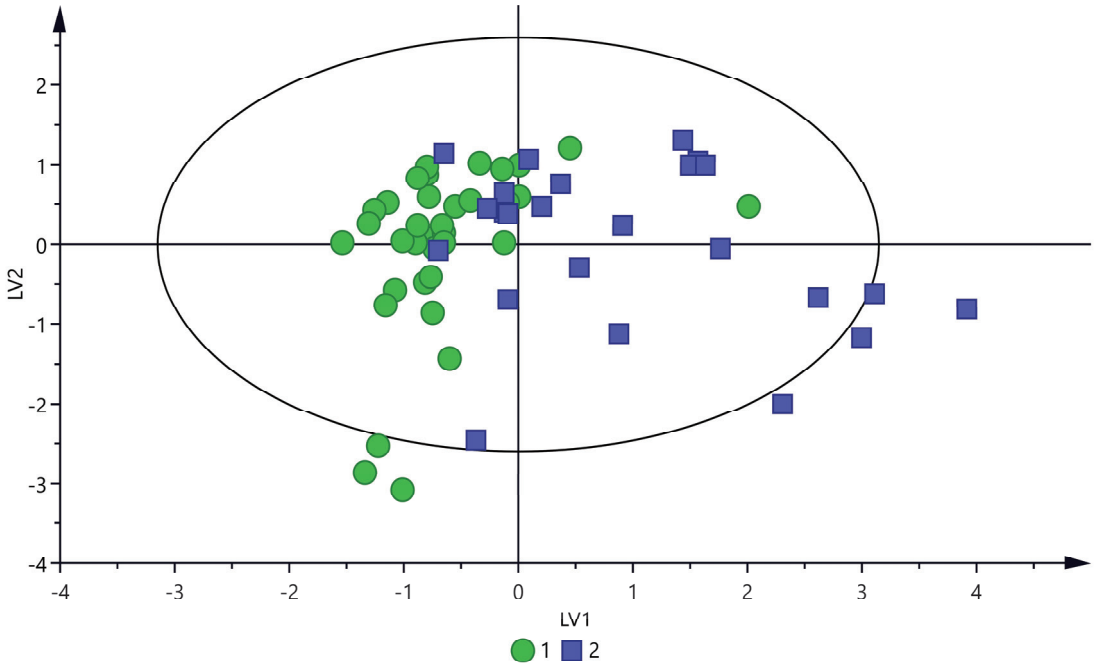


Figure 4.2. OPLS-DA score plot constructed with basic quality parameters showing their effects on harvest year

Table 4.3. Statistical parameters of OPLS-DA calibration and validation models of olive oils with respect to harvest year

Model	Number of samples	Basic quality parameters Pre-treatment: none, LVs: 1+1, R^2_{cal}: 0.42, R^2_{cv}: 0.37		
		2015/16	2016/17	%CC
Calibration				
2015/16	36	34	2	94
2016/17	24	11	13	54
Total	60	45	15	78
Validation				
2015/16	18	17	1	94
2016/17	13	5	8	62
Total	31	22	9	81

4.1.2. Fatty Acid Profile

Fatty acid profiles of the olive oil samples are presented in Table 4.4 and they were all in the ranges of European Standard for Olive Oils and Olive Pomace Oils (Commission Regulation (EC) 2002b). Individual fatty acid contents of the samples from different areas and harvest years were quite close to each other. Differences in oleic and linoleic acid contents were observed between consecutive harvest years as well as geographical regions in this study (Table 4.4) which is consistent with a previous report in literature (Gurdeniz, Ozen, and Tokatli 2008). Linoleic acid percentages were determined as 14.95% and 16.89% in two different harvest seasons in the same study while lower level of linoleic acid was found for all regions in the present study (Table 4.4). Linoleic acid contents of the olive oils from South regions were higher than the other two regions whereas the opposite is true for oleic acid content (Table 4.4). Except these major fatty acids, variations in other fatty acid compounds were not that significant. All the fluctuations observed between the years and regions could be attributed to the climatic conditions at different harvest years and differences in geographical locations of extracted oils.

Table 4.4. Individual fatty acid contents (%) of olive oils from Aegean region of Turkey

	North Aegean				South Aegean				Middle Aegean			
	2015/16 (n=19)		2016/17 (n =10)		2015/16 (n =25)		2016/17 (n=11)		2015/16 (n =10)		2016/17 (n =16)	
Fatty acids[±]	Average	Range	Average	Range	Average	Range	Average	Range	Average	Range	Average	Range
C16:0[1]	15.08	12.65-17.97	13.92	12.23-15.85	13.26	10.64-15.86	13.60	12.78-15.62	13.91	11.77-15.60	13.01	12.09-15.09
C16:1[2]	0.88	0.61-1.04	0.76	0.59-1.06	0.89	0.55-1.58	0.89	0.70-1.16	0.76	0.49-1.07	0.70	0.44-1.39
C17:0[3]	0.16	0.00-0.53	0.17	0.14-0.19	0.07	0.00-0.31	0.09	0.05-0.17	0.19	0.11-0.49	0.14	0.11-0.18
C17:1[4]	0.24	0.14-0.43	0.25	0.22-0.30	0.12	0.00-0.29	0.15	0.08-0.29	0.23	0.17-0.32	0.22	0.19-0.26
C18:0[5]	2.70	2.50-3.11	3.01	2.45-3.77	2.66	2.07-3.57	3.07	2.27-3.41	2.70	2.53-2.88	2.89	2.48-3.53
C18:1n9c[6]	68.31	65.91-71.34	69.58	67.65-74.32	71.85	65.44-76.99	69.78	65.92-72.47	66.24	64.83-67.58	66.70	64.09-70.75
C18:2n6c[7]	11.28	9.50-12.96	10.72	7.02-13.19	9.61	7.32-12.31	10.71	8.04-12.96	14.42	11.09-17.81	14.71	9.47-17.67
C20:0[8]	0.41	0.30-0.51	0.48	0.45-0.51	0.38	0.27-0.47	0.47	0.42-0.56	0.41	0.31-0.48	0.44	0.38-0.52
C18:3n3[9]	0.64	0.50-0.74	0.68	0.61-0.75	0.80	0.59-0.98	0.84	0.68-0.97	0.73	0.66-0.83	0.77	0.59-1.03
C20:1[10]	0.26	0.00-0.32	0.30	0.24-0.33	0.30	0.00-0.41	0.30	0.26-0.34	0.30	0.27-0.35	0.31	0.25-0.36
C22:0[11]	0.04	0.00-0.41	0.13	0.00-0.20	0.06	0.00-0.56	0.11	0.00-0.16	0.11	0.00-0.14	0.12	0.09-0.15

[±] Standard deviations: [1]0.02, [2]0,00, [3]0.01, [4]0.00, [5]0.01, [6]0.10, [7]0.04, [8]0.02, [9]0.07, [10]0.01, [11]0.01.

A multivariate data set of 11 fatty acid variables from 91 olive oil samples were used to examine the geographical location and harvest year effect on fatty acid profile. This data set was examined with OPLS-DA to observe the differences between locations as well as harvest year and statistical parameters for the constructed models could be found in Table 4.5 and Table 4.6. Model for geographical classification was constructed with 2 predictive and 1 orthogonal components and the first two LVs explained 67% of the total variance while 1 predictive and 2 orthogonal components were used in harvest year model in which the first two significant LVs explained 69% of the total variance. From the OPLS-DA score plot presented in Figure 4.3, it could be seen that all regions were separated well from each other except three specific samples from M region. These three oil samples were obtained from Ayvalik variety which is predominant in the north (N); hence, these samples were not well separated from the N samples. Therefore, these results reflected the effect of the cultivar in the olive oil classification based on the fatty acid composition, and they also confirm other reports in literature (D'Imperio et al. 2007). Rest of the samples were placed together with the characteristic varieties of the specified regions. Loading plot is presented in Figure 4.4 and this plot shows which fatty acids are responsible for differentiation. For this case, C16:1, C18:1n9c and C18:3n3c are the most effective variables on the separation of S region. Oils from M region are separated with respect to C18:0, C20:1, C22:0, C18:2n6c, while C16:0, C17:0, C17:1, and C20:0 are the fatty acids responsible for differentiation of N region. In the literature, three fatty acids as oleic, linoleic and palmitic were indicated as the fatty acids with high differentiation power (D'Imperio et al. 2007) and these three-fatty acids are also found effective in discrimination of S, M, and N regions in the present case. Parameters having variable importance projection (VIP) values greater than 1 are considered as the significant variables in the construction of the statistical models. From the VIP values (Figure 4.5), heptadecenoic and linolenic acids were also found effective in the discrimination of the olive oils in terms of growing locations besides the aforementioned fatty acids. Correct classification rates also proved the clear discrimination between each region with high success rates of 95% and 84% for calibration and validation data sets, respectively (Table 4.5). The details about misclassified samples in the external validation set is given in Figure 4.6 which explains how close the misclassified samples to the right classification in terms of percent probability difference. According to this plot, there are 15% and 7% differences between the right and wrong classification of 2 M samples as N samples while two other S samples are misclassified as N with 11-13 % difference.

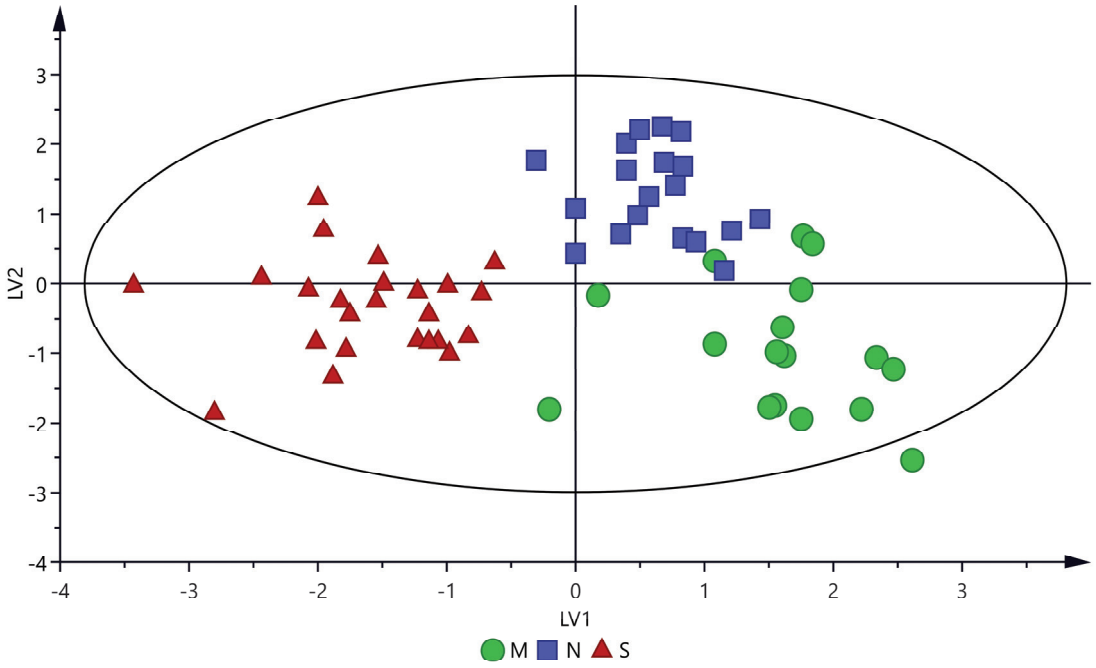


Figure 4.3. OPLS-DA score plot constructed with fatty acid profile for geographical location differentiation

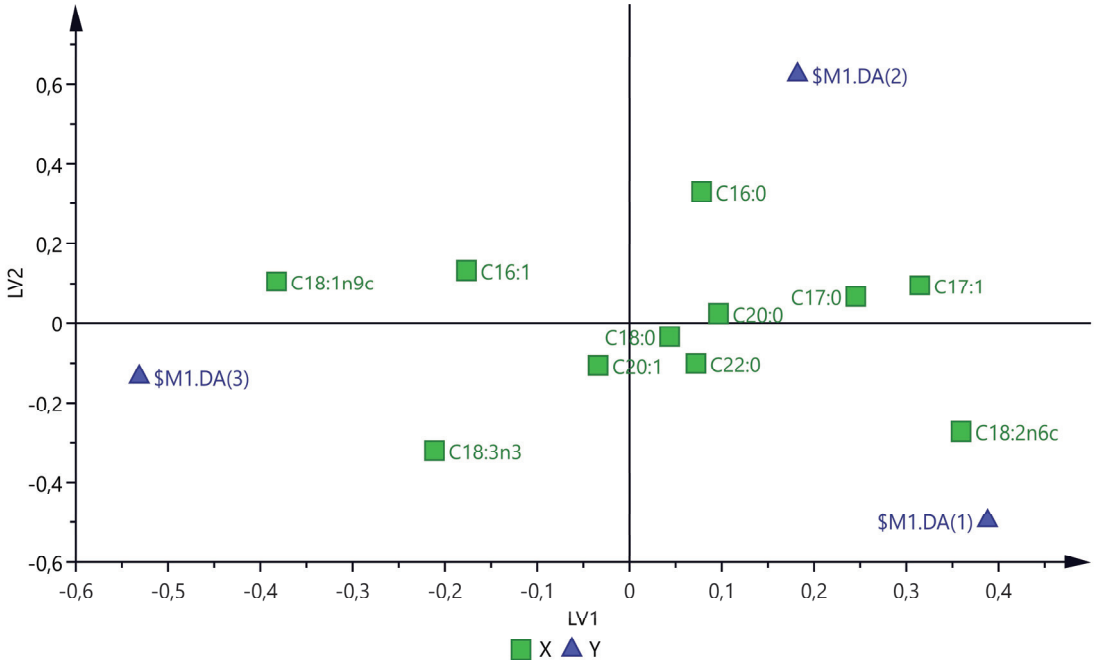


Figure 4.4. OPLS-DA loading plot constructed with fatty acid profiles for geographical location differentiation

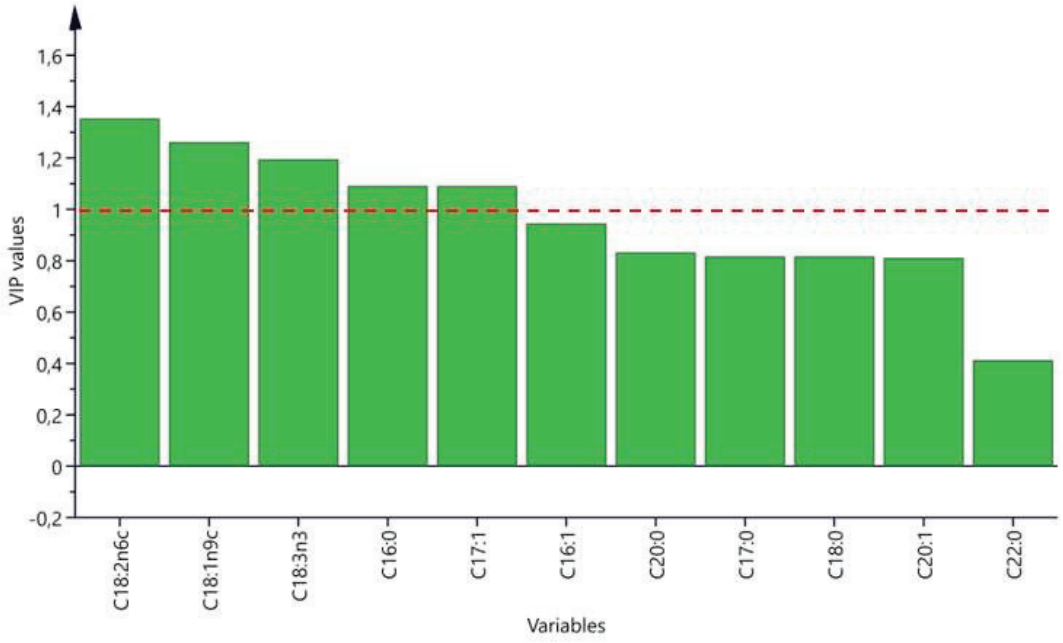


Figure 4.5. VIP values of OPLS-DA models with respect to geographical location

Table 4.5. Statistical parameters of OPLS-DA calibration and validation models of olive oils with respect to geographical location

Model	Number of samples	Fatty acid profile Pre-treatment: none, LVs: 2+4, R^2_{cal}: 0.72, R^2_{cv}: 0.62			
		M	N	S	%CC
Calibration					
M	17	14	3	0	82
N	19	0	19	0	100
S	24	0	0	24	100
Total	60	14	22	24	95
Validation					
M	9	7	2	0	78
N	10	0	9	0	90
S	12	0	2	10	83
Total	31	7	13	10	84

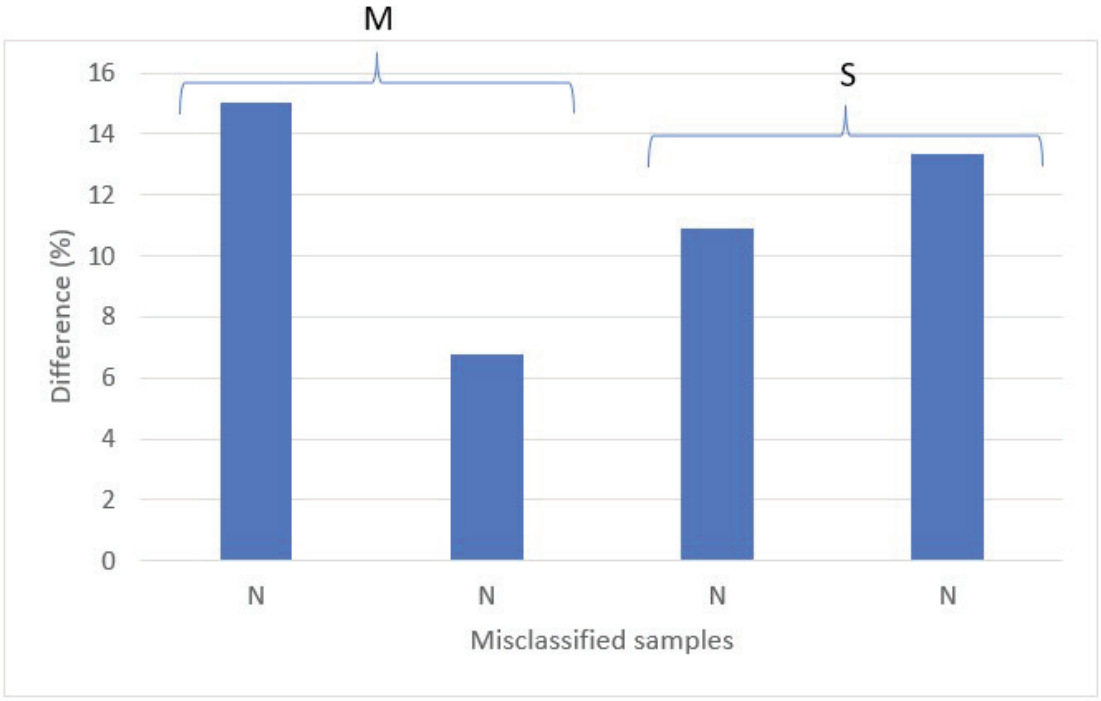


Figure 4.6. Percent probability differences between wrong and right classifications for the misclassified samples in the external validation set for geographical class model

Effect of harvest year was also investigated, and it was concluded that discriminatory power of harvest year was also successful up to an extent (Figure 4.7). It was observed that only two samples from the first harvest year were misclassified as the second harvest year, whereas the rest of the samples were correctly classified in the calibration set (Table 4.6). Details about misclassified samples in the external validation are given in Figure 4.10. From the loading plot (Figure 4.8), it was concluded that C16:0, C16:1, and C18:1n9c were successful in discrimination of the first-year samples, while the rest of the fatty acids were effective in the second harvest year differentiation. According to VIP values shown in Figure 4.9, C20:0, C18:0, and C18:2n6c were the most effective parameters in classification of the present models. In the literature, evaluation of fatty acid composition of olive oils obtained from M and N parts of Aegean region with principal component analysis revealed clear differentiation with respect to variety, geographical origin and harvest year (Gurdeniz, Ozen, and Tokatli 2008). In a similar study, a clear separation was obtained with fatty acid profile belonging to olive oils from N and S parts of Aegean Region (Gurdeniz, Ozen, and Tokatli 2010). In another study,

olive oil samples from very close geographical areas in the middle part of Aegean Region were discriminated with respect to their fatty acid profiles (Uncu and Ozen 2016).

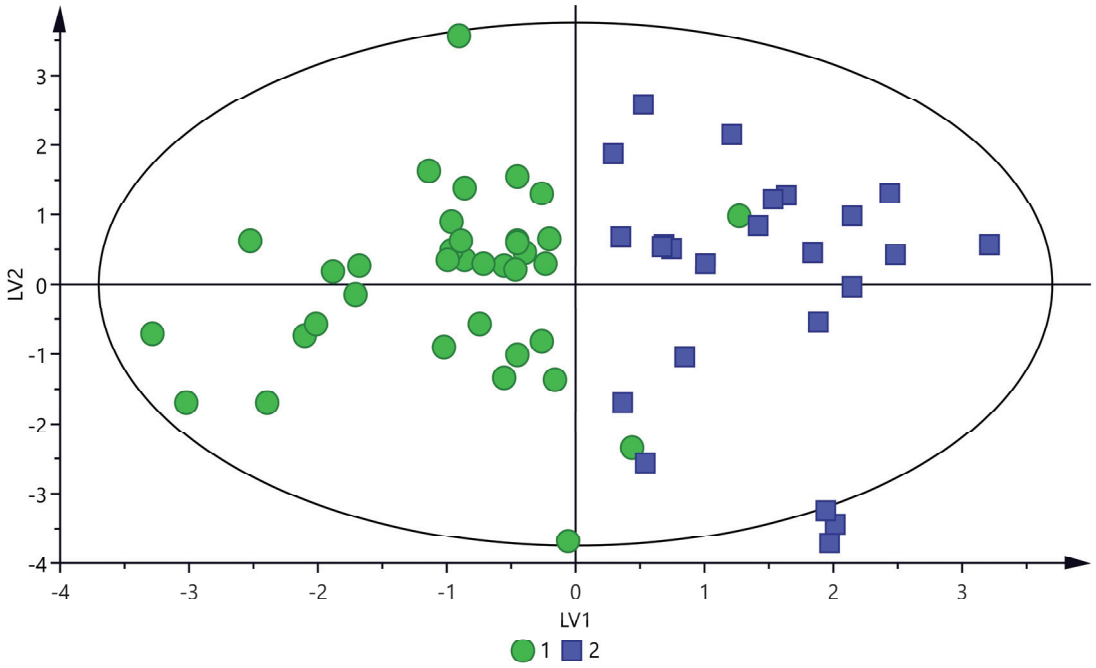


Figure 4.7. OPLS-DA score plot constructed with fatty acid profiles for harvest year differentiation

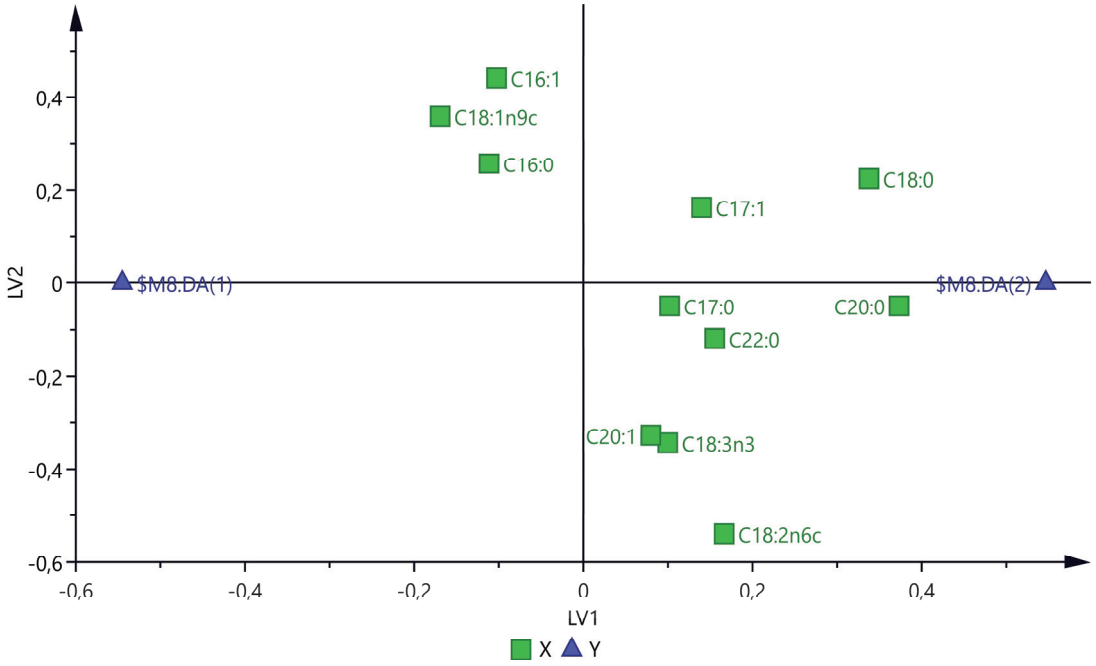


Figure 4.8. OPLS-DA loading plot constructed with fatty acid profiles for harvest year differentiation

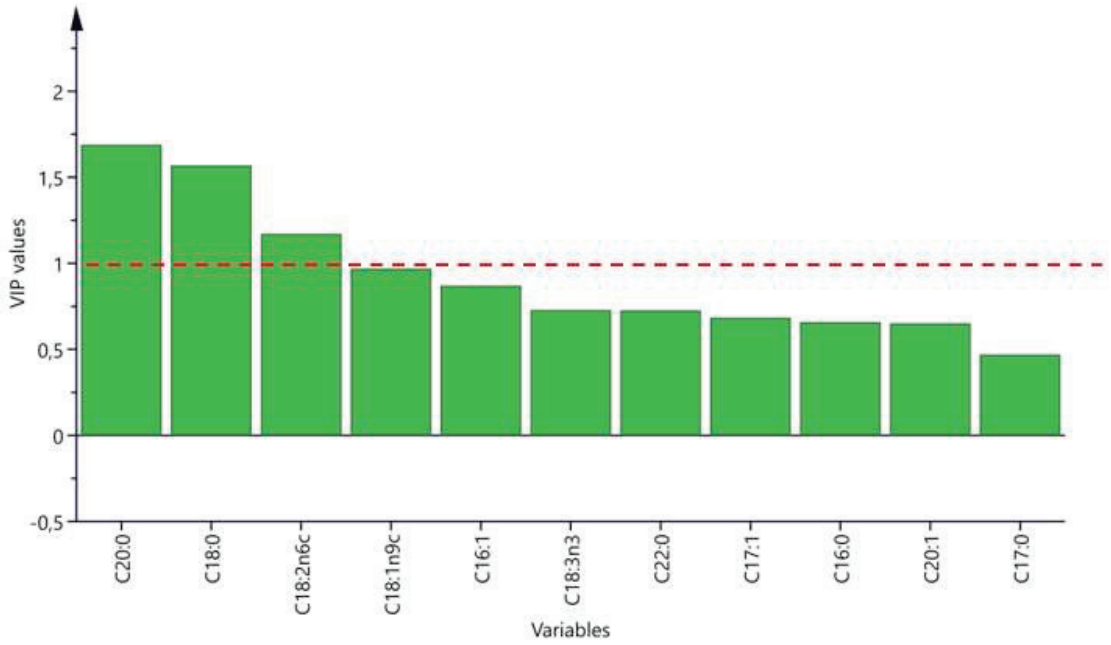


Figure 4.9. VIP values of OPLS-DA models with respect to harvest year

Table 4.6. Statistical parameters of OPLS-DA calibration and validation models of olive oils with respect to harvest year

Model	Number of samples	Fatty acid profile Pre-treatment: none, LVs: 1+2, R^2_{cal}: 0.69, R^2_{cv}: 0.61		
		2015/16	2016/17	%CC
Calibration				
2015/16	36	34	2	94
2016/17	24	0	24	100
Total	60	34	26	97
Validation				
2015/16	18	17	1	94
2016/17	13	5	8	62
Total	31	22	9	81

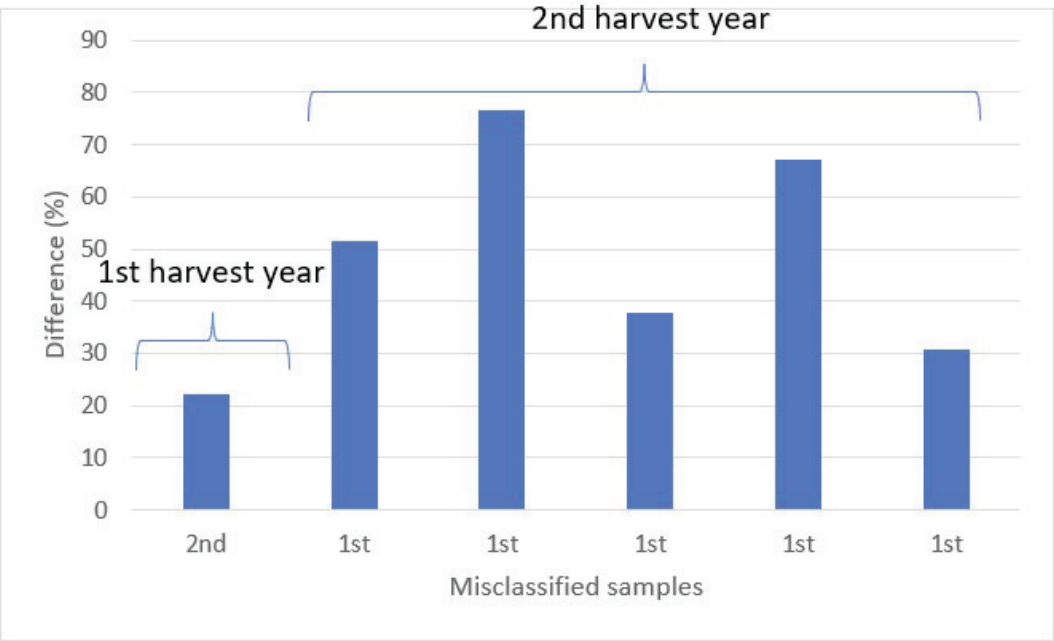


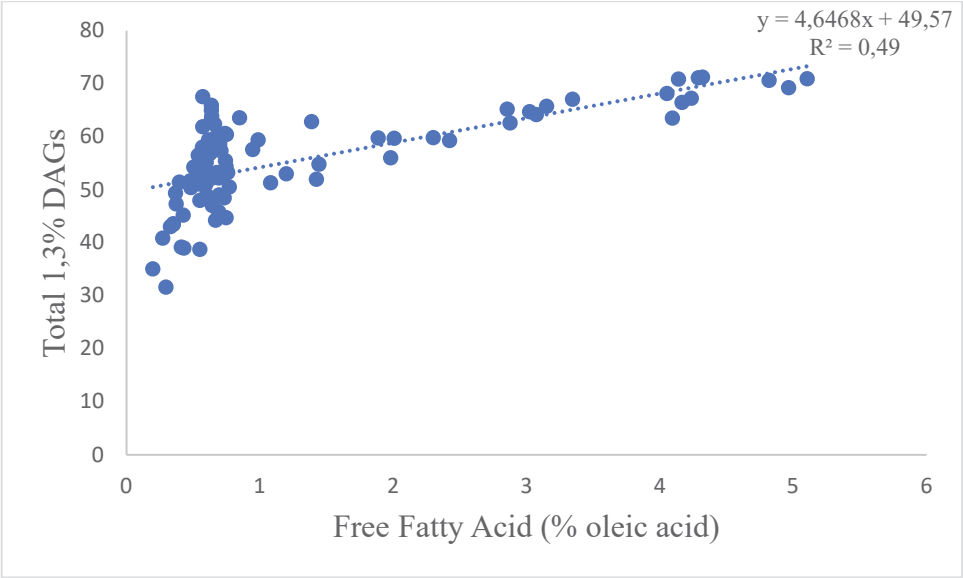
Figure 4.10. Percent probability differences between wrong and right classifications for the misclassified samples in the external validation set for harvest year classification

4.1.3. Diacylglycerols

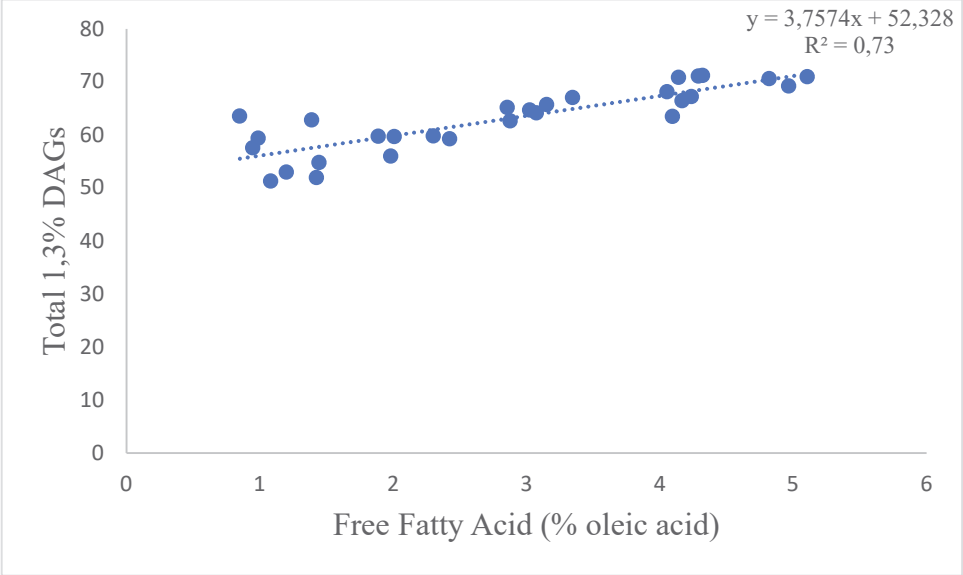
Diacylglycerols (DAG) are found in virgin olive oils in low amounts (between 1% and 3%) in the forms of intermediate products of the biosynthesis of triacylglycerols (1,2-isomers), or as products of enzymatic or chemical hydrolysis of triacylglycerols (1,3-isomers) which produced before or during the oil extraction process (Caponio et al. 2013). Healthy olive fruits yield oils containing almost exclusively 1,2-isomers whereas poor-quality ones produce oils with consistent amounts of 1,3-isomers and FFAs (Caponio et al. 2013). It is a known fact that during the storage 1,2-isomers undergo isomerization, yielding 1,3-isomers, that are thermodynamically more stable. Therefore, determination of the amounts of these isomeric forms could give information about the age and the freshness of virgin olive oils (Caponio et al. 2013). Therefore, DAG content could be an indicator of the quality of an olive oil. Some countries such as Australia, New Zealand, and California State of USA consider 1,2-DAGs and chlorophyll derivatives, as pyropheophytins (PPPs), as indicators of olive oil freshness (Bajoub et al. 2018).

There is a limited amount of study in the literature about DAG contents of Turkish olive oils. In one of these studies, 4 olive cultivars (Edremit, Gemlik, Domat and Sarıulak) from various locations in Turkey were characterized with regard to their composition of 1,2- and 1,3 DAGs (Matthäus and Musa Özcan 2011). 1,2- and 1,3-DAGs in olive oils varied between 27.5% to 49.2% and 50.8% to 72.5%, respectively. In the present study, 1,2 and 1,3 isomers of C32, C34 and C36 DAGs and total of 1,2 and 1,3 DAGs and their ratios were examined (Table 4.7). The results indicated that DAG composition was not constant and varying according to harvest year and geographical location. Olive oils from N region belonging to 2015 harvest year had average 43.37% total 1,2 DAG and 1,2 DAG content increased to 47.56% in 2016 harvest year. South region DAG composition did not change much between the years. On the other hand, total 1,2 DAG content of M region oils changed dramatically from 41.63% to 34.11% in consecutive harvest years. Effect of each parameter on classification are examined in detail in the following parts.

Correlation between free fatty acid content and DAG content was also investigated in this part of the study. According to Figure 4.11a there is no strong relation between olive oil acidity and DAG content as also indicated by Pérez-Camino et al. (2001). However, olive oil which possesses higher acidity value (≥1.0%) showed a better correlation with DAG content as the degradation reaction cause formation of more free fatty acid content (Figure 4.11b)



(a)



(b)

Figure 4.11. Correlation between free fatty acid content and diacylglycerol content of (a) all olive oil samples and (b) only samples with FFA ≥1.0

Table 4.7. Individual diacylglycerol contents (%) of olive oils from Aegean region of Turkey

	North Aegean				South Aegean				Middle Aegean			
	2015/16 (n=19)		2016/17 (n =10)		2015/16 (n =25)		2016/17 (n=11)		2015/16 (n =10)		2016/17 (n =16)	
Diacylglycerols[±]	Average	Range	Average	Range	Average	Range	Average	Range	Average	Range	Average	Range
[1]C32 1,2%	0.02	0.00-0.13	0.04	0.00-0.18	0.05	0.00-0.17	0.04	0.00-0.16	0.11	0.06-0.17	0.07	0.00-0.18
[2]C32 1,3%	0.14	0.00-0.29	0.17	0.00-0.43	0.13	0.00-0.30	0.10	0.00-0.31	0.24	0.19-0.34	0.29	0.16-0.47
3C34 1,2%	8.80	6.55-10.78	9.89	7.32-12.48	9.00	7.91-12.56	9.77	7.59-11.82	9.07	6,33-12.57	7.59	6.62-9.10
[4]C34 1,3%	11.75	7.91-14.00	11.40	5.97-14.86	9.39	6.45-12.23	11.08	8.45-12.88	12.61	10.37-16.19	14.60	12.65-17.27
[5]C36 1,2%	34.55	25.90-46.26	37.63	26.81-55.72	40.45	32.19-52.38	38.00	28.57-49.23	32.46	22.12-41.55	26.45	21.80-31.28
[6]C36 1,3%	44.74	34.88-53.28	40.86	25.40-52.10	40.98	27.94-47.10	41.01	30.29-50.66	45.51	34.97-55.06	51.00	45.79-55.37
[7]Total 1,2%	43.37	32.44-56.97	47.56	34.12-68.39	49.50	40.76-64.93	47.81	36.16-61.04	41.63	28.76-54.18	34.11	28.91-40.30
[8]Total 1,3%	56.63	43.03-67.56	52.44	31.61-65.88	50.50	35.07-59.24	52.19	38.96-63.84	58.37	45.82-71.24	65.89	59.70-71.09
[9]*D* value	0.79	0.48-1.32	1.00	0.52-2.16	1.01	0.69-1.85	0.96	0.57-1.57	0.76	0.40-1.18	0.52	0.41-0.68

[±] Standard deviations: [1]0.03, [2]0,06, [3]0.03, [4]0.68, [5]1.31, [6]0.52, [7]1.25, [8]1.25, [9]0.02.

There is not any report about any relation between DAG content of olive oils and olive variety and geographical origin of olives in literature. OPLS-DA model generated using DAGs profile have LVs= 2+1+0, R^2 = 0.31 and Q^2= 0.23. It was found that classification using DAG content alone was not successful with respect to geographical location (Figure 4.12). Also, there was no clear separation with respect to harvest year (Figure 4.13) according to OPLS-DA model with LVs= 1+3+0, R^2 = 0.32 and Q^2= 0.22. DAG is a quality parameter which could be associated with the oil extraction and storage conditions and it does not contain any markers to differentiate geographical location and/or harvest year. Therefore, it is confirmed that there is no direct correlation between the DAG content and the mentioned parameters. However, DAG content was used only in few discrimination studies which involved the use of combination of several parameters together rather than the individual form. DAGs combined with fatty acids, phenolics, total free sterols, free acidity, and iodine for geographical characterization of olive oils (Petrakis et al. 2008) and DAGs were also used together with aldehydes, phenolic compounds and terpenes for cultivar characterization (Özdemir, Dağ, Makuc, et al. 2018).

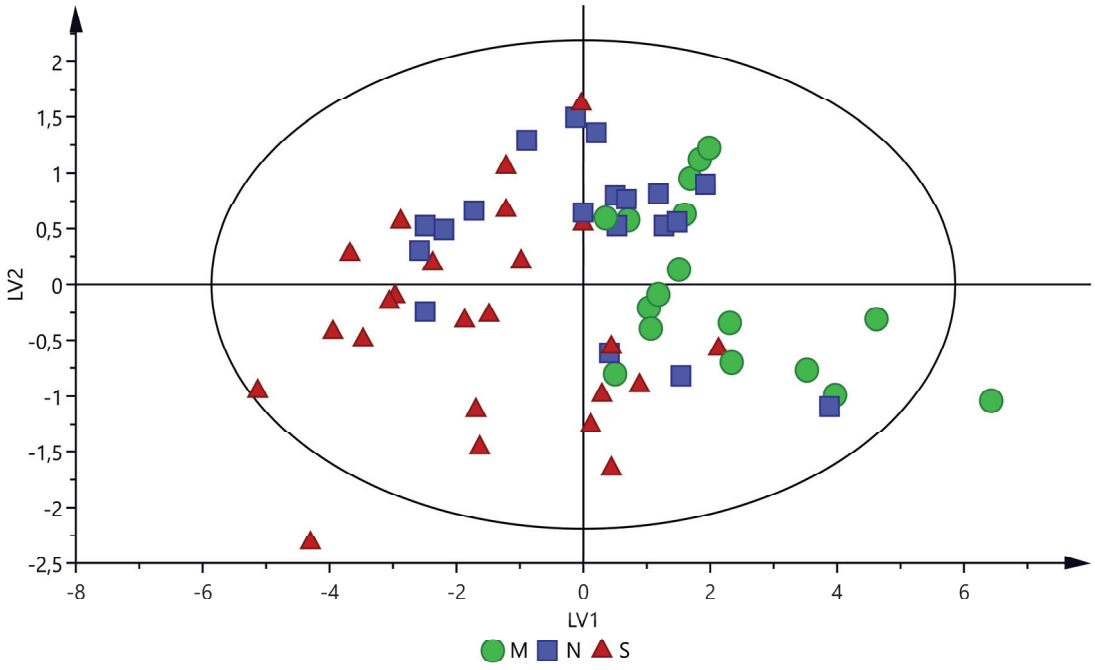


Figure 4.12. OPLS-DA score plot constructed using DAG profiles for geographical location differentiation

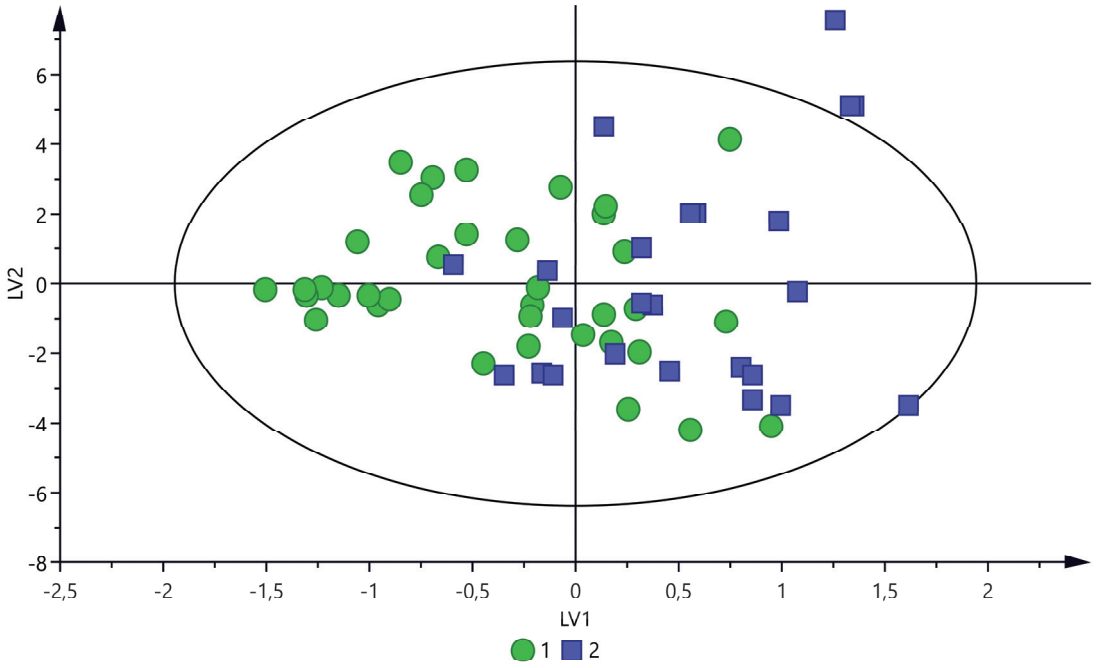


Figure 4.13. OPLS-DA loading plot of olive oil samples with respect to harvest year differentiation

4.1.4. Fatty Acid Alkyl and Ethyl Esters and Waxes

Fatty acid alkyl esters (FAAEs) as ethyl (FAEEs) and methyl esters (FAMEs) are a family of natural neutral lipids present in olive oils and formed by the esterification of FFAs with low molecular weight alcohols such as methanol and ethanol (Jabeur et al. 2015). They can easily form in an acid medium and their formation is catalyzed by certain enzymes (Jabeur et al. 2015). According to an early European Union regulation for FAAE content the limit was set at 75 mg/kg, but higher concentrations were allowed if they did not exceed 150 mg/kg and that the FAEE/FAME ratio was 1.5 at the maximum (Commission Regulation (EU) 2011; Gómez-Coca et al. 2016; International Olive Council (IOC) 2010). The knowledge that ethanol was produced as a metabolic by-product after alcoholic fermentation (Conte et al. 2014) drove to a conclusion that the presence of high concentration of both FAEE and ethanol could mean the use of sub-standard quality materials such as fermented olive fruits for oil extraction (Gómez-Coca et al. 2016).Therefore, new limits were officially published by the olive oil authorities due to the fact that FAEEs presence depended on the level of its substrate, ethanol, which

is produced chemically whereas FAMEs are associated with methanol content produced physiologically (Uncu and Ozen 2020; García-Vico et al. 2018). Moreover, only C16 FAEE and C18 FAEE were taken into consideration in the regulation to decide if a certain olive oil could be classified as extra virgin (Gómez-Coca et al. 2016). This decision was accompanied by a reduction of the maximum allowed limit to 40 mg/kg (2013–14 crop year) (Gómez-Coca et al. 2016). Limits regarding the fatty acid ethyl ester (FAEE) presence in extra virgin olive oil were further lowered to ≤ 35 mg/kg after 2016 harvest year (Uncu and Ozen 2020).

Other investigated quality parameter is wax content of olive oil. The straight chain wax esters are also shown to be useful indicators for the quality of olive oil. They are in the waxy surface layer of the olive and are poorly extracted by the oil derived from fruit pressing (Jabeur et al. 2015). Wax content has been also defined as a quality indicator and extra virgin olive oil wax content must not exceed 150 mg/kg according to the existing regulations (Commission Delegated Regulation (EU) 2016; International Olive Council (IOC) 2019).

There is not any study in the literature about FAAE, FAEE and wax contents of Turkish olive oils. In the present study, it was observed that olive oils from N and S region were within the set limit of FAEE in average for both harvest years while the samples from M region were not (Table 4.8). On the other hand, samples from all regions are below the limit of wax content for both harvest years. FAEE values of oils from all regions in 2016-17 harvest year increased compared to 2015-16.

It was found that there is a strong relationship between FFA value and alkyl ester formation as shown in Figure 4.14 (a) (b) (c) (R^2>0.80). This could be explained by the fact that FFAs promote ester formations (Biedermann et al. 2008). However, FFA content correlation with wax content is weak (Figure 4.14 (d)).

Table 4.8. Individual alkyl and wax esters of olive oil obtained from different areas of Aegean Region in two harvest year

	North Aegean				South Aegean				Middle Aegean			
	2015/16 (n=19)		2016/17 (n =10)		2015/16 (n =25)		2016/17 (n=11)		2015/16 (n =10)		2016/17 (n =16)	
Fatty acid alkyl esters±	Average	Range	Average	Range	Average	Range	Average	Range	Average	Range	Average	Range
C16:0 M[1]	4.00	1.86-7.69	3.62	0.72-5.94	4.29	1.83-13.55	3.84	0.90-5.64	12.81	3.54-25.29	20.41	4.40-87.63
C16:0 E[2]	1.71	0.31-4.49	3.04	0.69-8.09	3.19	0.44-11.12	4.82	0.45-8.82	15.62	1.44-41.29	22.94	5.11-35.77
C18 2 M[3]	1.86	1.04-3.70	2.17	0.35-3.46	1.85	0.90-5.23	2.88	1.49-6.22	9.73	1.26-22.48	26.24	3.39-199.44
C18 1 M[4]	10.13	3.82-19.71	13.18	1.78-25.04	13.22	2.58-44.46	14.66	1.56-24.00	49.44	8.70-109.05	81.40	18.77-237.38
C18:0 M[5]	0.92	0.61-1.54	0.80	0.29-1.39	0.93	0.51-1.60	0.82	0.29-1.10	2.25	0.74-4.53	6.09	1.13-45.18
C18:2 E[6]	1.03	0.31-2.71	1.85	0.42-3.75	1.65	0.00-7.59	3.75	0.66-8.26	12.28	0.68-32.64	22.43	2.55-37.37
C18:1 E[7]	6.90	1.04-19.98	11.14	1.70-29.25	14.51	1.52-55.49	18.51	1.46-34.74	64.37	3.75-163.62	89.31	13.13-144.68
C18 0 E[8]	0.14	0.00-1.17	0.83	0.43-1.62	0.26	0.00-1.48	1.23	0.46-1.91	2.48	0.00-6.04	3.99	0.71-6.53
C42[9]	5.73	0.00-14.91	6.46	1.98-11.03	4.25	0.00-20.57	6.02	3.01-16.42	12.47	4.76-20.79	15.42	4.83-25.55
C44[10]	8.46	3.38-15.71	13.69	3.37-21.51	6.62	1.28-25.07	9.36	3.18-15.33	12.84	4.11-25.83	25.98	8.43-43.08
C48[11]	6.46	0.00-10.84	3.71	2.12-5.27	5.89	1.61-21.43	3.97	1.68-8.00	15.33	7.87-26.69	8.91	1.72-24.54
FAEEs[12]	9.78	1.66-27.19	16.86	3.80-42.71	19.61	2.27-74.47	28.31	3.24-50.77	94.75	5.87-243.59	138.68	21.51-217.06
FAMEs[13]	16.91	7.36-31.89	19.77	3.14-35.76	20.29	6.28-64.85	22.20	4.24-36.87	74.23	14.62-161.35	134.15	27.96-539.04
FAAEs[14]	26.69	9.01-59.08	36.63	6.94-69.50	39.90	8.55-134.00	50.51	7.48-80.10	168.98	20.95-386.36	272.83	49.19-659.00
FAEEs/FAMEs[15]	0.54	0.22-1.33	0.87	0.34-1.59	0.86	0.35-2.61	1.25	0.59-1.78	1.10	0.38-2.26	1.33	0.22-2.37
Waxes[16]	20.64	8.56-30.62	23.86	11.47-35.22	16.77	5.26-61.40	19.35	12.05-25.69	38.32	19.75-60.57	50.30	18.11-89.59

± Standard deviations: [1]0.38, [2]0.11, [3]0.17, [4]1.10, [5]0.02, [6]0.07, [7]0.54, [8]0.18, [9]1.66,[10]3.38,[11]2.62, [12]0.86,[13]1.61,[14]2.47, [15]0.01,[16]0.56

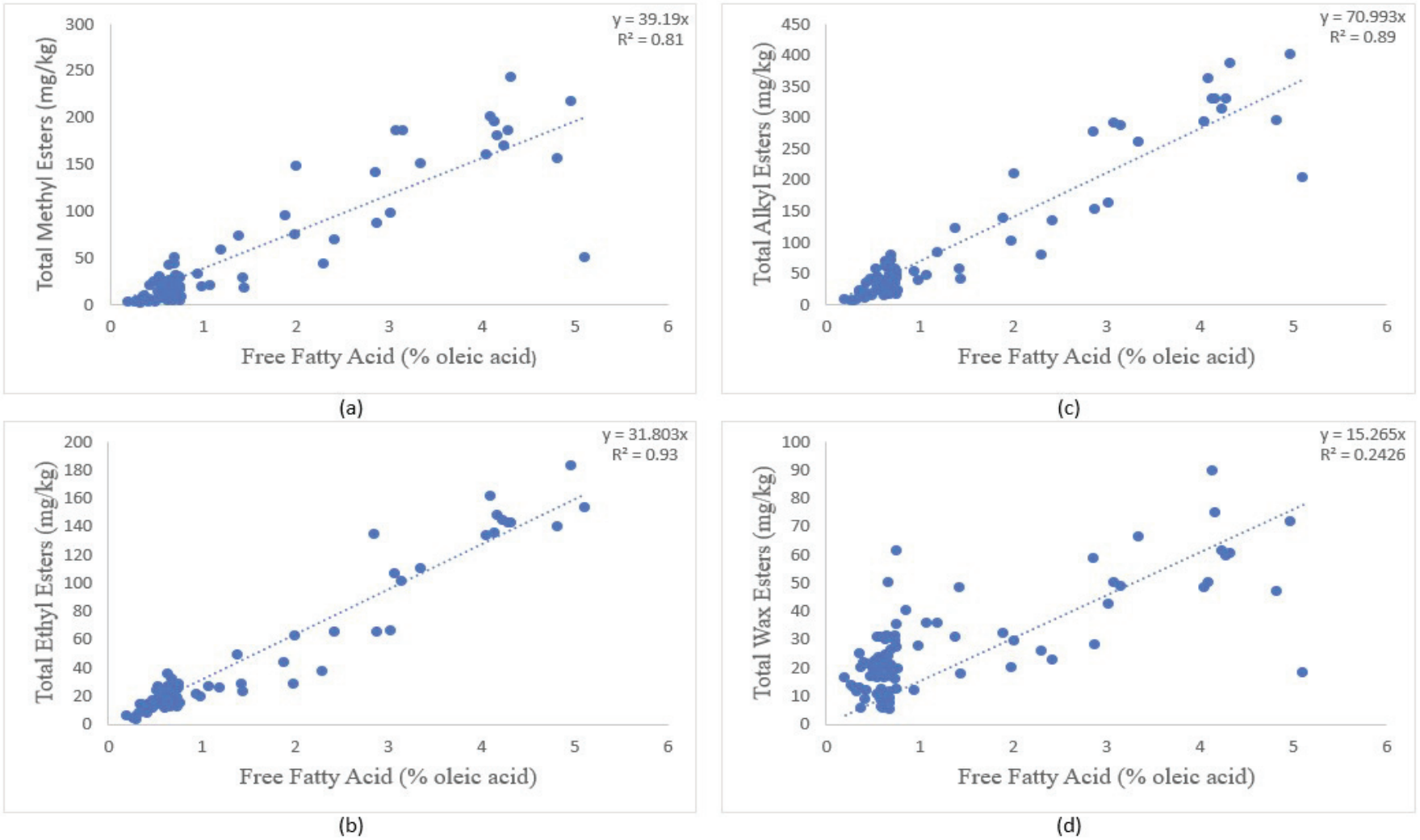


Figure 4.14. Correlation between free fatty acid and alkyl esters contents (a) (b) (c) and free fatty acid and wax contents (d) of olive oils.

Olive oil industry is facing strict demands regarding the fatty acid ethyl ester (FAEE) presence in extra virgin olive oil (≤35 mg/kg limit must be applied after 2016 harvest year). In a recent study, a relationship between the FAAEs concentration of olive oils and their sensory classification was evaluated. The results showed that there was a strong connection between the presence of high amounts of FAAEs and fermentative organoleptic defects (Gómez-Coca et al. 2016). FAAE has also been used for adulteration detection of olive oil with mild deodorized olive oil (Pérez-Camino et al. 2008; Jabeur et al. 2015). In addition to the fermentative effect of unhealthy olive fruits, it was revealed that ethanol formation is also triggered by the metabolism of the olive fruit itself which is highly related with cultivar (genotype) of the fruit (Beltrán et al. 2015). Accumulation is continued during fruit maturation on the olive tree (Beltrán et al. 2015). All of these make usage of FAEEs more complex since this parameter is affected by both quality and variety (Boudebouz et al. 2020). A different study also confirmed that ethanol is naturally found in the olive fruits, and it passes to the oils during extraction. As a result, it was detemined that concentration of the ethanol in the oil was a function of the cultivar, ripening stage and climate as well as growing conditions of the olives (García-Vico et al. 2018). Therefore, it was suggested that legislations on FAEEs should consider the basal levels of ethanol in the oils as it is quite high in many olive cultivars (García-Vico et al. 2018). Hence, it is not appropriate to use unique FAEE values for all olive varieties (Boudebouz et al. 2020). In a recent study, it was determined that not only ethanol but also methanol content and acetaldehyde as well as the ratio between them are characteristic to each olive variety (Boudebouz et al. 2020). Hence, it could be concluded that individual FAAEs and their specific ratio which have not been studied for the varietal determination before could possess a potential as an authentication tool for olive oils. In a literature study, Fourier transform infrared (FTIR) spectroscopy was used to separate virgin and non-virgin olive oils according to the FAEEs content (Squeo et al. 2019). In another recent work, alkyl esters content of Sicilian extra virgin olive oils having Protected Designation of Origin (PDO) was investigated from quality perspective only (Costa et al. 2017). However, there is not any authentication study in the literature focusing on alkyl esters alone from varietal point of view.

In the literature, composition of wax esters generally used for quality determination (Jabeur et al. 2015) and detection of adulteration made with lower quality olive oil or pomace oil (Jabeur et al. 2017). Individual and total wax esters of Spanish monovarietal olive oils with PDO were determined and it was found that significant

differences existed in C40, C44, C46 and total wax esters content (Aragón et al. 2011). These findings were also supported by a study performed with Italian cultivars and it was proven that wax ester content was influenced by cultivar and harvest year (Giuffrè 2013) as well as ripening (Giuffrè 2014). Results of these studies were also confirmed by a research in which wax esters, DAGs, TAGs, triterpenic acids, and aldehydes were shown to be strongly dependent on the olive cultivar (Vichi et al. 2016).

However, there is no study in the literature that aims at determining the effect of FAAEs and wax content of olive oils on differentiation based on geographical origin and/or harvest year. In the present study, OPLS-DA statistical model (Figure 4.15 with LVs: 2+1, R^2_{cal}: 0.67, R^2_{cv}: 0.62) indicates that M, N, and S samples were correctly classified according to the geographical location with some exceptions as explained in the misclassification table (Table 4.9). Model was built with 2 predictive and 1 orthogonal components and particularly the first two significant LVs explained 67% of the total variance. According to the score plot (Figure 4.15), M region samples were successfully separated in the right (positive) side of LV1 whereas N and S region samples were located on the opposite side. Moreover, these two regions (N and S) were separated from each other in the upper side (positive) of the first quarter and the lower side (negative) of the fourth quarter of LV2, respectively. Loading plot (Figure 4.16) showed that M region was placed apart from the rest in terms of the higher amounts of all the studied parameters. It can be concluded that S and N regions were more similar in terms of alkyl ester and wax profile while M region was more apart than the rest (Table 4.8). N and S region olive oils contain lower amounts of FAAEs, and wax esters compared to M region olive oils. Considering only N and S regions, S region samples had slightly higher amounts of individual alkyl esters. However, still there is an obvious separation between N and S region (Figure 4.15) oils although they have quite similar basic quality parameters (Table 4.1). Although FAAEs are quite related with the quality of the oil, a strong relation between these parameters and varietal factors are also well established with the recent study (Boudebouz et al. 2020). This differentiation between oils of these two regions could be also related to the effect of olive variety since Ayvalik is the olive type in N part while Memecik is the dominant variety in S part.

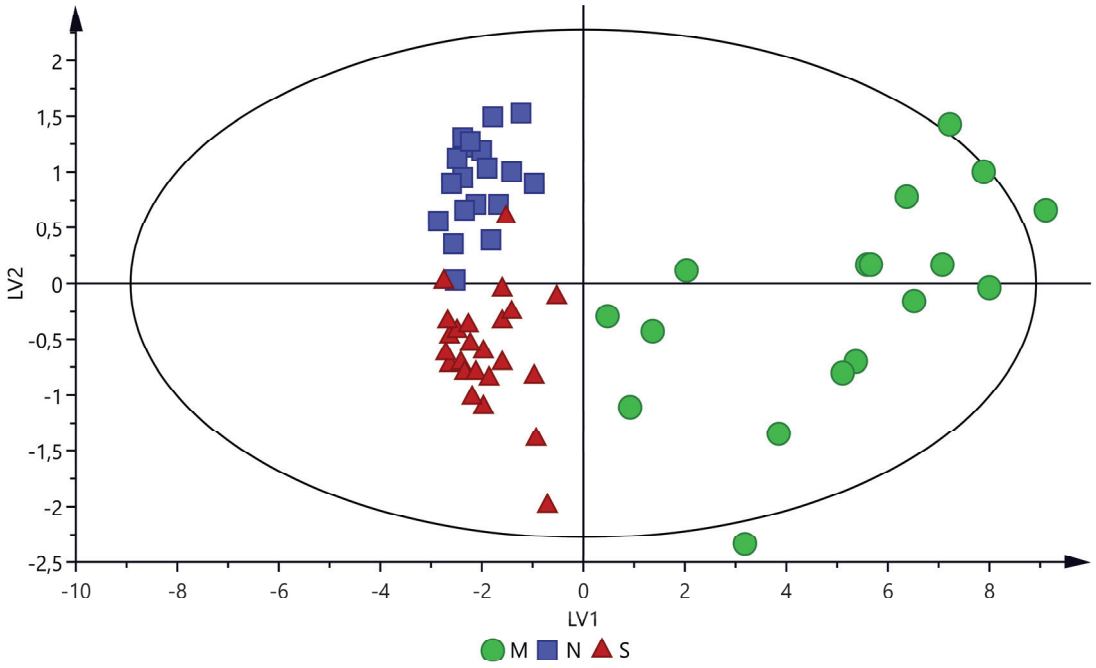


Figure 4.15. OPLS-DA score plot constructed using alkyl esters and wax profiles for geographical location differentiation

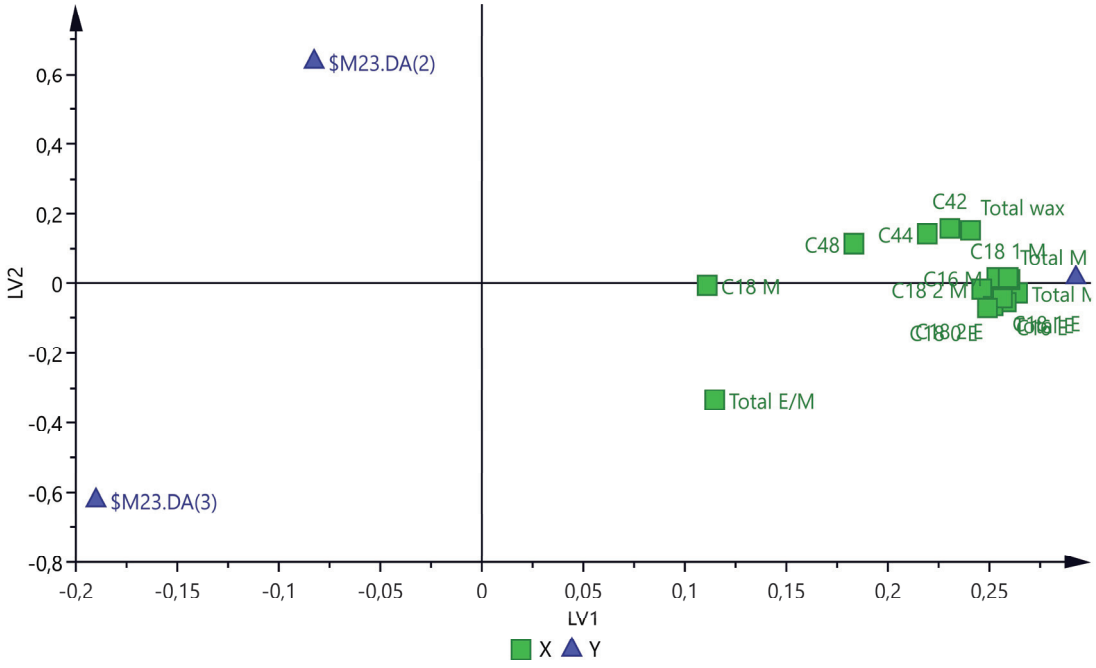


Figure 4.16. OPLS-DA loading plot of olive oil samples with respect to geographical location differentiation

In the present study, FAEE/FAME (total E/M), total wax as well as individual C42 and C44, C16E, total FAEEs and FAAEs (total of FAME + FAEE), having VIP values larger than 1 were found significant in discrimination of oils (Figure 4.17). Lastly, the classification model efficiency was determined with calibration and external validation data sets. As it could be seen from Table 4.9, OPLS-DA model revealed good discrimination ability with the average correct classification rate of 92% (out of 60 samples; 4 samples misclassified as S and 1 sample misclassified as N) and 74% (out of 31 samples; 3 samples were misclassified as S and 5 samples were misclassified as N) in calibration and validation data sets, respectively. Probability of misclassification was lower than 50% for the misclassified samples in the external set (Figure 4.18).

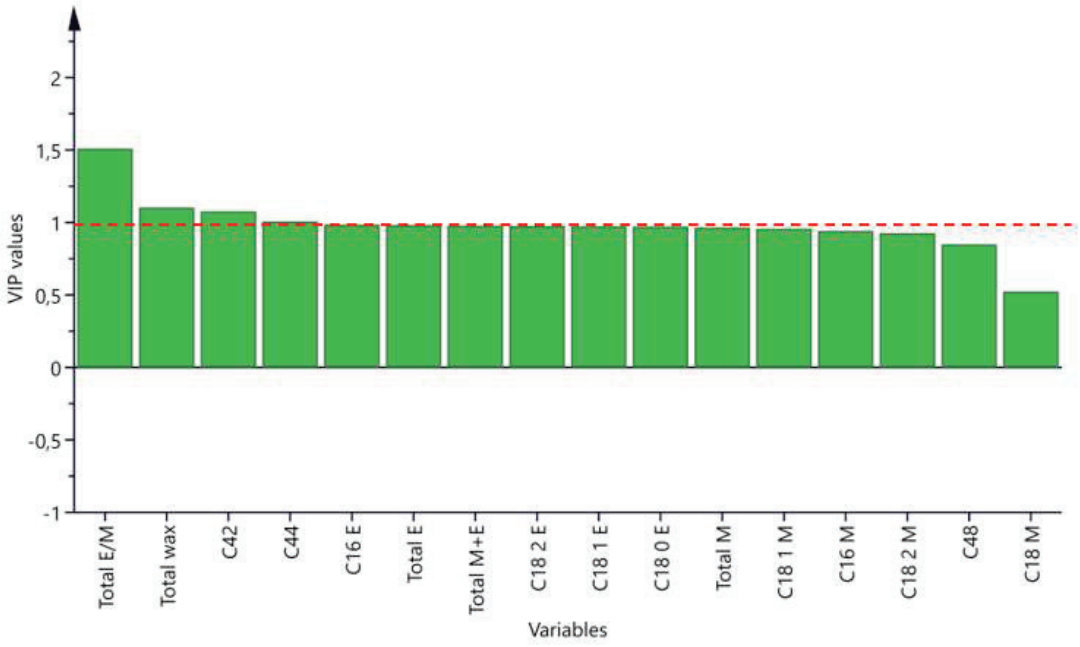


Figure 4.17. VIP values of OPLS-DA models with respect to geographical origin

Table 4.9. Statistical parameters of OPLS-DA calibration and validation models of olive oils with respect to geographical location

Model	Number of samples	FAAEs and waxes Pre-treatment: none, LVs: 2+1, R^2_{cal}: 0.67, R^2_{cv}: 0.62			
		M	N	S	%CC
Calibration					
M	17	14	0	3	82
N	19	0	18	1	95
S	24	0	1	23	96
Total	60	14	19	27	92
Validation					
M	9	7	1	1	78
N	10	0	8	2	80
S	12	0	4	8	67
Total	31	7	13	11	74

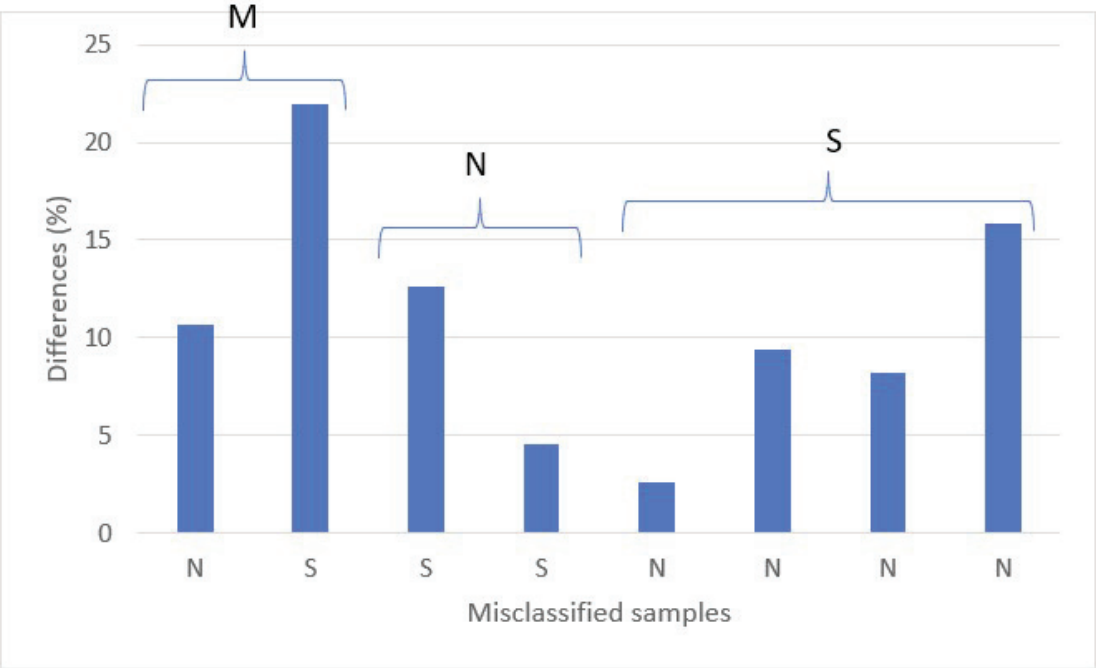


Figure 4.18. Percent probability differences between wrong and right classifications for the misclassified samples in the external validation set for geographical region

Finally, the effect of harvest year on the classification of olive oils in terms of individual alkyl ester profiles and waxes was also examined. It was observed that the first year samples were clearly differentiated from the second year (Figure 4.19) since 2015 harvest year olive oils from all three region contained lower amounts of alkyl ester and wax compared to 2016 harvest year samples (Table 4.8). This observation was also

supported with loading plot (Figure 4.20) in which all the components except C48 were grouped opposite of 2015 harvest year meaning that they were differentiated from the second harvest year in terms of lower alkyl and wax esters. Fly attacks reported for 2016 harvest year might have led to defects in fruit quality which was ultimately causing higher amounts of alkyl ester formation. In the literature, clear effects of climatic conditions of harvest year on the ethanol content of two main olive cultivars grown in Spain was shown (García-Vico et al. 2018). In the present study, the same situation also holds true for Turkish olive oils. Average correct classification rates of 97% for calibration (out of 60 samples; 2 samples misclassified as first harvest year) and 90% for validation data sets (out of 31 samples; 1 sample was misclassified as first harvest year and 2 samples was misclassified as second harvest year) further confirmed the robustness of the OPLS-DA model (Table 4.10). Model was generated with 1 predictive and 2 orthogonal components and particularly the first two significant LVs explained 69% of the total variance. The VIP values were found significant for FAEEs, C18:2E, C18:0E, C16:0 E, total E/M, total E, total M+E and wax, C44 (Figure 4.21). Difference between right and wrong classification for misclassified samples was found lower than 20% for external validation (Figure 4.22).

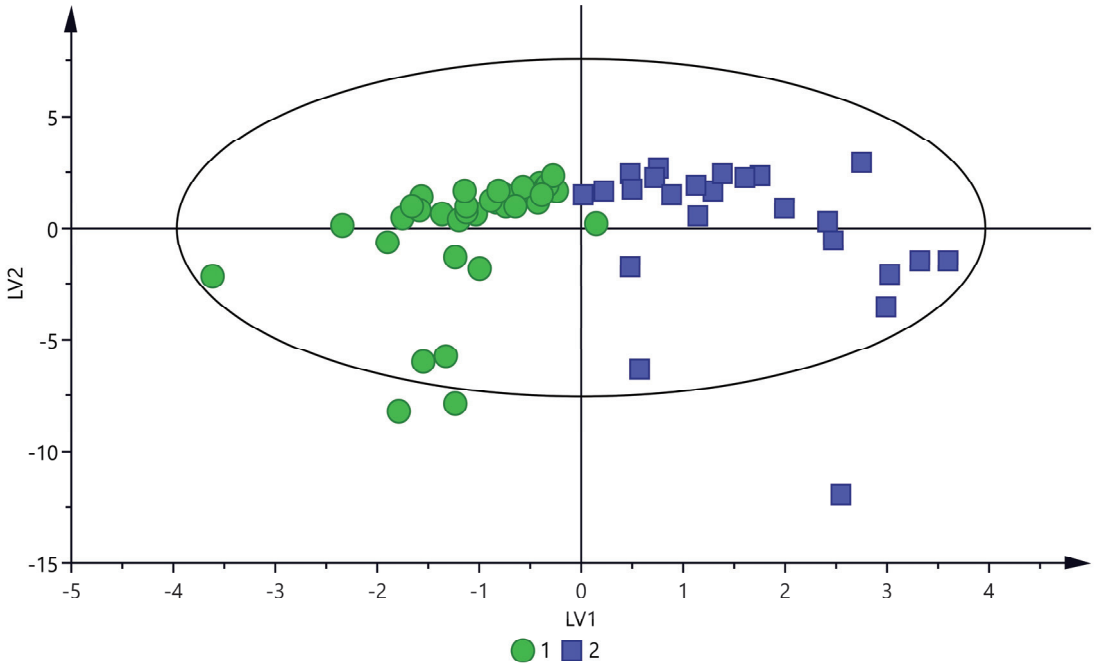


Figure 4.19. OPLS-DA score plot constructed using alkyl esters and wax profiles for harvest year differentiation

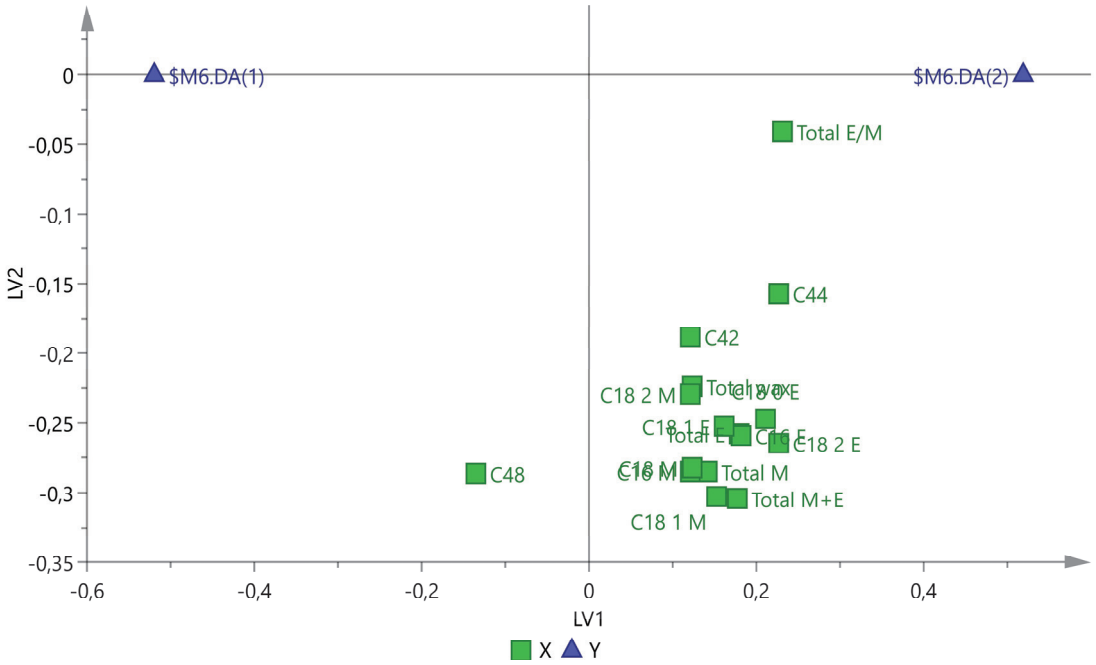


Figure 4.20. OPLS-DA loading plot of olive oil samples with respect to harvest year differentiation

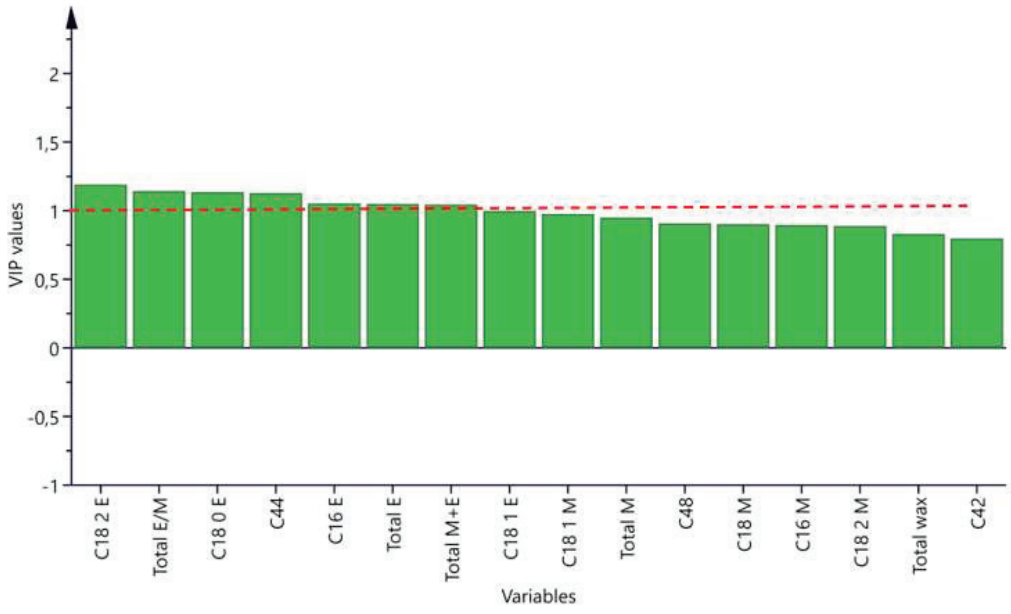


Figure 4.21. VIP values of OPLS-DA models with respect to harvest year

Table 4.10. Statistical parameters of OPLS-DA calibration and validation models of olive oils with respect to harvest year

Model	Number of samples	FAAEs and waxes Pre-treatment: none, LVs: 1+2, R^2_{cal}: 0.69, R^2_{cv}: 0.61		
		2015/16	2016/17	%CC
Calibration				
2015/16	36	36	0	100
2016/17	24	2	22	92
Total	60	38	22	97
Validation				
2015/16	18	16	2	89
2016/17	13	1	12	92
Total	31	17	14	90

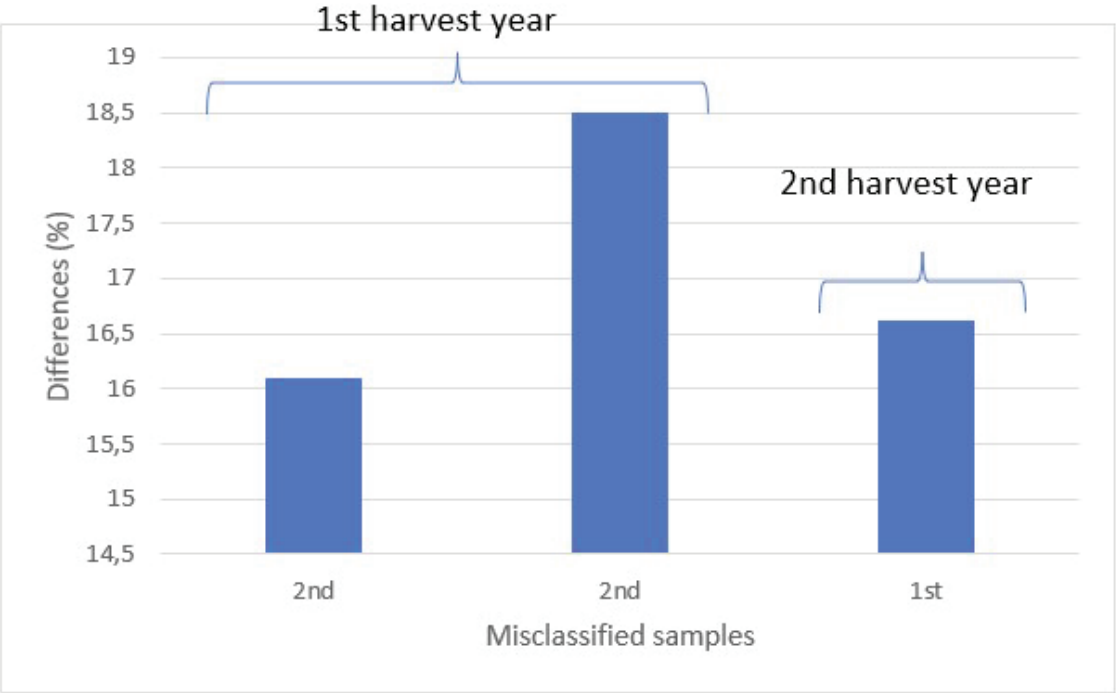


Figure 4.22. Percent probability differences between wrong and right classifications for the misclassified samples in the external validation set for harvest year

It was seen that alkyl esters along with wax content have a potential in olive oil authentication with respect to geographical location and harvest year. Moreover, the results obtained from the correct classification tables (Table 4.9 and Table 4.10) was comparable with fatty acid profile results (Table 4.5 and Table 4.6). Therefore, the fatty

acid esters and waxes could be a promising alternative in authentication purposes of olive oils.

4.1.5. Pigment Content

Well known minor compounds such as polyphenols (Alkan, Tokatli, and Ozen 2012; Bajoub et al. 2016; Nescatelli et al. 2014; Mohamed et al. 2018), sterols (Mohamed et al. 2018; Giacalone et al. 2015) and volatiles (Pouliarekou et al. 2011) have already been used successfully in many PDO studies. However, a limited number of studies in the literature deal with the classification of olive oil by using the pigment profile. Varietal discrimination of olive oil was accomplished by determining the content of some chlorophyll and carotenoid compounds (Cichelli and Pertesana 2004). The effect of harvest year on the main pigments of Italian olive oils was investigated in a recent study (Lazzerini and Domenici 2017). There are also studies based on the determination of total chlorophyll and carotenoid contents (Uncu and Ozen 2016) and/or overall color features (Becerra-Herrera et al. 2018; Borges et al. 2017) in combination with other chemical parameters for geographical classification. However, there are no reports in the literature about using detailed major pigment fractions along with their derivatives in the classification of olive oils with respect to geographical location and/or harvest year.

Nineteen different pigment compounds (11 from carotenoids and 8 from chlorophyll group) including their derivatives were identified and quantified for each olive oil samples from 3 geographical areas in 2 harvest years. A representative pigment profile for an olive oil sample obtained from HPLC analysis is provided before in Figure 3.4 in Materials and Methods section and this profile does not have a peak for β-carotene since it was determined spectrophotometrically. The qualitative pigment profile was the same for all samples, whereas quantitative differences were observed with respect to geographical origin and harvest year. Amounts of the pigments for the samples with respect to their geographical origin and harvest year are listed in Table 4.11.

Table 4.11. Individual pigment profiles of olive oils with respect to geographical location and harvest year

	North Aegean				South Aegean				Middle Aegean			
	2015/16 (n=19)*		2016/17 (n =10)*		2015/16 (n =25)*		2016/17 (n=11)*		2015/16 (n =10)*		2016/17 (n =16)*	
Pigments (mg/kg)[±]	Average	Range	Average	Range	Average	Range	Average	Range	Average	Range	Average	Range
[1]Pheophytin *a*	4.87	0.16-10.69	5.98	1.62-11.37	8.98	3.62-33.04	8.33	3.67-12.97	5.42	1.17-12.81	2.78	1.09-7.05
[2]Pheophytin *a* der.	0.72	0.03-1.77	0.57	0.12-1.40	1.22	0.38-4.46	1.00	0.27-2.59	0.78	0.13-1.76	0.49	0.13-1.35
[3]Chlorophyll *a*	0.02	0.01-0.03	0.04	0.02-0.10	0.05	0.01-0.18	0.04	0.02-0.08	0.02	0.01-0.04	0.07	0.02-0.26
[4]Chlorophyll *a* der.	0.04	0.02-0.06	0.06	0.03-0.10	0.04	0.02-0.12	0.07	0.02-0.12	0.02	0.00-0.03	0.04	0.02-0.09
[5]Pheophytin *b*	0.09	0.04-0.18	0.19	0.06-0.61	0.23	0.05-0.79	0.25	0.09-0.44	0.12	0.06-0.21	0.21	0.08-0.58
[6]Pheophytin *b* der.	0.09	0.02-0.17	0.22	0.04-0.73	0.25	0.05-0.88	0.25	0.06-0.52	0.12	0.04-0.22	0.18	0.04-0.48
[7]Chlorophyll *b*	0.25	0.10-0.54	0.47	0.17-1.40	0.84	0.25-2.96	0.91	0.25-1.57	0.44	0.24-0.67	0.34	0.19-0.72
[8]Chlorophyll *b* der.	0.06	0.02-0.16	0.10	0.03-0.34	0.20	0.04-0.80	0.18	0.05-0.35	0.08	0.03-0.12	0.07	0.03-0.16
[9]Total xanthophylls	1.40	0.26-2.41	1.07	0.54-2.02	1.45	0.56-5.11	1.21	0.60-1.52	0.81	0.30-1.68	0.61	0.40-0.94
[10]Lutein	1.49	0.60-2.01	2.13	1.08-4.64	3.32	1.38-11.00	4.07	1.44-5.89	1.19	0.66-1.72	1.89	1.00-2.86
[11]Lutein der.	0.24	0.06-0.44	0.42	0.18-0.61	0.48	0.08-1.56	0.82	0.26-1.35	0.19	0.10-0.37	0.29	0.16-0.59
[12]Lutein second der.	0.14	0.05-0.23	0.27	0.15-0.67	0.31	0.15-1.12	0.56	0.17-1.38	0.13	0.09-0.20	0.25	0.16-0.53
[13]*β*-carotene	3.18	1.39-6.60	2.40	1.16-3.74	3.56	1.70-7.29	3.13	1.35-5.75	3.13	0.66-4.98	3.30	1.54-6.33
[14]Total chlorophylls	6.14	0.40-13.60	7.63	2.09-16.05	11.81	4.42-43.23	11.03	4.43-18.64	7.00	1.68-15.86	4.18	1.60-10.69
[15]Total carotenoids	6.45	2.36-11.69	6.29	3.11-11.68	9.12	3.87-26.08	9.79	3.82-15.89	5.45	1.81-.8.95	6.34	3.26-11.25

[±]Standard deviations: 0.52[1], 0.11[2], 0.01[3], 0.01[4], 0.02[5], 0.02[6], 0.05[7], 0.02[8], 0.10[9], 0.24[10], 0.08[11], 0.03[12], 0.82[13], 0.75[14], 1.27[15], *n: number of samples

There are limited number of studies in the literature about Turkish olive oil pigment profiles, and existing studies only focused on total color pigment concentrations as total chlorophyll and/or total carotenoid contents rather than single pigments (Uncu and Ozen 2016; Dıraman and Dibeklioğlu 2009). Turkey is one of the major olive oil producer countries in the world, and the locations where the olive oils were obtained in this study are the areas where most of the production is done. North and South Aegean Regions have national designated origin specifications, and Middle Aegean Region oils have unique characteristics. The PDO area might have oils obtained from a single olive cultivar or various cultivars might exist in the same region (Becerra-Herrera et al. 2018). Whereas North and Middle Region oils were from a single variety, South region oils were produced predominately from a particular variety along with some local cultivars. In the present study, it was observed that the averages of total chlorophyll concentrations (mg/kg) of South region olive oils for the first (11.81 mg/kg) and the second harvest year (11.03 mg kg^{-1}) were higher than both harvest years of North (6.14 and 7.63 mg/kg) and Middle region oils (7.00 and 4.18 mg/kg) (Table 4.11). The same observation is also true for average total carotenoids amounts. Whereas South region oils have 9.12 and 9.79 mg/kg total carotenoids in two harvest years, samples belonging to North region have 6.45 and 6.29 mg/kg and the Middle region have 5.45 and 6.34 mg/kg total carotenoids in the consecutive harvest periods, respectively. The average total chlorophyll (4.18-11.81 mg/kg) and carotenoid (5.45-9.79 mg/kg) concentrations in the present study were found to be lower than two different studies investigating Italian olive oil samples. In the first study, the amounts determined were 24.95-31.97 mg/kg chlorophyll and 18.32-27.44 mg/kg carotenoids (Giuffrida et al. 2007), whereas the other study reported 1.00-26.64 mg/kg chlorophyll and 4.19-16.12 mg/kg carotenoids (Giuffrida et al. 2011).

Pheophytin *a* (2.78-8.98 mg/kg) was determined as the major chlorophyll pigment for all regions, followed by lutein (1.19-4.07 mg/kg), *β*-carotene (2.40-3.56 mg/kg), total xanthophylls (0.61-1.45 mg/kg) and pheophytin *a′* (0.49-1.22 mg/kg) whereas the concentrations of the other components were minor in the current research. A study from Sicilian region of Italy (Giuffrida et al. 2007) revealed a similar trend, except there were higher concentrations of pheophytin *a* as the major pigment (19.36–25.04 ppm) and *β*-carotene (8.06–16.27 ppm). Pheophytin *a′* (2.92–4.17 ppm) and lutein (2.28–4.49 ppm) concentrations were close to the findings of the current study. The quantitative differences could be attributed to the harvest periods in each season rather than weather conditions

(Criado et al. 2008) as well as genetic factors and/or geographical differences (Giuffrida et al. 2007).

Multivariate analyses of chromatographic and spectral data were conducted with OPLS-DA rather than PLS-DA due to the proven efficiency of this method in the previous classification studies (Mohamed et al. 2018). First, the olive oil samples were classified with OPLS-DA models (Figure 4.23a-b) according to their geographical location as Middle (M), North (N), and South (S) regardless of the harvest year to observe the effect of individual pigment concentrations on geographical origin determination. Then, the changes in the amounts of the pigments in the first and second harvest years were also investigated by grouping the samples as the first and the second harvest year in OPLS-DA (Figure 4.23c-d) analysis regardless of geographical location.

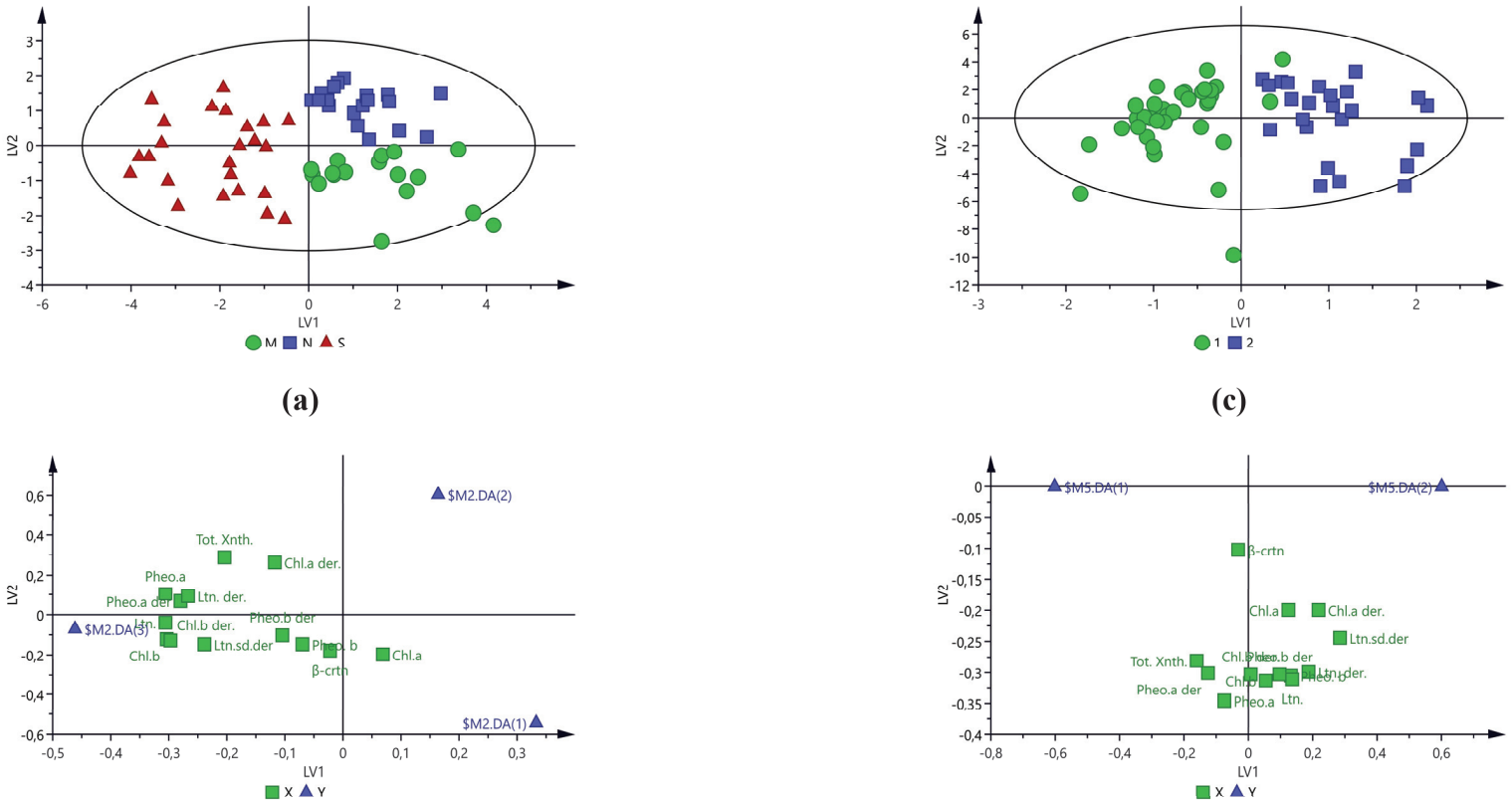


Figure 4.23. OPLS-DA score (a), (c) and loading (b), (d) plots of individual pigment profiles of olive oil samples with respect to geographical location (●M: Middle, ■N: North, ▲S: South) and harvest year (●1: 2015/16, ■2: 2016/17), respectively.

Table 4.12 and Table 4.13 present the statistical parameters of pretreatment type, correct classification rates (%), number of LVs used, and coefficients of determination R^2 for OPLS-DA classification models with respect to both geographical origin and harvest year for individual pigments. Also, percent differences between right and wrong classification for misclassified samples are given in Figure 4.24 and Figure 4.25 for geographical origin and harvest year, respectively.

Table 4.12. Statistical parameters of OPLS-DA calibration and validation models of olive oils with respect to geographical location by using individual pigments

Model	Number of samples	Individual pigments Pre-treatment: none, LVs: 2+2, R^2_{cal}: 0.63, R^2_{cv}: 0.53			
		M	N	S	%CC
Calibration					
M	17	16	0	1	94
N	19	0	19	0	100
S	24	0	1	23	96
Total	60	16	20	24	97
Validation					
M	9	6	2	1	67
N	10	1	7	2	70
S	12	2	1	9	75
Total	31	9	10	12	71

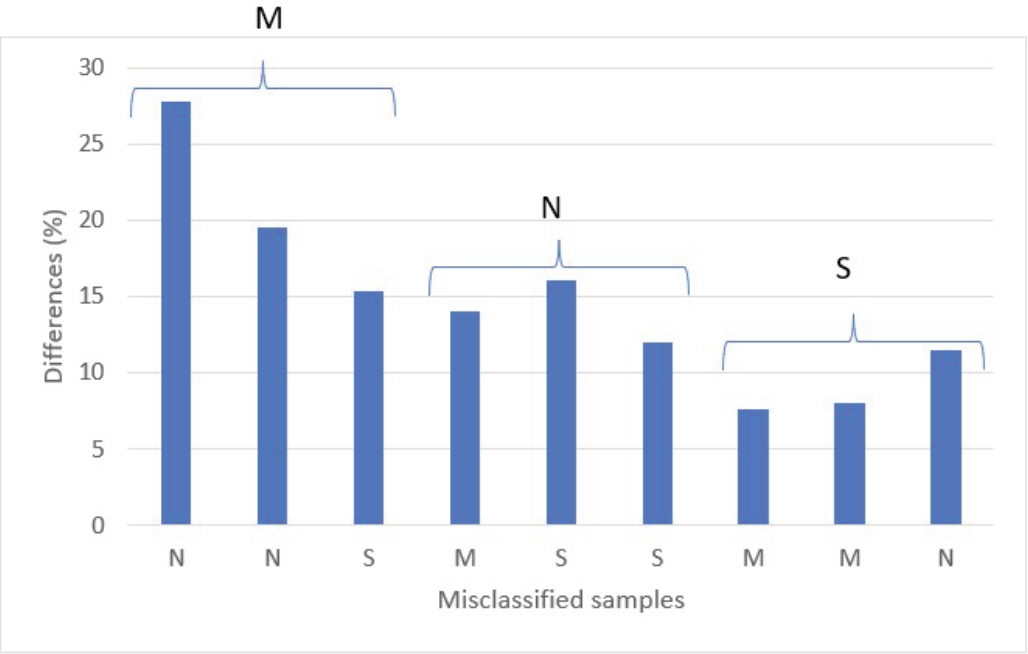


Figure 4.24. Percent probability differences between wrong and right classifications for the misclassified samples in the external validation set for geographical location

Table 4.13. Statistical parameters of OPLS-DA calibration and validation models of olive oils with respect to harvest year by using individual pigments

Model	Number of samples	Individual pigment Pre-treatment: none, LVs: 1+5, R^2_{cal}: 0.74, R^2_{cv}: 0.54		
		2015/16	2016/17	%CC
Calibration				
2015/16	36	34	2	94
2016/17	24	0	24	100
Total	60	34	26	97
Validation				
2015/16	18	18	0	100
2016/17	13	1	12	92
Total	31	19	12	97

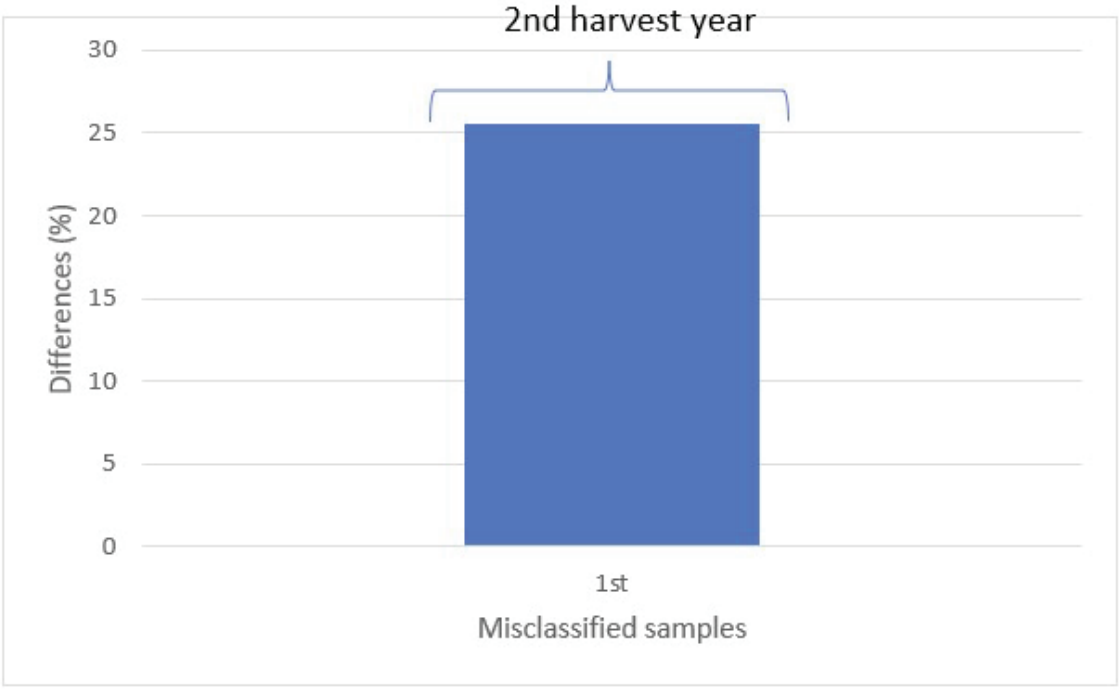


Figure 4.25. Percent probability difference between wrong and right classification for the misclassified samples in the external validation set for harvest year

OPLS-DA score plot (Figure 4.23a), which is generated with raw pigment data only treated with univariate scaling and mean centering, indicated complete discriminations between olive oil samples from S, N, and M regions. OPLS-DA analysis resulted in the classification model with 2 predictive and 2 orthogonal components; in particular, the first two significant LVs explained 63% of the total variance (Table 4.12). As can be seen from the score plot (Figure 4.23a), the S region samples were successfully separated in the left (negative) side of LV1, explaining 53% of the total variation, whereas the N and M region samples were located on the opposite side. Moreover, the latter two regions were separated from each other, with the N region scattered in upper side (positive) of the first quarter of LV2 and the M region in the lower side (negative) of the fourth quarter of LV2. The loading plot (Figure 4.23b) showed that S region was placed apart from the rest in terms of the higher amounts of all pigments except chlorophyll *a*, which is responsible for scattering of the samples of the N region. It can be concluded that M and N regions were more similar in terms of pigment profile while S region was more apart than the rest (Table 4.11).

Furthermore, VIP values were determined to reveal the most significant pigments in differentiation with respect to geographic origin. In multivariate analysis, the VIP

scores are used to summarize the contribution of any variable making the OPLS-DA model, and are based on a weighted sum of the squared correlations between the OPLS-DA components and the original variables (Mohamed et al. 2018). Moreover, VIP values are the most compact model interpretation alternatives in OPLS and PLS evaluation (Galindo-Prieto, Eriksson, and Trygg 2015). In the present study, VIP values of total xanthophylls (1.18), chlorophyll *b* (1.11), pheophytin *a* (1.10), chlorophyll *a* and *b* derivatives (1.08), lutein (1.07), and pheophytin *a* derivative (1.01), which are > 1, were found effective. Lastly, the classification model efficiency was determined with calibration and external validation datasets. As it could be seen from Table 4.12, OPLS-DA model revealed good discrimination ability, with an average correct classification rate of 97% (out of 60 samples; 1 sample was misclassified as S and 1 sample was misclassified as N) and 71% (out of 31 samples; 3 samples for each region were misclassified as N, S, and M) in the calibration and validation datasets, respectively. There are limited number of studies in the literature about the classification and/or differentiation of olive oils with respect to harvest year and/or geographical location by using pigment profile. Most of the studies are based on overall approach which aims at determining the effect of total chlorophyll and carotenoid contents combined with some other minor and major compounds (acidity, peroxide value, K232, K270, ΔK indices, trace elements, fatty acids) on classification (Karabagias et al. 2013). In only one study there were a limited number of components (chlorophylls, pheophytin *a*, pheophytin *b*, lutein) used to observe the discrimination of olive oils according to variety, and some success was obtained with these variables (Cichelli and Pertesana 2004). There is no comparable study about the use of detailed pigment profiles on the determination of geographical origin.

Lastly, the effect of harvest year on the classification of olive oils was also examined. As seen from the OPLS-DA score plot (Figure 4.23c), samples were differentiated clearly with respect to the first LV (except 2 misclassified samples), explaining about 74% of the total variance (LVs= 1+5, R^2_{cal}: 0.74, R^2_{cv}: 0.54) (Table 4.13). From the loading plot (Figure 4.23d), it was observed that the first harvest year samples were separated according to their higher content of *β*-carotene, chlorophyll *b* and its derivative, pheophytin *a* and its derivative, and total xanthophylls located in the negative side of LV1, whereas the second harvest year samples were differentiated with respect to higher amounts of chlorophyll *a* and its derivative, lutein and its derivatives, pheophytin *b* and its derivative, which are scattered in the positive side of LV1. In Table

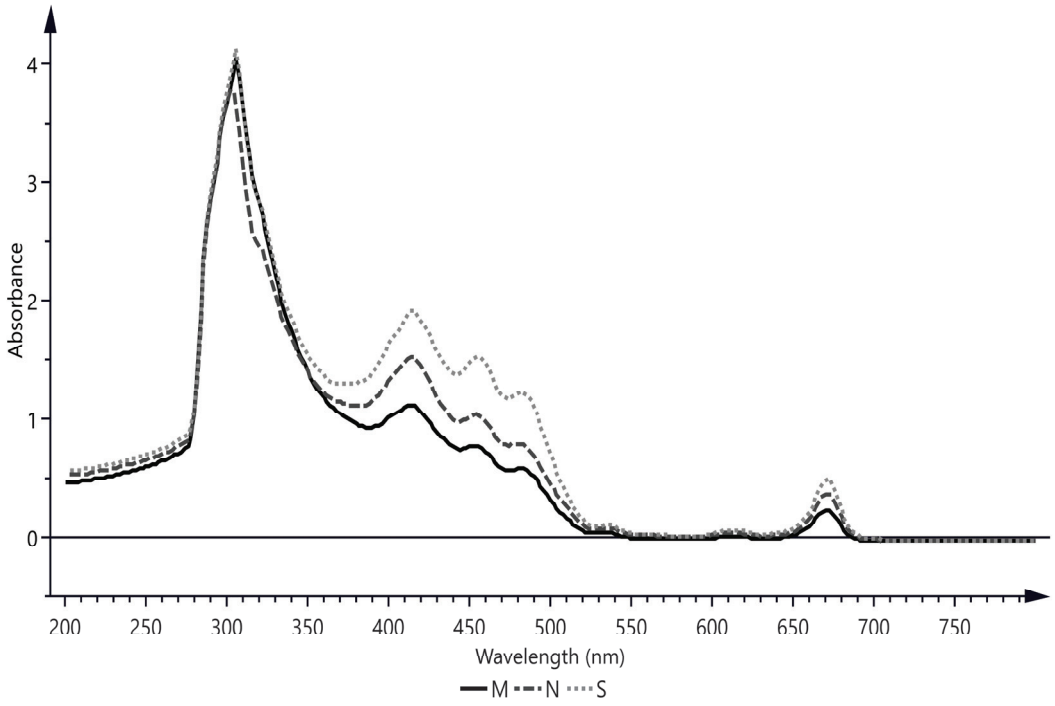
4.13, average correct classification rates of 97% for both calibration (out of 60 samples; 2 samples misclassified as second harvest year) and validation datasets (out of 31 samples; 1 sample was misclassified as first harvest year), further confirming the robustness of the OPLS-DA model. The VIP values were found notable for lutein second derivative (1.55), chlorophyll *a* (1.28), lutein derivative (1.15), total xanthophyll (1.07), and lutein (1.00). In the literature, there is only one recent study that focused on the major pigments *β*-carotene, lutein, pheophytin *a* and *b* to investigate the effect of harvest year (2012, 2013, and 2014) on pigment concentration (Lazzerini and Domenici 2017). The findings of that study showed that olive oils harvested in 2014 could be distinguished successfully with respect to the previous years.

4.1.6. FTIR and UV-Visible Spectroscopic Methods

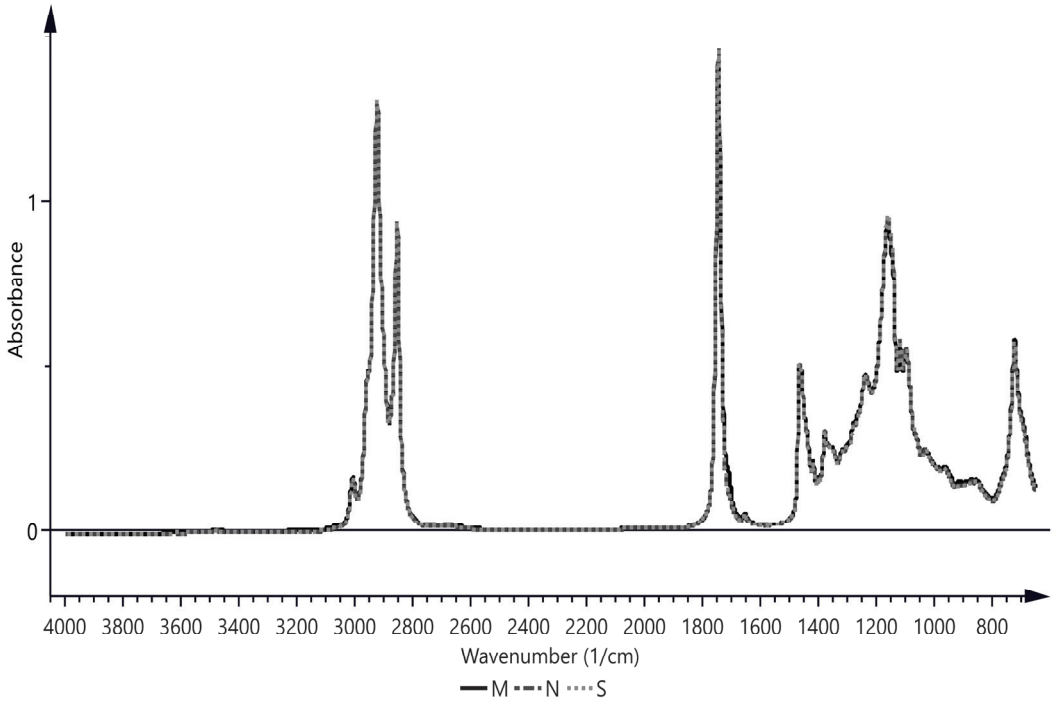
As an alternative to methods based on wet chemistry (Esteki, Shahsavari, and Simal-Gandara 2019; 2020), UV-visible spectroscopy has been preferred in measuring the amounts of absorbing species in food analysis due to its ease of use and non-destructive nature as well as its good sensitivity and accuracy (Esteki, Shahsavari, and Simal-Gandara 2018; Torrecilla et al. 2010a; 2015). However, this technique has been rarely used in geographical classification (Lazzerini, Cifelli, and Domenici 2017) and in harvest year differentiation of olive oils (Lazzerini and Domenici 2017). In a very recent study, fatty acid profiling was compared with UV-visible fingerprints for classification of Moroccan Argan oils (Kharbach et al. 2019), and another study used visible spectroscopy fused with basic quality parameters in classification of Spanish extra virgin olive oils (Pizarro et al. 2013). In addition, as a fast and robust application, FTIR spectroscopy in mid-IR (MIR) region was also compared (Bevilacqua et al. 2012) and combined (Dupuy et al. 2010) with near-IR (NIR) spectroscopy to determine the origin of virgin olive oils. These spectroscopic methods in individual and combined forms were compared with fatty acid profile in characterization of Italian PDO olive oils (Casale et al. 2012). However, FTIR spectroscopy was not directly compared with UV-visible spectroscopy to classify olive oil samples according to harvest year.

Raw UV-visible spectra of the studied samples are shown in Figure 4.26a. Absorption spectra are known to be highly correlated with the mainly pigment profile and the polyphenol content of olive oil. The bands between 300-400 nm are associated with polyphenol contents (Mignani et al. 2012). In addition, maximum absorption for carotenoids were found at 486, 455, and 432 nm for lutein; and 490 and 462 nm for *β*-carotene. Absorptions at 670 and 414 nm; 657 and 437 nm were correlated with pheophytin *a* and *b*, respectively (Domenici et al. 2014).

Raw FTIR spectra of all the studied olive oil samples are presented in Figure 4.26b. Major peaks at distinct wavenumbers 2924, 2852, 1743, 1463, 1377, 1238, 1163, 1114, 1099 and 721 cm^{-1} are correlated with certain vibration modes of the molecular bonds. Absorbances at 2924 cm^{-1} and 2852 cm^{-1} wavelengths are the result of -CH_2 asymmetric and symmetric stretching vibrations, respectively (Sinelli et al. 2007). A sharp peak around 1745 cm^{-1} is associated with C=O stretching vibration of carbonyl groups of the triacylglycerols and known as ester peak (de la Mata et al. 2012) while smaller peaks at 1463 cm^{-1} and 1377 cm^{-1} are known for CH_2 and CH_3 scissoring vibrations, respectively (Sinelli et al. 2007). The rest of the peaks are mainly correlated with C-O stretching vibration.



(a)



(b)

Figure 4.26. Average raw spectra of olive oil samples obtained from M: Middle, N: North, S: South regions by using (a) UV-visible and (b) FTIR spectroscopy.

The classification power of FTIR and UV-visible spectroscopy separately and in combined form with regard to geographical origin and harvest year differentiation of olive oil samples was also investigated. OPLS-DA models (Figure 4.30) were constructed to see the success of each of these techniques for the differentiation. Statistical parameters of each spectroscopic technique for geographic location and harvest year are given in Table 4.14-Table 4.16 and Table 4.17-Table 4.19, respectively. Also, percent differences between right and wrong classification for the misclassified samples for external validation sets were given in Figure 4.27-Figure 4.29.

Table 4.14. Statistical parameters of OPLS-DA calibration and validation models of olive oils with respect to geographical location by using UV-visible spectroscopy

Model	Number of samples	UV-visible Pre-treatment: SD:SNV, LVs: 2+3, R^2_{cal}:0.77, R^2_{cv}: 0.61			
		M	N	S	%CC
Calibration					
M	17	14	2	1	82
N	19	1	18	0	95
S	24	0	0	24	100
Total	60	15	20	25	93
Validation					
M	9	6	2	1	67
N	10	1	8	1	80
S	12	0	3	9	75
Total	31	7	13	11	74

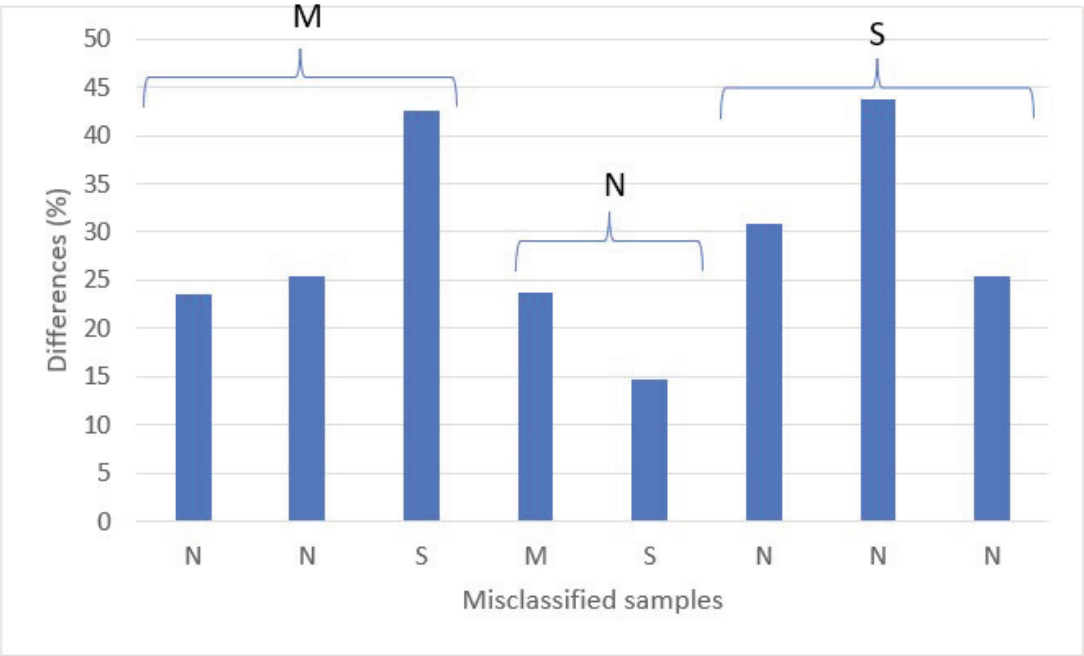


Figure 4.27. Percent probability differences between the wrong and right classifications for the misclassified samples in the external validation set of UV-vis data for geographical location

Table 4.15. Statistical parameters of OPLS-DA calibration and validation models of olive oils with respect to geographical location by using FTIR

Model	Number of samples	FTIR Pre-treatment: SD, LVs: 2+1, R^2_{cal}: 0.90, R^2_{cv}: 0.55			
		M	N	S	%CC
Calibration					
M	17	17	0	0	100
N	19	0	19	0	100
S	24	0	0	24	100
Total	60	17	19	24	100
Validation					
M	9	7	1	1	78
N	10	0	7	3	70
S	12	0	1	11	92
Total	31	7	9	15	81

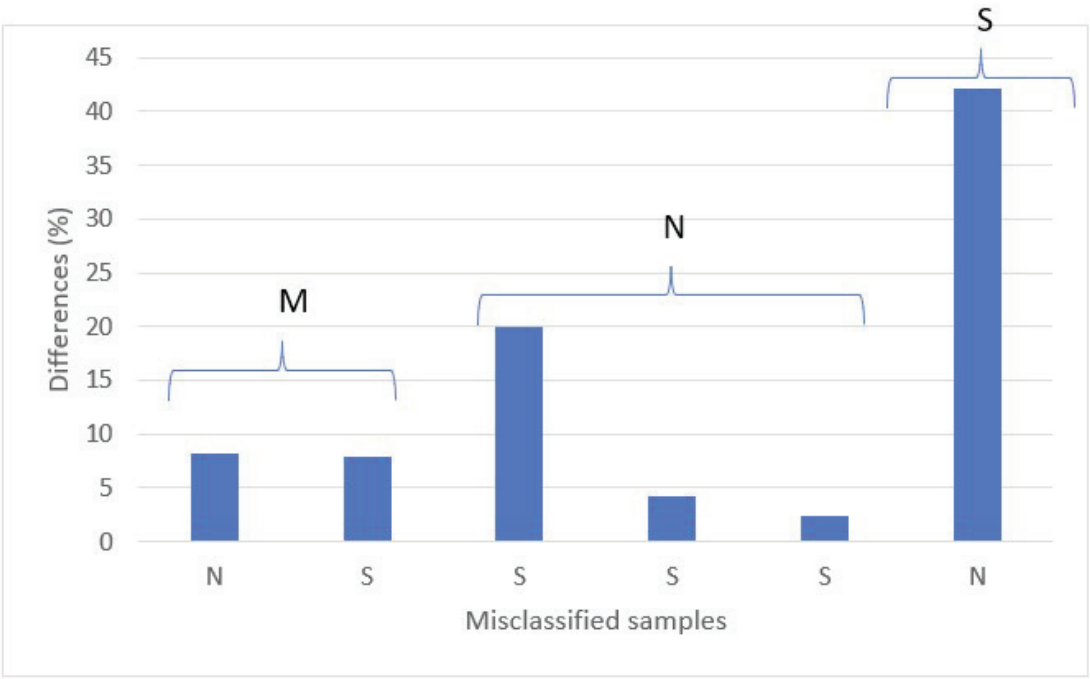


Figure 4.28. Percent probability differences between the wrong and right classifications for the misclassified samples in the external validation set of FTIR data for geographical location

Table 4.16. Statistical parameters of OPLS-DA calibration and validation models of olive oils with respect to geographical location by using FTIR+UV-visible

Model	Number of samples	FTIR+UV-visible Pre-treatment: SD, LVs: 2+2, R^2_{cal}: 0.91, R^2_{cv}: 0.57			
		M	N	S	%CC
Calibration					
M	17	17	0	0	100
N	19	0	19	0	100
S	24	0	0	24	100
Total	60	17	19	24	100
Validation					
M	9	8	1	0	89
N	10	0	9	1	90
S	12	1	0	11	92
Total	31	9	10	12	90

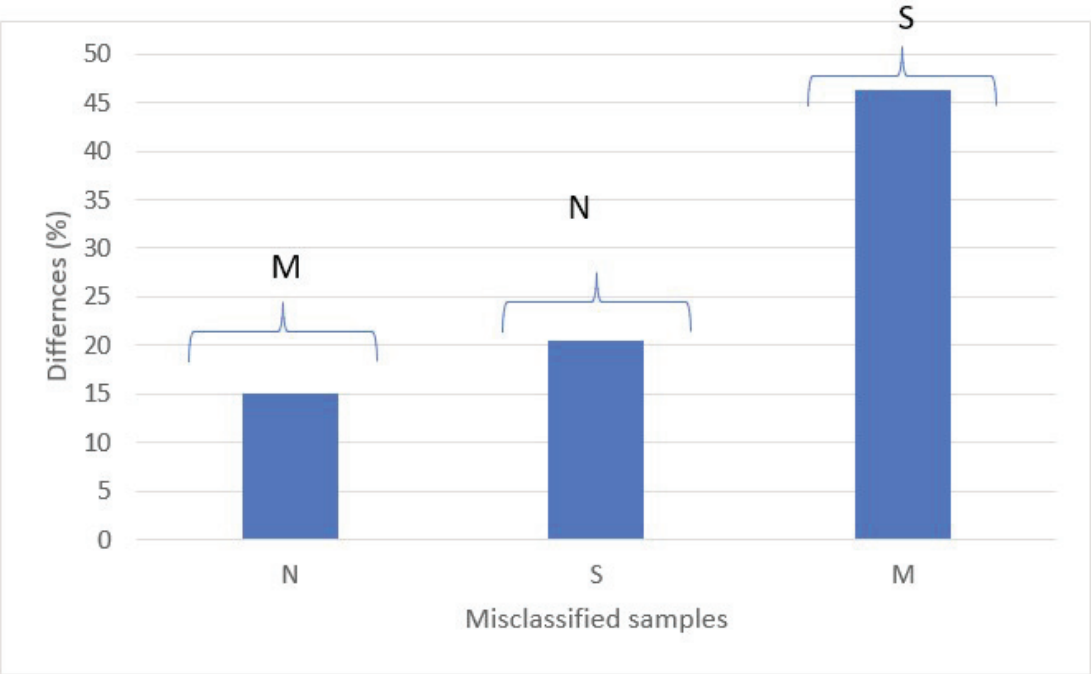


Figure 4.29. Percent probability differences between the wrong and right classifications for the misclassified samples in the external validation set of UV-vis+FTIR data for geographical location

Table 4.17. Statistical parameters of OPLS-DA calibration and validation models of olive oils with respect to harvest year by using UV-visible

Model	Number of samples	UV-visible Pre-treatment: SD:SNV, LVs: 1+4, R^2_{cal}: 0.99, R^2_{cv}: 0.95		
		2015/16	2016/17	%CC
Calibration				
2015/16	36	36	0	100
2016/17	24	0	24	100
Total	60	36	24	100
Validation				
2015/16	18	18	0	100
2016/17	13	0	13	100
Total	31	18	13	100

Table 4.18. Statistical parameters of OPLS-DA calibration and validation models of olive oils with respect to harvest year by using FTIR

Model	Number of samples	FTIR Pre-treatment: SD, LVs: 1+2, R^2_{cal}: 0.99, R^2_{cv}: 0.76		
		2015/16	2016/17	%CC
Calibration				
2015/16	36	36	0	100
2016/17	24	0	24	100
Total	60	36	24	100
Validation				
2015/16	18	18	0	100
2016/17	13	0	13	100
Total	31	18	13	100

Table 4.19. Statistical parameters of OPLS-DA calibration and validation models of olive oils with respect to harvest year by using FTIR+UV-visible

Model	Number of samples	FTIR+UV-visible Pre-treatment: SD, LVs: 1+2, R^2_{cal}: 0.99, R^2_{cv}: 0.85		
		2015/16	2016/17	%CC
Calibration				
2015/16	36	36	0	100
2016/17	24	0	24	100
Total	60	36	24	100
Validation				
2015/16	18	18	0	100
2016/17	13	0	13	100
Total	31	18	13	100

Derivatized and transformed (SD:SNV) spectra of the olive oil samples were used in UV-visible spectroscopic data evaluation. As OPLS-DA models created for geographic origin (LVs= 2+3, R^2_{cal} = 0.77, and R^2_{cv} = 0.61, Table 4.14) and harvest year (LVs= 1+4, R^2_{cal} = 0.99, and R^2_{cv} = 0.95, Table 4.17) indicated, this spectroscopic technique was found successful in the classification of olive oils using these variables.

The first two LVs of OPLS-DA model from UV-visible data (Figure 4.30a) used in the geographical origin differentiation explained 77% of the total variance, with 93% and 74% correct classification for calibration and external validation, respectively, and this classification pattern was found very similar to one with the pigment profile (Figure

4.23a). This could be attributed to high correlation between UV-visible data and the pigment profile. Table 4.14 showed 93% (out of 60 samples; 1 sample misclassified as S, 2 sample misclassified as N, and 1 sample misclassified as M) and 74% (out of 31 samples; 2 sample misclassified as S, 5 sample misclassified as N, and 1 sample misclassified as M) correct classification for calibration and external validation datasets, respectively. In detail, according to the score plot (Figure 4.30a), all S samples were correctly classified (100%) in the left (negative) side of LV1, whereas the N samples were successfully scattered (95%) in the first quartile with respect to positive LV2 except one misclassified sample. The M samples were also correctly classified up to an extent (82%) and they are placed on the fourth quartile of negative LV2, and 2 samples and 1 sample being misclassified as N and S, respectively (Table 4.14).

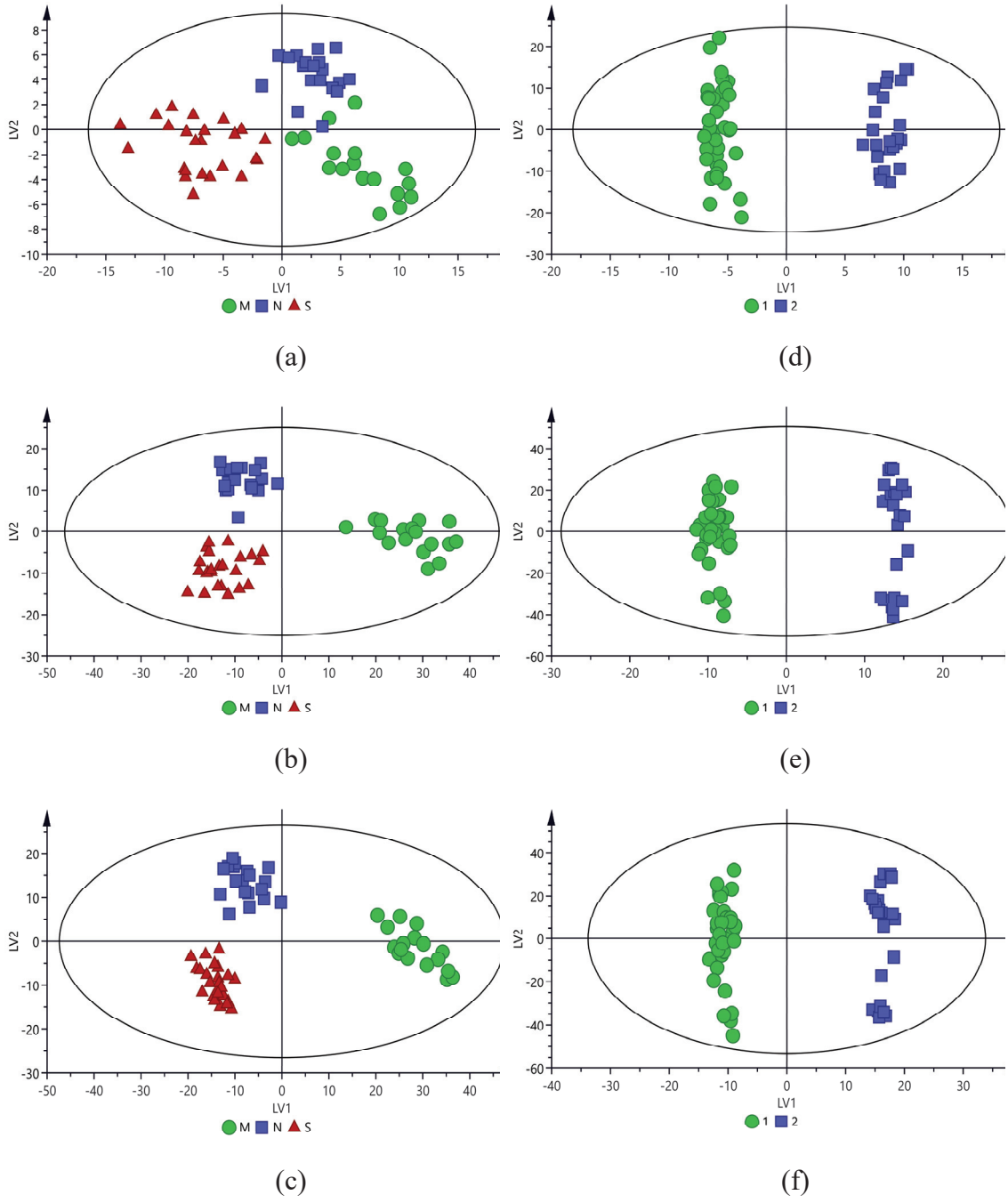


Figure 4.30. OPLS-DA score plots of olive oil samples with respect to geographical location ((a), (b), and (c)) as ●M: Middle, ■N: North, ▲S: South and harvest year ((d), (e), and (f)) as ●1: 2015/16, ■2: 2016/17 by using UV-visible, FTIR and FTIR+UV-visible spectroscopy, respectively.

It is also important to figure out which wavenumbers and/or wavelengths are important in classification. Therefore, VIP values for the corresponding spectral data were also investigated. Owing to the clustered information in the OPLS-DA loading plots of the spectral data, it was not easy to interpret results by visual inspection of the plot.

Hence, VIP values of the corresponding spectra were evaluated. The highest VIP values were observed at some specific bands between 300-400 and 430-460 nm and also the peak at 670 nm. These bands were correlated with a variety of polyphenols (300-400nm), color pigments, carotenoids (430-460 nm), and chlorophylls and their derivatives (670 nm) (Mignani et al. 2012).

The ability of UV-visible spectra to differentiate olive oils with respect to harvest year was also investigated. Clear separation was observed for the first and the second harvest year samples according to LV1 scattered in the left (negative) and right (positive) side of the OPLS-DA plot (Figure 4.30d), respectively. The first two LVs explained 91% of the total variance. There were no misclassified samples from each geographical region in either the calibration or validation datasets; therefore, a 100% correct classification rate is achieved (Table 4.17). Examination of the VIP values resulted in the same spectral region described in the above geographical classification. In the literature, UV-visible spectroscopy has been used in harvest year classification of olive oils only in few studies (Lazzerini and Domenici 2017), and there is no comparison of it with pigment profile in terms of differentiation power for geographical origin and/or harvest year.

The second derivative (SD) of FTIR spectroscopic data was also used in geographical classification of the samples. Score plot presented in Figure 4.30b explained 90% of the total variance with perfect separation (100%) for all of the regions studied according to the third quartile (lower) of negative LV2 for S, second quartile (upper) of positive LV2 for N, and right side of positive LV1 for M regions. The model was constructed with 2 predictive and 1 orthogonal component having R^2 of 0.90 and 0.55 for calibration and cross validation, respectively (Table 4.15). Statistical values of OPLS-DA models (Table 4.15) indicated robust discrimination ability with an average correct classification rate of 100% (out of 60 samples; none of the samples were misclassified) and 81% (out of 31 samples; 2 samples misclassified as N and 4 samples misclassified as S) in the calibration and validation data sets, respectively. The highest VIP values, which are >1 in the fingerprint region (1464–983 cm^{-1}), are attributed to the common bending and rocking vibrations of functional groups (Jolayemi et al. 2017) as well as C=O double bond stretching (≈1700 cm^{-1}), C-H bending (650–750 cm^{-1}), and C-H stretching (2800–3100 cm^{-1}) (Bevilacqua et al. 2012).

FTIR spectroscopy was also studied and found successful in the differentiation of olive oils with respect to harvest year. The first two variables of the OPLS-DA model explained 94% of the variance with clear separation of the first and the second harvest

years, which are placed on the left (negative) and the right side (positive) of LV1, respectively (Figure 4.30e). Both calibration and external validation models confirmed the differentiation clearly at 100% correct classification with LVs: 1+2, R^2_{cal}: 0.99, R^2_{cv}: 0.76 (Table 4.18). The same VIP values explained above were applicable also in harvest year differentiation. As a result, FTIR spectroscopy was better at discriminating olive oils with respect to geographical origin and similar in harvest year differentiation when compared with pigment profile and UV-visible spectroscopy. In the literature, this technique was also successfully used in classification of North and South Aegean olive oils according to harvest year and geographical location (Gurdeniz, Ozen, and Tokatli 2010) and, high discriminatory power of FTIR was also proven for smaller regions (Uncu and Ozen 2016). However, it has never been compared with UV-visible spectroscopy and/or pigment profile before.

It is also useful to couple several spectroscopic methods as a simple unique model to get as much information as possible from each model. This technique is called as low-level data fusion (Borràs et al. 2015). In the present study, UV-visible and FTIR spectroscopic data were combined after the application of SD. When compared to the previous results, efficiency of models in classification according to geographical location (Figure 4.30c with LVs: 2+2, R^2_{cal}: 0.91, R^2_{cv}: 0.57) and harvest year (Figure 4.30f with LVs: 1+2, R^2_{cal}: 0.99, R^2_{cv}: 0.85) increased with fused data as indicated in Table 4.16 and Table 4.19, respectively.

The findings were further confirmed with the first two LVs used in the OPLS-DA model for geographical differentiation (Figure 4.30c). Two LVs explained 91% of the total variance, and samples belonging to M, N, and S regions were flawlessly classified with respect to the right (positive) of LV1, the second quartile (positive) of upper LV2, and the third quartile (negative) of lower of LV2, respectively. In addition, robust average correct classification rates supporting the clear separation in both calibration (100%) and external validation (90%) were obtained, as shown in Table 4.16.

The effect of harvest year was also investigated with combined spectroscopic data. It was seen that perfect separation was achieved according to the OPLS-DA plot (Figure 4.30f), in which the first two LVs explained 96% of the total variance and the samples from the first and the second harvest year are located at the left (negative) and the right (positive) of LV1, respectively. 100% of the samples were classified correctly in both calibration and external validation sets (Table 4.19).

In the literature, there are few classification studies using combination of spectroscopic methods as well as their comparison with wet chemical data. Recently, the potentials of fused FT-NIR and FTIR spectroscopy, and electronic nose (e-nose) on varietal classification of Turkish olive oils were demonstrated (Jolayemi et al. 2017). In another study, MIR, NIR and UV–visible spectroscopic data were used to classify olive oils from Italy in comparison with their gas chromatographic fatty acid profile (Casale et al. 2012). Nevertheless, FTIR and UV-visible spectroscopies were not fused and/or compared with pigment data before in literature.

To sum up, FTIR as a fingerprinting technique has a slightly better differentiation ability than the wet chemical method in classification studies. The same type of conclusion was also reached by Dais and Hatzakis (2013) and it was indicated that metabolic fingerprinting of the unsaponifiable fraction of olive oils had better discriminatory characteristics than metabolic profiling of the same fraction of the olive oils.

4.2 Conclusions

In this part, basic quality parameters, fatty acid profile, DAGs, FAAEs and waxes, pigments and spectroscopic profiles were used to characterize and differentiate Aegean region olive oil samples in terms of geographic location and harvest year. Results revealed that FTIR+UV-vis spectroscopy was the most successful tool in both types of classification. Whereas pigment profile has comparable results with fatty acid profile and alkyl esters including wax content in differentiation of olive oils with respect to geographical origin and harvest year. Therefore, it could be concluded that spectroscopic methods offer advantages over wet chemical techniques in authentication purposes. However, the classification power of wet chemical techniques in combination form should not still be underestimated.

CHAPTER 5

RESULTS AND DISCUSSION

PREDICTION OF OLIVE OIL CHEMICAL PARAMETERS WITH SPECTROSCOPIC METHODS

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5.1. Prediction of Chemical Parameters

The standard analysis methods used in determination of the measured chemical parameters in the previous chapter were based on high-cost wet chemistry techniques which produce waste and have long analysis time. Rapid, environmentally friendly and non-destructive spectroscopic analysis techniques such as mid-infrared (mid-IR) spectroscopy have been used to determine various important quality and/or purity parameters of olive oils such as fatty acid profile (Gurdeniz, Ozen, and Tokatli 2010; Uncu and Ozen 2015), oxidative stability, phenolic profile and total color pigments (Uncu and Ozen 2015). UV-visible (UV-vis) spectroscopy has been also used in authentication

studies of olive oil (Casale et al. 2007) while there are quite limited studies about its application as a quality tool for olive oil (Gonçalves et al. 2018).

Mid-IR spectroscopy was used in the prediction of the total chlorophyll and carotenoid contents of olive oils rather than individual color pigments (Uncu and Ozen 2015). A recent study successfully correlated near UV-vis spectroscopy measurements with chromatographic results of main color pigments of olive oil (Lazzerini, Cifelli, and Domenici 2017). However, there is no study in the literature that predicts the individual color pigment profile of olive oil with FTIR and/or UV-vis spectroscopy. A preliminary study that successfully quantified FAAE content and the ratio between ethyl and methyl esters of olive oil using mid-IR spectroscopy with limited number of samples was also conducted (Valli et al. 2013). Techniques such as near-infrared spectroscopy (Garrido-Varo et al. 2017; Cayuela 2017) and time domain reflectometry (Berardinelli et al. 2013) were used to predict FAEE and FAME content in some recent studies. However, no studies found in the literature regarding the estimation of 1,2 DAGs in oils with mid-IR and UV-vis spectroscopic techniques.

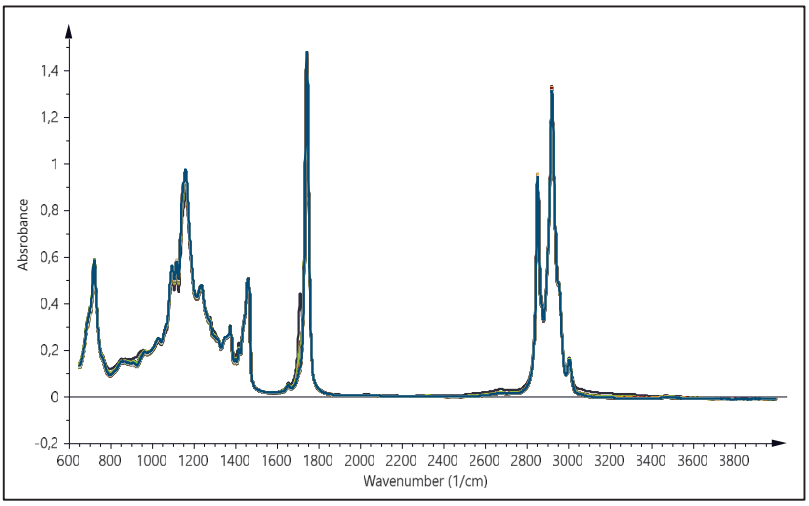
Aim of the present part is to predict several measured chemical parameters (DAGs, FAEEs, wax and individual pigment profile) of olive oil from UV-vis and mid-IR spectroscopic data as well as their fused form in combination with multivariate statistical methods. As a result, these chemical parameters could be determined simultaneously with a single measurement by using the developed methodology.

5.1.1. Chemical Interpretation of Spectral Data

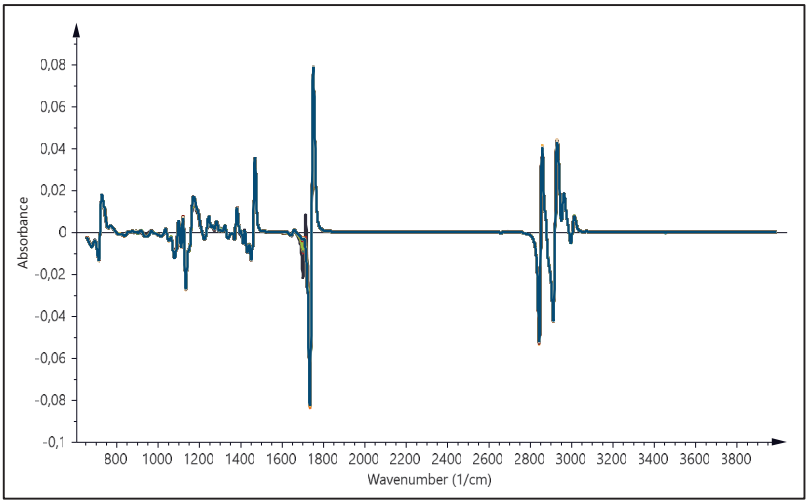
Raw and transformed forms of FTIR spectra of olive oil samples are shown in Figure 5.1a-c. Major peaks in the spectra and vibration modes of corresponding functional groups could be summarized as follows; band at 3009 cm^{-1} is due to C-H stretching of olefinic double bonds attributed to unsaturated fatty acids, while bands centered at 2924 and 2854 cm^{-1} known as methylene absorbance peaks are associated with antisymmetric and symmetric stretching vibrations of aliphatic C-H in $-CH_2$ and terminal $-CH_3$ groups, respectively (Niu et al. 2017). In addition, sharp peak at about 1745 cm^{-1} is an ester peak because of C=O stretching vibration of carbonyl groups of the triacylglcerols while weak

band at 1654 cm^{-1} is attributed to stretching vibration of the C=C group of cis-olefins (de la Mata et al. 2012). Bands in fingerprint region of 1464–983 cm^{-1} are assigned to bending vibrations of -CH_2 and -CH_3 aliphatic groups as well as rocking vibrations (de la Mata et al. 2012; Jolayemi et al. 2017). Symmetric H-C-H bending at 1377 cm^{-1} could be attributed to glycerol group, O-CH_2 (mono-, di- and triglycerides) (Rabelo et al. 2015). CH_2 scissoring are observed at 1462 cm^{-1} whereas band between 1125 and 1095 cm^{-1} wavenumber is due to the stretching vibration of C=O ester groups and -CH_2 wag (de la Mata et al. 2012). The last major peak located near 723 cm^{-1} could be associated with overlapping of the $(CH_2)_n$ rocking vibration and out of plane vibration (-CH wag) of cis-di-substituted olefins (de la Mata et al. 2012).

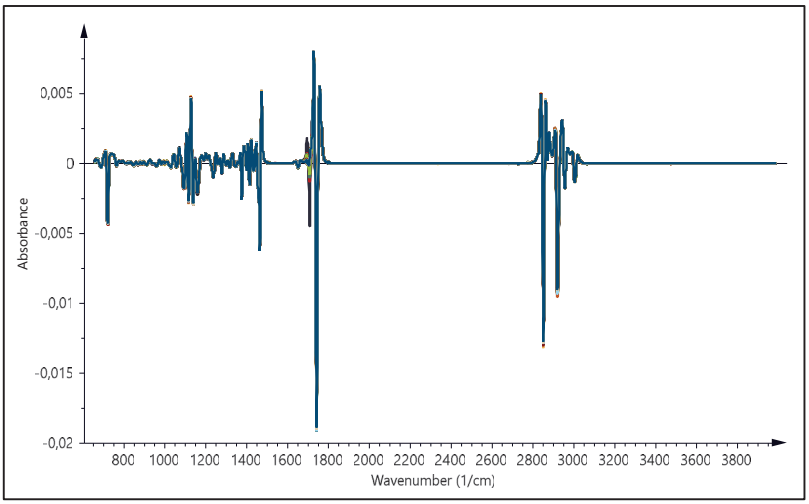
Typical UV-vis spectra of olive oil and their transformed forms are shown in Figure 5.2 and they are highly correlated with pigment profile. Especially, pigments (chlorophyll and carotenoid) of olive oil dominate the light absorption between 390−720 nm. Maximum absorption for lutein, *β*-carotene, pheophytin *a* and pheophytin *b* were detected in the following wavelengths: 486, 455, and 432 nm; 490 and 462 nm; 670 and 414; 657 and 437 nm, respectively (Domenici et al. 2014).



(a)



(b)



(c)

Figure 5.1 (a) Raw, (b) first derivative and (c) second derivative of FTIR spectra of olive oil samples.

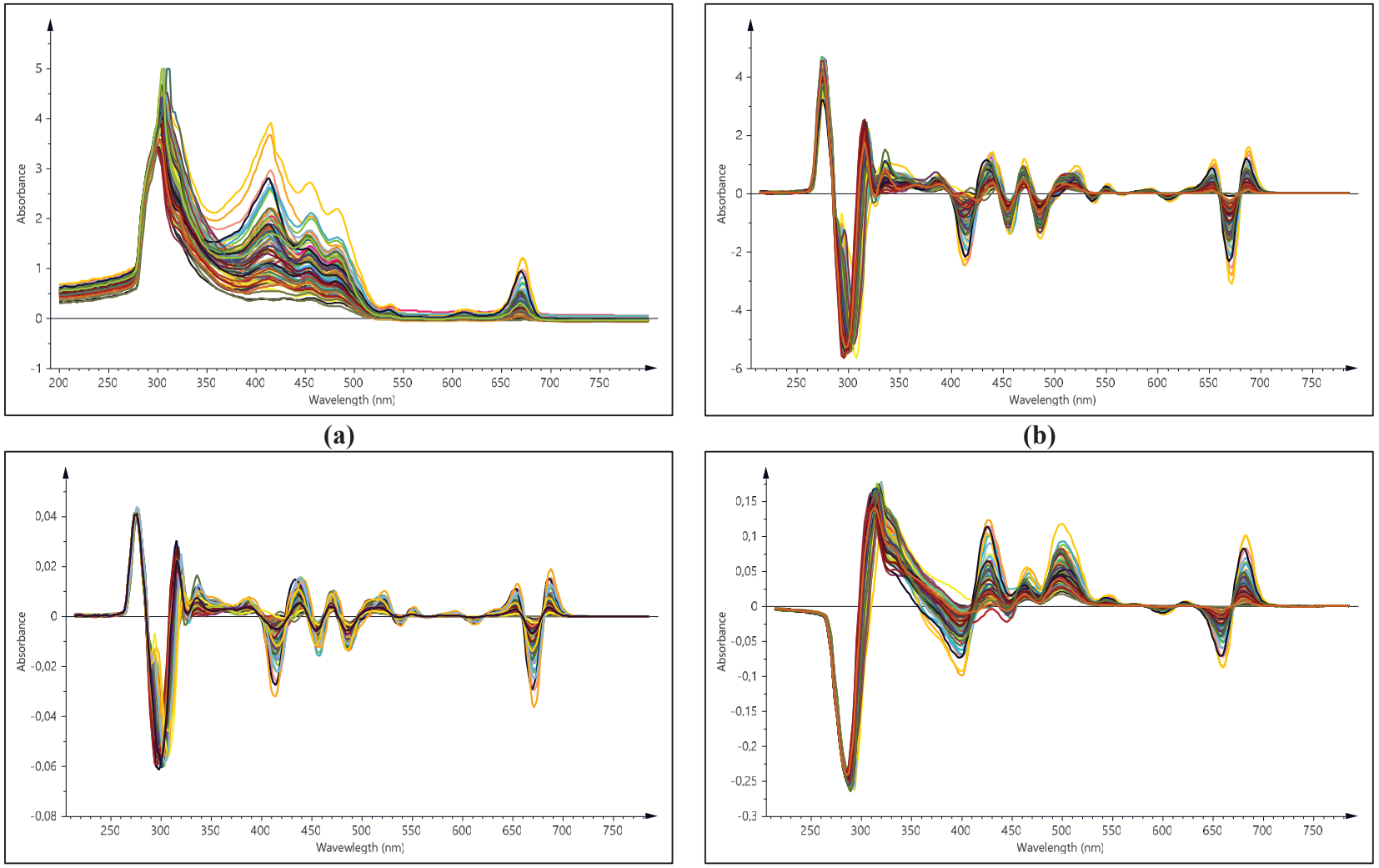


Figure 5.2. (a) Raw, (b) first derivative, (c) second derivative and (d) second derivative + MSC + SNV of UV-vis spectra of olive oil samples.

5.1.2. Prediction of FAMEs, FAEEs, FAAEs and Waxes by PLS regression

FAAE and wax contents of the olive oil samples were quantified with the reference methods. Then, PLS regression analysis of FTIR, UV-vis, and fused spectral data was performed to predict FAAEs including FAMEs and FAEEs as well as wax content of olive oils. Ranges and means of reference data are presented in Table 5.1. These values are comparable with the ranges found in the literature (Cayuela 2017; Garrido-Varo et al. 2017). Second-order derivative of FTIR spectra is shown in Figure 5.1c and, second order derivative + MSC + SNV were used in alkyl ester and wax prediction from UV-vis (Figure 5.2d) and FTIR+UV-vis spectral data since they resulted in the development of the best models.

Table 5.1. Range and mean of fatty acid alkyl esters and wax (mg/kg) of olive oil samples.

Measured parameters	Range	Mean	Standard deviation
FAMEs	3.14-539.04	46.44	68.89
FAEEs	1.66-243.59	48.45	62.14
FAAEs	6.94-659.00	94.88	120.42
Waxes	5.26-89.59	26.96	17.84

Statistical parameters of regression models for each spectroscopic approach are listed in Table 5.2. FTIR spectral data were found successful in quantification of FAMEs (3.14–539.04 mg/kg) with 3 LVs explaining 99.4% and 92.6% of the total variance in the calibration and prediction data set, respectively. In addition, R^2_{cal} and R^2_{pred} have high values of 0.99 and 0.93, respectively also with a high RPD value (3.1) required for a successful prediction model. Moreover, RMSEC and RMSEP values (6.06 and 16.97, respectively) were reasonable when compared with the range and magnitude of FAMEs.

UV-vis spectra of olive oil samples were also used to predict FAMEs. The regression models showed that UV-vis spectra were not that successful compared to FTIR spectral data in prediction with lower statistical values (R^2_{cal} =0.72, R^2_{cv}=0.60, R^2_{pred}=

0.47, and RPD=1.3) including 2 LVs which explains 71.8% and 46.8% of calibration and prediction sets, respectively.

Table 5.2. Statistical parameters of PLS regression models for prediction of fatty acid alkyl ester and wax contents of olive oils by different spectroscopic methods.

Method	Parameter (mg/kg)	Transformation	LVs[1]	R^2_{cal}[2]	R^2_{cv}[3]	R^2_{pred}[4]	RMSEC[5]	RMSECV[6]	RMSEP[7]	RPD[8]	Slope
FTIR	FAMEs	2nd order derivative	3	0.99	0.87	0.93	6.06	41.63	16.97	3.1	0.99
	FAEEs	2nd order derivative	4	0.99	0.85	0.88	4.92	27.43	23.64	2.8	0.99
	FAAEs	2nd order derivative	3	0.99	0.87	0.96	13.73	60.10	26.69	4.1	0.99
	Waxes	2nd order derivative	4	0.99	0.77	0.71	1.80	9.45	11.70	1.7	0.99
UV-vis	FAMEs	2nd order+MSC+SNV	2	0.72	0.60	0.47	24.41	28.54	77.86	1.3	0.72
	FAEEs	2nd order+MSC+SNV	3	0.77	0.63	0.78	30.62	37.55	30.49	2.1	0.77
	FAAEs	2nd order+MSC+SNV	3	0.78	0.61	0.74	59.27	78.49	61.07	1.9	0.78
	Waxes	2nd order+MSC+SNV	2	0.71	0.60	0.61	10.32	11.95	11.01	1.4	0.71
FTIR+UV-vis	FAMEs	2nd order+MSC+SNV	3	0.99	0.89	0.91	5.39	40.39	17.83	2.9	0.99
	FAEEs	2nd order+MSC+SNV	4	0.99	0.84	0.90	5.76	26.52	21.98	3.0	0.99
	FAAEs	2nd order+MSC+SNV	2	0.96	0.86	0.96	26.78	54.85	32.45	3.4	0.96
	Waxes	2nd order+MSC+SNV	3	0.95	0.64	0.75	3.90	10.74	9.75	1.9	0.95

[1]LVs: latent variables, [2]R^2_{cal}: regression coefficient for calibration, [3]R^2_{cv}: regression coefficient for cross validation, [4]R^2_{pred}: regression coefficient for prediction, [5]RMSEC: root mean square error of calibration, [6]RMSECV: root mean square error of cross-validation, [7]RMSEP: root mean square error of prediction, [8]RPD: residual predictive deviation

Combination of FTIR and UV-vis spectra were also used to investigate if there was any improvement of the constructed models. It was observed that FTIR+ UV-vis data provided similar prediction ability on determination of FAMEs content of olive oils compared to FTIR spectroscopy alone (Table 5.2). FAMEs could be predicted well with robust model parameters (R^2_{pred}= 0.91 and RPD = 2.9). There is no study in the literature related with direct determination of total methyl ester content of olive oils with FTIR and/or UV-vis spectroscopy while some other studies applied other methods such as near-infrared (NIR) spectroscopy and time domain reflectometry (TDR). A recent study showed that NIR spectroscopy in combination with PLS regression could predict FAMEs content of olive oils quite successfully with high R^2 value for both calibration (0.95) and validation (0.92) set (Cayuela 2017). Also, TDR was found as a promising method in quantification of FAMEs content of olive oils with PLS regression model having good R^2 value for both calibration (0.996) and external validation (0.905) (Berardinelli et al. 2013).

FAEE is a chemical parameter that is used in regulations about the quality of olive oil (Commission Delegated Regulation (EU) 2016) and it was also predicted using different spectral techniques. It was found that FTIR was successful in predicting total ethyl esters (1.66–243.59 mg/kg) found in olive oil samples with 4 LVs explaining 99.4% and 87.7% of calibration and prediction models, respectively. R^2_{cal} of 0.99, R^2_{cv} of 0.85 and R^2_{pred} of 0.88 were determined and these values indicate good prediction ability (Table 5.2). The model performance was also supported by tolerable error values of RMSE for calibration (4.92), cross validation (27.43), and prediction (23.64) with robust RPD of 2.8 and slope of 0.99 values.

The regression models developed using UV-vis spectra for the prediction of FAEEs content have average statistical values (R^2_{cal} =0.77, R^2_{pred}= 0.78, and RPD=2.1) with 3 LVs which explains 76.8% and 77.7% of calibration and prediction sets, orderly (Table 5.2).

Combination of FTIR+UV-vis spectral data performs well and is slightly better than FTIR in quantification of FAEEs with higher R^2_{pred}= 0.90 and RPD=3.0 as well as lower RMSEP value of 21.98 (Table 5.2). The FTIR and fused data findings were comparable with the literature in which NIR spectroscopy and TDR were used. Two different studies conducted by NIR spectroscopy reveals that NIR could be used in FAEEs prediction promisingly (Cayuela 2017; Garrido-Varo et al. 2017). PLS model parameters in a study using NIR spectroscopy (Cayuela 2017) resulted in R^2_{pred}=0.88 and 0.89 values

and generated models in another study with NIR spectroscopy had R^2_{cv}=0.73 and RPD=1.92 (Garrido-Varo et al. 2017). TDR provided a robust PLS regression model for alkyl ester determination (R^2_{pred}= 0.923) with very limited number of samples (Berardinelli et al. 2013).

Total alkyl ester (FAMEs + FAEEs) content of olive oils was also determined by FTIR spectroscopy in combination with PLS regression. Oil samples used in this study has a wide range of FAAE content (6.94–659.00 mg/kg). As in other parameters FTIR provided successful quantification of FAAEs. Constructed PLS model contains 3 LVs explaining 98.9%, 87.3%, and 95.7% of the total variation of calibration, cross validation, prediction sets, respectively. Additionally, the model possesses quite high regression coefficients (0.99, 0.87, 0.96) and RPD (4.1) value with a very reliable slope (0.99) (Table 5.2). Obtained results are in accordance with the finding of a study in literature in which FTIR spectroscopy was applied to the limited number of olive oil samples with narrow FAAE range but still good R^2_{cal} of 0.98 was obtained in the prediction of FAMEs + FAEEs (Valli et al. 2013). However, UV-vis spectroscopy was not as good as FTIR spectroscopy for FAAEs determination, and it only provided average prediction power with R^2_{cal} =0.78, R^2_{pred}= 0.74, and RPD=1.9 values. On the other hand, combination of FTIR and UV-vis data resulted in a robust prediction model (R^2_{cal}=0.96, R^2_{pred}= 0.96, and RPD=3.4). NIR spectroscopy (Cayuela 2017; Garrido-Varo et al. 2017) and TDR (Berardinelli et al. 2013) have been also used in quantification of total alkyl esters with promising results. In the present study, variable importance for the projection (VIP) values were determined for FTIR and UV-vis models to see the importance of variable effect on methyl, ethyl and alkyl esters prediction model explanation. It was observed that in FTIR related models the bands between 1700-1800 cm^{-1} and fingerprint region (1464–983 cm^{-1}) have the highest VIP values and the observed peaks could be attributed to the stretching of C=O as typical of esters and contain distinct peaks correlated with the amount of methyl ester and ethyl ester, respectively (Rabelo et al. 2015; Niu et al. 2017). Also, VIP values of the constructed models with UV-vis data revealed that peaks between 200-300 nm comprising absorption of conjugated dienes and trienes were important.

Total wax content (5.26–89.59 mg/kg) was also estimated with FTIR and the obtained PLS model possessed average quantification power with R^2_{cal} =0.99, R^2_{pred}= 0.71, and RPD=1.7 (Table 5.2). UV-vis spectral data were not good enough to estimate total wax content because of low R^2 and other statistical parameters (Table 5.2). However, FTIR+UV-vis data allowed better prediction of wax content of olive oils compared to

only FTIR spectral data. FTIR+UV-vis spectra have average prediction power for total wax quantification with tolerable statistical parameters (R^2_{cal}=0.95, R^2_{pred}= 0.75, and RPD=1.9) (Table 5.2). Despite low prediction ability of the proposed model, it could still be used for screening purposes of olive oil quality to distinguish low, medium and high values of waxes. To the best of our knowledge there is no comparable literature that predicts wax content of olive oils with any spectroscopic techniques. However, TDR was used unsuccessfully in the same type of investigation (Berardinelli et al. 2013).

5.1.3. Prediction of DAGs content by PLS regression

Ranges and means of DAG content of the olive oil samples are shown in Table 5.3. C32 values for 1,2 and 1,3 DAG isomers were also quantified but they were in negligible amounts (data not shown). Similar ranges of DAG content of Turkish olive oils obtained from 4 distinct olive cultivars were reported (Matthäus and Musa Özcan 2011).

Table 5.3. Range and mean of diacylglycerols (%) (mg/kg) of olive oil samples.

Measured parameters	Range	Mean	Standard deviation
C34 1,2	6.33-12.57	8.89	1.50
C34 1,3	5.97-18.65	11.71	2.51
C36 1,2	20.77-55.72	34.96	7.52
C36 1,3	25.40-55.37	44.21	6.44
Total 1,2	28.14-68.39	43.90	8.77
Total 1,3	31.61-71.86	56.10	8.77
Ratio	0.39-2.16	0.83	0.33

According to the Australian and Californian standards, total 1,2% DAG content is a representative parameter for the quality of olive oil. Consequently, it was focused on total 1,2 DAG% (28.14-68.39%) in this investigation rather than other individual DAGs.

Model parameters for each spectroscopic technique and their combination are given in Table 5.4. PLS model developed with the first order derivative of FTIR spectral data (b) for the prediction of total 1,2 DAG content have 5 LVs which explains 98.6%, 79.2%, and 70.9% of total variations with respect to calibration, cross-validation and external validation models. R^2_{cal} (0.99), R^2_{cv} (0.79) and R^2_{pred} (0.71) values further confirmed the goodness of the models for 1,2% DAGs from chemical data. Close RMSEC (1.13), RMSECV (5.09), and RMSEP (4.29) values indicate a robust model with no over fitting. Slope of the calibration curve (0.99) is good for high reliability with RPD value of 1.9. For the other individual DAG parameters similar performance values were obtained (R^2_{cal}=0.88-0.99, R^2_{cv}=0.62-0.83, R^2_{pred}= 0.40-0.80, and RPD=1.3-2.2). The highest VIP value of the corresponding model is around 1360 cm^{-1} accompanied with 3500 cm^{-1} which are highly correlated with diglyceryl compounds. Thus, FTIR spectroscopy could be used for screening of olive oil quality according to a threshold value of 35 mg/kg for 1,2 DAGs.

However, first order derivative of UV-vis spectroscopy (Figure 5.2b) and FTIR+UV-vis combinations were not successful compared to FTIR spectral data alone in predicting total 1,2 DAGs content with lower performance parameters, R^2_{pred}=0.51 and RPD=1.4 for UV-vis and R^2_{pred}=0.64 and RPD=1.7 for fused data. Negligible contribution of UV-vis spectrum to the generated models of DAGs could be because of no relation of pigmented compounds with DAG content (Cayuela 2017).

In the literature, there is no study about quantification of DAGs by FTIR spectroscopy. However, NMR spectroscopy have been used in qualitative and quantitative analysis of the diglyceride content (Hatzakis et al. 2011). Neverthless, NMR study was based on direct determination of target compounds rather than prediction of them.

Table 5.4. Statistical parameters of PLS regression models for prediction of DAGs by different spectroscopic methods.

Method	Parameter (%)	Transformation	LVs[1]	R^2_{cal}[2]	R^2_{cv}[3]	R^2_{pred}[4]	RMSEC[5]	RMSECV[6]	RMSEP[7]	RPD[8]	Slope
FTIR	C34 1,2	1st order derivative	3	0.88	0.62	0.66	0.55	1.07	0.81	1.7	0.88
	C34 1,3	1st order derivative	5	0.99	0.83	0.80	0.31	1.26	1.03	2.2	0.99
	C36 1,2	1st order derivative	5	0.99	0.79	0.73	0.94	4.29	3.59	1.9	0.99
	C36 1,3	1st order derivative	5	0.98	0.77	0.66	0.89	4.02	3.42	1.7	0.98
	Total 1,2	1st order derivative	5	0.99	0.79	0.71	1.13	5.09	4.29	1.9	0.99
	Total 1,3	1st order derivative	5	0.99	0.79	0.71	1.13	5.09	4.29	1.9	0.99
	Ratio	1st order derivative	5	0.99	0.82	0.40	0.03	0.16	0.29	1.3	0.99
UV-vis	C34 1,2	1st order derivative	2	0.40	0.27	0.31	1.18	1.27	1.28	1.2	0.40
	C34 1,3	1st order derivative	2	0.66	0.58	0.65	1.52	1.65	1.51	1.6	0.66
	C36 1,2	1st order derivative	2	0.62	0.56	0.54	4.73	4.97	5.17	1.5	0.62
	C36 1,3	1st order derivative	2	0.52	0.44	0.47	4.51	4.71	4.84	1.4	0.52
	Total 1,2	1st order derivative	2	0.59	0.52	0.51	5.74	5.99	6.26	1.4	0.59
	Total 1,3	1st order derivative	2	0.59	0.52	0.51	5.74	5.99	6.26	1.4	0.59
	Ratio	1st order derivative	2	0.53	0.43	0.51	0.23	0.24	0.24	1.4	0.53
FTIR+UV-vis	C34 1,2	1st order derivative	3	0.88	0.60	0.69	0.55	1.11	0.77	1.8	0.88
	C34 1,3	1st order derivative	6	0.99	0.88	0.83	0.20	1.22	0.95	2.4	0.99
	C36 1,2	1st order derivative	4	0.98	0.76	0.66	1.16	4.51	4.04	1.7	0.98
	C36 1,3	1st order derivative	6	0.99	0.82	0.56	0.48	4.01	4.03	1.5	0.99
	Total 1,2	1st order derivative	4	0.98	0.74	0.64	1.41	5.39	4.78	1.7	0.98
	Total 1,3	1st order derivative	4	0.98	0.74	0.64	1.41	5.39	4.78	1.7	0.98
	Ratio	1st order derivative	6	0.99	0.80	0.36	0.02	0.17	0.30	1.3	0.99

[1]LVs: latent variables, [2]R^2_{cal}: regression coefficient for calibration, [3]R^2_{cv}: regression coefficient for cross validation, [4]R^2_{pred}: regression coefficient for prediction, [5]RMSEC: root mean square error of calibration, [6]RMSECV: root mean square error of cross-validation, [7]RMSEP: root mean square error of prediction, [8]RPD: residual predictive deviation

5.1.4. Prediction of chlorophyll and carotenoid content by PLS regression

Details about the concentration ranges of the pigments in olive oil samples are provided in Table 5.5. However, it might not be very easy to compare the results with the literature since pigment concentration is variable depending on cultivar, geographic origin, maturity of olives, climate and storage conditions (Lazzerini, Cifelli, and Domenici 2017). In the present study, pheophytin *a* (0.16-19.21 mg/kg), total xanthophylls (0.24-3.35 mg/kg), lutein (0.60-6.29 mg/kg), and *β*-carotene (0.66-6.79 mg/kg) were determined as the major pigments while the rest of the pigments have lower quantities (Table 5.5).

Table 5.5. Range and mean of color pigments (mg/kg) of olive oil samples

Measured parameters	**Range**	**Mean**	**Standard deviation**
Pheophytin *a*	0.16-19.21	5.89	3.53
Pheophytin *a* der.	0.03-2.59	0.80	0.49
Chlorophyll *a*	0.01-0.26	0.04	0.04
Chlorophyll *a* der.	0.00-0.12	0.04	0.03
Pheophytin *b*	0.04-0.65	0.17	0.12
Pheophytin *b* der.	0.02-0.73	0.17	0.14
Total Xanthophyll	0.24-3.35	0.98	0.57
Lutein	0.60-6.29	2.28	1.25
Lutein der.	0.06-1.35	0.39	0.28
Lutein second der.	0.05-1.38	0.26	0.18
Chlorophyll *b*	0.10-1.70	0.51	0.36
Chlorophyll *b* der.	0.03-0.39	0.12	0.09
β-carotene	0.66-6.79	3.18	1.29

Statistical parameters for prediction models developed for chlorophylls and carotenoids using FTIR, UV-vis and their combination data are listed in Table 5.6. Second-order derivative of each spectroscopic data was used in individual chlorophyll and carotenoid predictions. Second derivative of UV-vis spectroscopy (Figure 5.2c) was more successful compared to the second derivative of FTIR (Figure 5.1c) in prediction of individual color pigments. FTIR measurement might not be sensitive enough to small amounts of pigments present in olive oil; therefore, predictive power of the models developed with the data from this spectroscopic technique might be low. However, data fusion improves the prediction ability of UV-vis spectroscopy. In addition, reliable prediction models for *β*-carotene with any studied spectroscopic techniques could not be obtained. The range of *β*-carotene concentrations for the studied samples was very limited and multivariate regression techniques generally provide better models with samples having wider concentration ranges.

UV-vis spectroscopy provided relatively promising results in prediction of individual pigments. The best regression models were obtained for the pigments with the highest concentrations, lutein and its derivative, pheophytin *a* and its derivative, and total xanthophylls. As can be seen from Table 5.6, regression coefficients R^2_{cal}, R^2_{pred} and RPD values were found in the range of (0.62-0.86), (0.65-0.84), and (1.7-2.5), respectively indicating successful prediction. In addition, constructed models were not overfitted since they have close and low error values for each parameter. According to a study in the literature near-UV-vis spectra of olive oils were also highly correlated with the main pigments of olive oil (Domenici et al. 2014). The highest VIP values of the proposed models for the present study were around 450 and 480 nm for lutein and its derivative and also around 670 nm for pheophytin *a* and its derivative which are comparable with the previous study.

UV-vis spectral data provided moderate prediction for the rest of the pigments. The reason for lower prediction ability than that of major pigments could be because of the lower amount of these pigments in olive oil. These pigments include chlorophyll *a* and its derivative (R^2_{pred}=0.66 and 0.46, RPD=1.1 and 1.3, respectively), pheophytin *b* and its derivative (R^2_{pred}=0.55 and 0.61, RPD=1.5 and 1.2, respectively), lutein second derivative (R^2_{pred}=0.66 and RPD=1.5), chlorophyll *b* and its derivative (R^2_{pred}=0.67 and 0.60, RPD=1.4 and 1.6, respectively). One recent study in the literature successfully correlated four main pigments of olive oil, *β*-carotene, lutein, pheophytin *a* and pheophytin *b* with near-UV-vis spectroscopy using very limited number of samples

(Lazzerini, Cifelli, and Domenici 2017). Fluorescence spectroscopy was also used in successful determination of chlorophyll *a* and *b* and pheophytins *a* and *b* content of 42 olive oil samples in combination with PLS regression (Galeano Díaz et al. 2003).

Data fusion approach was found slightly better, in general, on prediction of major pigments compared to UV-vis alone. The statistics presented in Table 5.6 showed that major pigments (pheophytin *a*, total xanthophyll, and lutein) including their derivatives (pheophytin *a* der., lutein der., and lutein second der.) were successfully predicted with higher $R^2_{cal} \geq 0.96$ and higher in range of $R^2_{cv}=0.71\text{-}0.85$ and $R^2_{pred}=0.70\text{-}0.84$ compared to UV-vis. However, minor pigments (chlorophyll *a*, pheophytin *b*, and chlorophyll *b*) with their derivatives were not predicted that successfully with lower model performance parameters ($R^2_{cv}=0.60\text{-}0.76$, $R^2_{pred}=0.42\text{-}0.62$).

Table 5.6. Statistical parameters of PLS regression models for prediction of individual color pigments of olive oil by different spectroscopic methods.

Method	Parameter (mg/kg)	Transformation	LVs[1]	R^2_{cal}[2]	R^2_{cv}[3]	R^2_{pred}[4]	RMSEC[5]	RMSECV[6]	RMSEP[7]	RPD[8]	Slope
FTIR	Pheophytin *a*	2nd order derivative	5	0.99	0.72	0.18	0.19	2.42	3.60	1.1	0.99
	Pheophytin *a* der.	2nd order derivative	5	0.99	0.70	0.03	0.03	0.32	0.57	1.0	0.99
	Chlorophyll *a*	2nd order derivative	5	0.99	0.75	0.18	0.00	0.03	0.02	0.9	0.99
	Chlorophyll *a* der.	2nd order derivative	5	0.99	0.66	0.36	0.00	0.02	0.02	1.2	0.99
	Pheophytin *b*	2nd order derivative	5	0.99	0.71	0.04	0.01	0.10	0.13	1.0	0.99
	Pheophytin *b* der.	2nd order derivative	3	0.94	0.49	0.00	0.03	0.12	0.15	0.9	0.94
	Total Xanthophyll	2nd order derivative	4	0.99	0.61	0.46	0.07	0.41	0.37	1.2	0.99
	Lutein	2nd order derivative	5	0.99	0.75	0.41	0.08	0.71	1.27	1.2	0.99
	Lutein der.	2nd order derivative	4	0.96	0.54	0.49	0.02	0.20	0.21	1.4	0.96
	Lutein second der.	2nd order derivative	5	0.99	0.79	0.60	0.01	0.12	0.12	1.5	0.99
	Chlorophyll *b*	2nd order derivative	3	0.96	0.72	0.24	0.07	0.21	0.37	1.1	0.96
	Chlorophyll *b* der.	2nd order derivative	5	0.99	0.68	0.30	0.01	0.07	0.08	1.2	0.99

[1]LVs: latent variables, [2]R^2_{cal}: regression coefficient for calibration, [3]R^2_{cv}: regression coefficient for cross validation, [4]R^2_{pred}: regression coefficient for prediction, [5]RMSEC: root mean square error of calibration, [6]RMSECV: root mean square error of cross-validation, [7]RMSEP: root mean square error of prediction, [8]RPD: residual predictive deviation

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Table 5.6. (cont.) Statistical parameters of PLS regression models for prediction of individual color pigments of olive oil by different spectroscopic methods.

Method	Parameter (mg/kg)	Transformation	LVs[1]	R^2_{cal}[2]	R^2_{cv}[3]	R^2_{pred}[4]	RMSEC[5]	RMSECV[6]	RMSEP[7]	RPD[8]	Slope
UV-vis	Pheophytin *a*	2nd order derivative	2	0.77	0.64	0.75	1.61	1.99	2.00	2.0	0.77
	Pheophytin *a* der.	2nd order derivative	1	0.62	0.60	0.65	0.28	0.28	0.34	1.7	0.62
	Chlorophyll *a*	2nd order derivative	3	0.69	0.45	0.66	0.02	0.03	0.02	1.1	0.69
	Chlorophyll *a* der.	2nd order derivative	3	0.75	0.56	0.46	0.01	0.02	0.02	1.3	0.75
	Pheophytin *b*	2nd order derivative	2	0.67	0.55	0.55	0.07	0.08	0.08	1.5	0.67
	Pheophytin *b* der.	2nd order derivative	3	0.86	0.67	0.61	0.06	0.09	0.09	1.2	0.86
	Total Xanthophyll	2nd order derivative	3	0.86	0.78	0.84	0.22	0.28	0.22	2.5	0.86
	Lutein	2nd order derivative	3	0.84	0.74	0.76	0.55	0.67	0.57	2.0	0.84
	Lutein der.	2nd order derivative	3	0.83	0.72	0.83	0.13	0.16	0.10	2.3	0.83
	Lutein second der.	2nd order derivative	2	0.55	0.38	0.66	0.14	0.16	0.09	1.5	0.55
	Chlorophyll *b*	2nd order derivative	4	0.83	0.65	0.67	0.17	0.24	0.16	1.4	0.83
	Chlorophyll *b* der.	2nd order derivative	3	0.72	0.51	0.60	0.05	0.06	0.06	1.6	0.72

[1]LVs: latent variables, [2]R^2_{cal}: regression coefficient for calibration, [3]R^2_{cv}: regression coefficient for cross validation, [4]R^2_{pred}: regression coefficient for prediction, [5]RMSEC: root mean square error of calibration, [6]RMSECV: root mean square error of cross-validation, [7]RMSEP: root mean square error of prediction, [8]RPD: residual predictive deviation

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Table 5.6. (cont.) Statistical parameters of PLS regression models for prediction of individual color pigments of olive oil by different spectroscopic methods.

Method	Parameter (mg/kg)	Transformation	LVs[1]	R^2_{cal}[2]	R^2_{cv}[3]	R^2_{pred}[4]	RMSEC[5]	RMSECV[6]	RMSEP[7]	RPD[8]	Slope
FTIR+ UV-vis	Pheophytin *a*	2nd order derivative	5	0.99	0.80	0.76	0.26	1.87	2.08	1.9	0.99
	Pheophytin *a* der.	2nd order derivative	5	0.99	0.82	0.77	0.04	0.28	0.26	1.8	0.99
	Chlorophyll *a*	2nd order derivative	4	0.99	0.69	0.56	0.00	0.03	0.02	1.4	0.99
	Chlorophyll *a* der.	2nd order derivative	5	0.99	0.69	0.53	0.00	0.02	0.02	1.4	0.99
	Pheophytin *b*	2nd order derivative	3	0.97	0.60	0.57	0.02	0.08	0.11	1.4	0.97
	Pheophytin *b* der.	2nd order derivative	4	0.99	0.65	0.62	0.02	0.10	0.07	1.5	0.99
	Total Xanthophyll	2nd order derivative	4	0.99	0.85	0.84	0.06	0.28	0.22	2.5	0.99
	Lutein	2nd order derivative	3	0.96	0.71	0.70	0.22	0.65	1.05	1.5	0.96
	Lutein der.	2nd order derivative	4	0.99	0.78	0.76	0.02	0.16	0.16	1.8	0.99
	Lutein second der.	2nd order derivative	5	0.99	0.76	0.82	0.01	0.13	0.07	2.0	0.99
	Chlorophyll *b*	2nd order derivative	3	0.96	0.76	0.54	0.07	0.21	0.31	1.3	0.96
	Chlorophyll *b* der.	2nd order derivative	3	0.95	0.66	0.42	0.02	0.06	0.09	1.2	0.95

[1]LVs: latent variables, [2]R^2_{cal}: regression coefficient for calibration, [3]R^2_{cv}: regression coefficient for cross validation, [4]R^2_{pred}: regression coefficient for prediction, [5]RMSEC: root mean square error of calibration, [6]RMSECV: root mean square error of cross-validation, [7]RMSEP: root mean square error of prediction, [8]RPD: residual predictive deviation

5.2 Conclusions

Several chemical quality parameters of olive oils including FAEE, DAGs and chlorophyll and carotenoid pigments were predicted from FTIR and UV-vis spectral data as well as their combination using multivariate regression. The results showed that FTIR+UV-vis and FTIR could be used to predict not only FAAEs but also FAMEs and FAAEs content of olive oil successfully. FTIR+UV-vis spectroscopy could quantify wax esters less accurately. FTIR spectroscopy was found as a promising alternative to a wet chemical method based on tedious and expensive extraction as well as derivatization steps for determination of DAG content of olive oils. The other examined parameters were individual pigment contents of olive oil which are especially important for authenticity studies. Both UV-vis and FTIR+UV-vis spectroscopy had good prediction ability for quantification of major pigments along with their derivatives while moderate prediction was obtained for minor pigments and their derivatives.

This part of the study showed that spectroscopic techniques offered advantages over classical methods in determination of several chemical quality parameters of olive oils since they are faster, relatively cheaper and environmentally friendly compared to wet chemical methods.

CHAPTER 6

RESULTS AND DISCUSSION

ADULTERATION DETECTION OF FRESH OLIVE OILS WITH OLD OLIVE OILS

Redrafted and modified, from:

Uncu, Oguz, and Banu Ozen. 2019. "A comparative study of mid-infrared, UV–Visible and fluorescence spectroscopy in combination with chemometrics for the detection of adulteration of fresh olive oils with old olive oils." *Food Control* 105: 209-218. https://doi.org/10.1016/j.foodcont.2019.06.013.

6.1. Adulteration Study

Although there are several successful examples of olive oil adulteration detection studies using different spectroscopic methods, differentiation of mixtures of olive oils such as mixtures from different olive varieties, mixtures of refined and extra virgin olive oils or mixtures of fresh and old olive oils is generally a more challenging task. Therefore, it is important to test the capabilities of these techniques for these cases. To the best of our knowledge, there is a few preliminary studies in the literature about the detection of adulteration concerning mixing of old olive oils with fresh olive oils. FTIR was used to detect limited number of adulterated samples in one study (Hirri et al. 2015) and laser

diode-based fluorescence spectroscopy was also used in another research (Torreblanca-Zanca et al. 2019). In addition, a recent study employed different classification methods in analyzing fluorescence spectra to determine freshness of olive oils as expired or non-expired (Dankowska and Kowalewski 2019). However, there is not any study which compares the performances of different spectroscopic approaches about this emerging issue.

Purpose of this part of the study is to differentiate fresh olive oil from old olive oil in a mixture by using fluorescence, FTIR and UV-vis and the combination of FTIR and UV-vis spectroscopies; moreover, quantification of different levels of adulterant is also possible with these spectroscopic methods when they are used along with multivariate statistical approaches. Therefore, it was also aimed to investigate the effectiveness of different spectroscopic techniques individually and also in combination to detect this type of fraud in a fast way with minimal chemical waste.

6.1.1. Chemical Characteristics of Olive Oil

Free fatty acid and specific extinction (K232 and K270) values of the olive oil samples were determined to evaluate the general quality of the samples. Average acidity (%), K232, and K270 values of fresh olive oil samples used in mixing studies were 0.40±0.12, 2.18±0.21, and 0.20±0.01, respectively while the same parameters for the old olive oil samples were 0.92±0.29, 2.30±0.32, and 0.19±0.10, orderly.

Average major fatty acids values (%) of fresh olive oil samples were determined as follows; palmitic acid 13.79±0.97, stearic acid 3.04±0.34, oleic acid 69.63±1.83, linoleic acid 10.74±1.46, linolenic acid 0.76±0.11, SFA 17.56±1.46, MUFA 70.94±2.10, and PUFA 11.50±1.57. While the same parameters for old olive oil samples were determined as 14.09±2.05% palmitic acid, 2.68±0.13% stearic acid, 68.94±3.16% oleic acid, 11.56±3.24% linoleic acid, 0.74±0.07% linolenic acid, 17.41±2.30% SFA, 70.29±3.39% MUFA, and 12.30±3.31% PUFA.

All the studied samples were in the limits of quality standards of European Union regulation on olive oil (Commission Delegated Regulation (EU) 2016). Fresh olive oil

samples were graded as extra virgin olive oils while old olive oil samples were at lower grades.

6.1.2. Spectral Evaluation

Typical spectra of all the studied olive oil samples obtained with different spectroscopic techniques are shown in Figure 6.1. The FTIR spectra of the samples (Figure 6.1a) are dominated by the peaks at distinct wavelengths of 2924, 2852, 1743, 1463, 1377, 1238, 1163, 1114, 1099 and 721 cm^{-1} (Sinelli et al. 2007). Absorbances at 2924 and 2852 cm^{-1} wavelengths are due to -CH_2 asymmetric and symmetric stretching vibrations, respectively. The major peaks at 1743 cm^{-1} followed by 1463 and 1377 cm^{-1} are associated with C=O stretching, CH_2 and CH_3 scissoring vibrations, respectively. The rest of the peaks at 1238, 1163, 1114, 1099 cm^{-1} are relevant with C-O stretching vibration while a small peak at 721 cm^{-1} are correlated with CH_2 rocking mode (Sinelli et al. 2007).

UV-vis spectra of the olive oil samples are shown in Figure 6.1b. Absorption spectra of the olive oil samples have specific peaks around 230-270 nm indicating the presence of conjugated dienes and trienes of unsaturated fatty acids. Moreover, 300-400 nm band was correlated with a variety of polyphenols (Mignani et al. 2012). A shift in the positions of the peaks and/or the absence of the peaks in the current study compared to above assignments could be related to differences in the quality, varietal and geographical differences of olive oil samples with respect to investigated samples in the literature as well as measurement parameters. In addition, carotenoids as one of the color pigments are responsible for the absorption between 430–460 nm and peak at 670 nm is attributed to chlorophylls and their derivatives (Mignani et al. 2012).

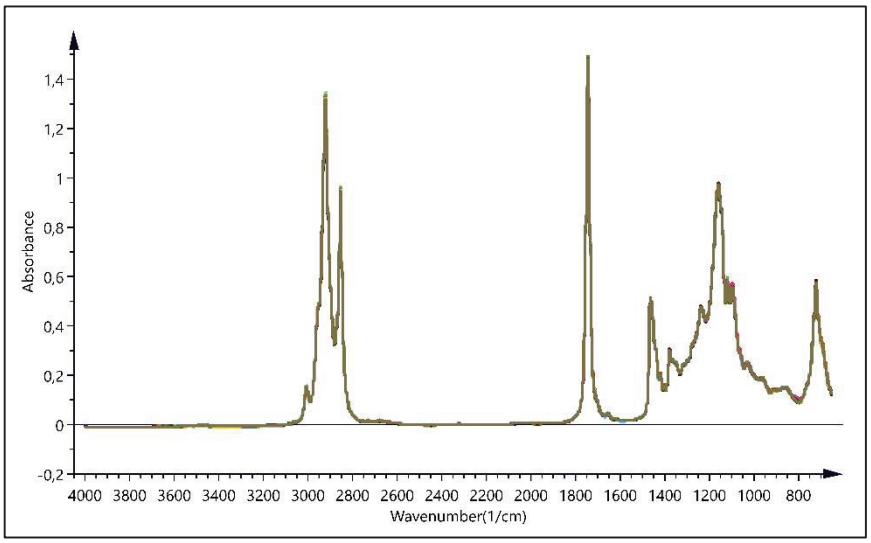
Fluorescence emission spectra of the olive oil samples are shown in Figure 6.1c and these spectra reveal three regions of interest around 350 nm due to specific excitation together with 400-600 nm, and 650-750 nm. Bands between 600-700 nm in emission possessed well known relationship with chlorophylls a and b and pheophytins *a* and *b*. Bands at 250-400 nm are correlated with α-tocopherol and phenolic compounds while 400-600 nm emission spectral range could be attributed to vitamin B_2 and carotenoids as

well as oxidation products of fatty acids, especially conjugated hydroperoxides, are found in the range of 440–470 nm (Dupuy et al. 2005; Ali et al. 2018).

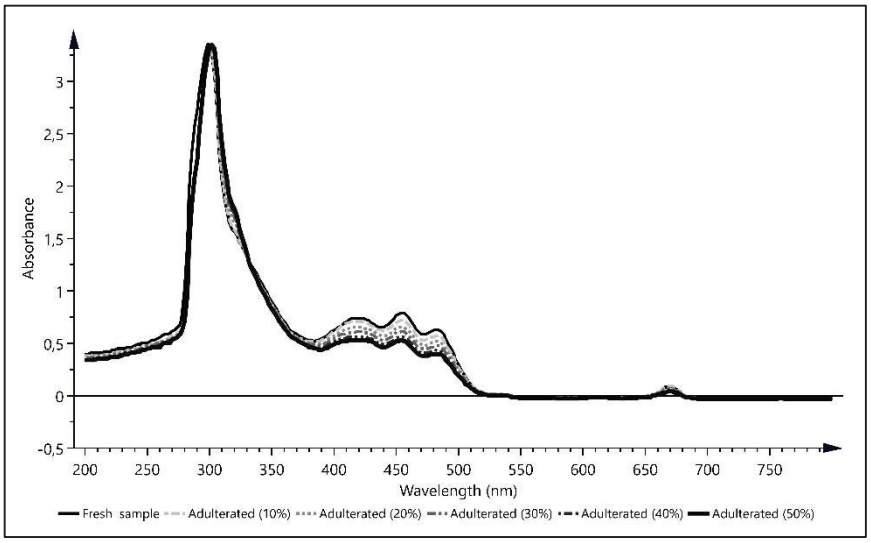
Spectra obtained from each of these spectroscopic methods were further investigated to observe for any visual differences between a fresh sample and adulterated ones. The differences in FTIR spectra were not easy to recognize visually. On the other hand, visual inspection revealed noticeable differences between the spectra of adulterated and fresh olive oil samples obtained with UV-vis and fluorescence spectroscopy.

Main differences in UV-vis spectra are observed in the peaks attributed to carotenoids (400-500 nm) and chlorophylls (670 nm) (Figure 6.1b). Both chlorophylls and carotenoids are pigments which are affected from environmental conditions such as light and temperature and are converted into other forms and/or degraded during storage. Therefore, differences in UV-vis spectra of old oil containing samples could be associated with the changes in the pigment composition of the samples. The changes in pigment composition could be attributed to the oxidation of these compounds during storage (Ali et al. 2018).

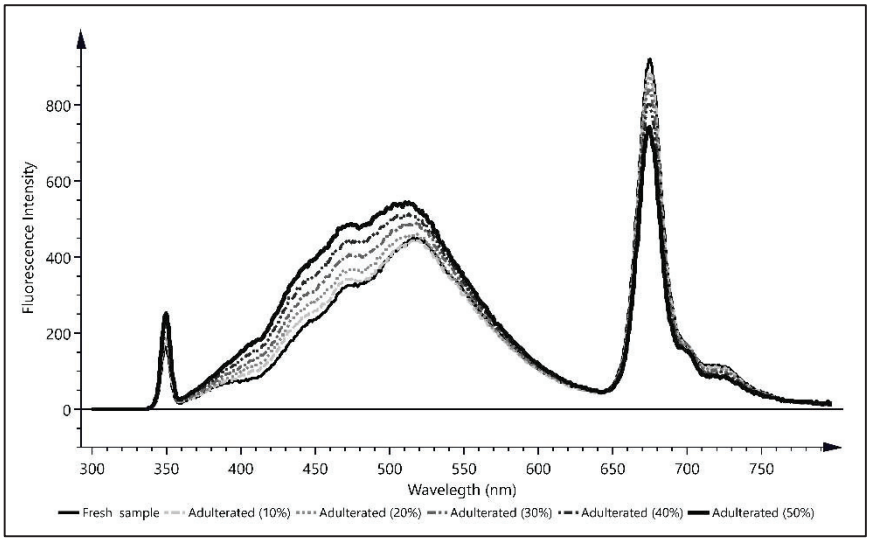
Fluorescence emission spectra of the olive oil samples at varying adulteration levels are provided in Figure 6.1c. Fluorescence intensity at distinct wavelengths (400-500 nm) increased with increasing adulteration level and this could be correlated with the formation of oxidation products of fatty acids such as hydroperoxides emitted around 450 nm (Lleó et al. 2016). However, fresh olive oil samples have higher intensity at 650-750 nm compared to adulterated ones and this difference could be attributed to change in chlorophyll content having negative linear relationship with oxidation products (Hernández-Sánchez et al. 2017).



(a)



(b)



(c)

Figure 6.1. (a) FT-IR, (b) UV-vis and (c) fluorescence spectra of the olive oil samples

6.1.3. Discrimination of Fresh Olive Oils from Adulterated Oils

OPLS-DA models were created with the data from each spectroscopic technique and with the combination of FTIR and UV-vis spectral data and Table 6.1 shows the results of statistical parameters for the models obtained with the application of various spectral pre-treatments. The best models were obtained with the first derivative (FD) of FTIR, FTIR+UV-vis and fluorescence spectroscopy data while the second derivative (SD) of UV-vis spectral data resulted in the most successful differentiation. Each model was comprising a calibration and external validation set and the number of the samples in calibration and validation is 80 and 40 out of total 120 samples (100 adulterated and 20 fresh samples), respectively (Table 6.1). Although it might look like there is an unbalance between the numbers of adulterated (100) and non-adulterated (20) samples there is still enough number of non-adulterated samples to form a class in OPLS-DA model. Classification could be also performed by using each adulteration percentages as a different class. However, it was thought that assigning all adulteration levels to a single class is a more realistic approach. This is because of that it is generally impossible to know the adulteration concentrations of external samples that are brought to the control laboratories and constructed model allows detection of mixing regardless of adulteration percentages. OPLS-DA score plots of each calibration model are provided in Figure 6.2 which shows the scattering of two classes as adulterated and fresh samples (non-adulterated).

As it could be seen from Table 6.1, OPLS-DA model of FD of FTIR spectra provided the best differentiation of fresh olive oil samples from adulterated ones with the average correct classification rate of 100% and 93% (out of 40 sample; 1 sample misclassified as adulterated and 2 samples misclassified as fresh samples) in calibration and validation sets, respectively. The OPLS-DA model was built with 1 predictive and 3 orthogonal components. Other statistical parameters such as high R^2 values for calibration and cross-validation sets further confirm the classification ability of the model (Table 6.1). According to the score plot (Figure 6.2a), fresh samples located on the right side of this plot are separated from adulterated samples with respect to the first latent variable (LV1) explaining 49% of the total variation. Furthermore, variable importance for the projection (VIP) values are also evaluated to determine the most significant wavelengths

in differentiation of adulteration. VIP parameter is increasingly preferred in the model evaluation since it provides the most compact model interpretation compared to loading weights and regression coefficients (Galindo-Prieto, Eriksson, and Trygg 2015). VIP values greater or close to 1 are considered as influential in the explanation of classification and prediction models (Uncu and Ozen 2015). The highest VIP values are obtained at around 1723 cm^{-1} which could be associated with stretching of C=O (free fatty acids) groups (Hirri et al. 2016) as well as fingerprint region (1464–983 cm^{-1}) and around 723 cm^{-1} (Jolayemi et al. 2017). In the literature, there is only one study in which limited number of old olive oil samples (lampante) were separated from fresh (extra virgin) samples by using discriminant analysis (PLS-DA) of FTIR data (Hirri et al. 2015).

Table 6.1. OPLS-DA models of different spectroscopic methods in classification of adulterated and fresh samples (the number of samples are shown in parenthesis)

Method	**Pre-treatment[a]**	**LVs**	**R^2_{cal}**	**R^2_{cv}**	**%CC_{cal}[b] (n=80)**	**%CC_{pred}[c] (n=40)**
FTIR	FD	1+3	0.98	0.53	100	93
	WDTs:FD	1+3	0.97	0.58	100	85
	SD	1+2	0.97	0.42	100	90
UV-vis	FD	1+3	0.98	0.98	100	83
	WDTs:FD	1+3	0.98	0.97	100	83
	SD	1+4	0.99	0.98	100	100
FTIR+UV-vis	FD	1+4	0.99	0.66	100	98
	WDTs:FD	1+3	0.98	0.65	100	85
	SD	1+2	0.97	0.58	100	88
Fluorescence	FD	1+8	0.95	0.70	100	90
	WDTs:FD	1+8	0.98	0.71	100	95
	SD	1+7	0.90	0.68	100	89

[a]FD: first derivative, SD: second derivative, WDTs:FD combination of wavelet denoising techniques and first derivative, [b]average correct classification rate for calibration, [c]average correct classification rate for prediction (external validation)

Score plot of OPLS-DA model constructed with SD of UV-vis absorbance spectra is shown in Figure 6.2b. A clear separation was obtained between fresh and adulterated samples in the calibration set (100%) as well as in the external validation set with correct prediction rate of 100% (Table 6.1). LV1 was effective in the classification by separating each class of olive oil samples to the left and the right of the score plot (Figure 6.2b). The highest VIP values for the constructed model are found as around 260-290, 470 and 680 nm and these values correspond to the presence of conjugated dienes and trienes (oxidation products), carotenoids and chlorophyll derivatives, respectively. To the best of our knowledge, there is no comparable literature about the differentiation of old and fresh olive oil samples by using UV-vis spectroscopy. Until so far, studies with UV-vis spectroscopy have been based on the quantification of the adulteration of extra virgin olive oil with lower grade olive oils (Torrecilla et al. 2010a) as well as binary and ternary mixtures of monovarietal extra virgin olive oils (Aroca-Santos et al. 2016).

Combination of two spectroscopic methods as FTIR+UV-vis is also investigated for any improvement that could be attributable to the data fusion in the classification of the samples. Prior to combining the data, FD of both spectra were taken individually and then they were fused. The fused data set provided the best OPLS-DA model and score plot of this model is shown in Figure 6.2c. According to the statistical results listed in Table 6.1, combined data have higher classification power than the model of FTIR spectroscopic data and also have comparable success with UV-vis data. The model was built with 1 predictive and 4 orthogonal components explaining 56% of the overall model according to LV1. The measure of fit for calibration and cross validation are 99% and 66%, respectively. The OPLS-DA model correctly separated all samples from two classes in the calibration set (100%) and also correctly predicted all samples for each class in the external validation set except one misclassified sample from the adulterated set (98%) (Table 6.1).

Fluorescence spectroscopy was also used in the differentiation of adulterated samples. De-noised fluorescence spectra were further pre-treated with FD transformation prior to model construction. The OPLS-DA score plots (Figure 6.2d) revealed a good separation between adulterated and fresh olive oil samples which are scattered in the negative and positive sides of the LV1, respectively. The correct classification rates for both calibration and validation sets are satisfactorily high as 100% and 95% (2 samples misclassified as fresh samples), respectively. Certain wavenumbers around 435-500 nm and 670 nm could be correlated with higher VIP values in comparison to the rest of the

wavelengths. These bands could be attributed to conjugated hydroperoxides and chlorophyll content, respectively (Ali et al. 2018); therefore, these compounds are most likely responsible for the differentiation of fresh olive oil from adulterated ones. As far as we know, there was only two very recent studies in the literature using laser diode induced excitation to differentiate fresh and old olive oil samples successfully (Torreblanca-Zanca et al. 2019; Lastra-Mejías et al. 2019). Most of the fluorescence studies have been focused on the detection of lower grade olive oil (Durán Merás et al. 2018) and authenticity confirmation and geographical origin determination (Jiménez-Carvelo, Lozano, and Olivieri 2019).

To sum up, all of the studied models are found to be quite successful in differentiation of adulteration with old olive oil samples. All the calibration models built with different spectroscopic techniques are 100% successful in adulteration detection while external validation models are also promising with decreasing order of correct classification rate for UV-vis, FTIR+UV-vis, fluorescence, and FTIR as 100%, 98%, 95%, and 93%, respectively. Presence of oxidation products and change in the pigment content caused differentiation of fresh olive oils adulterated with old olive oil from fresh olive oils.

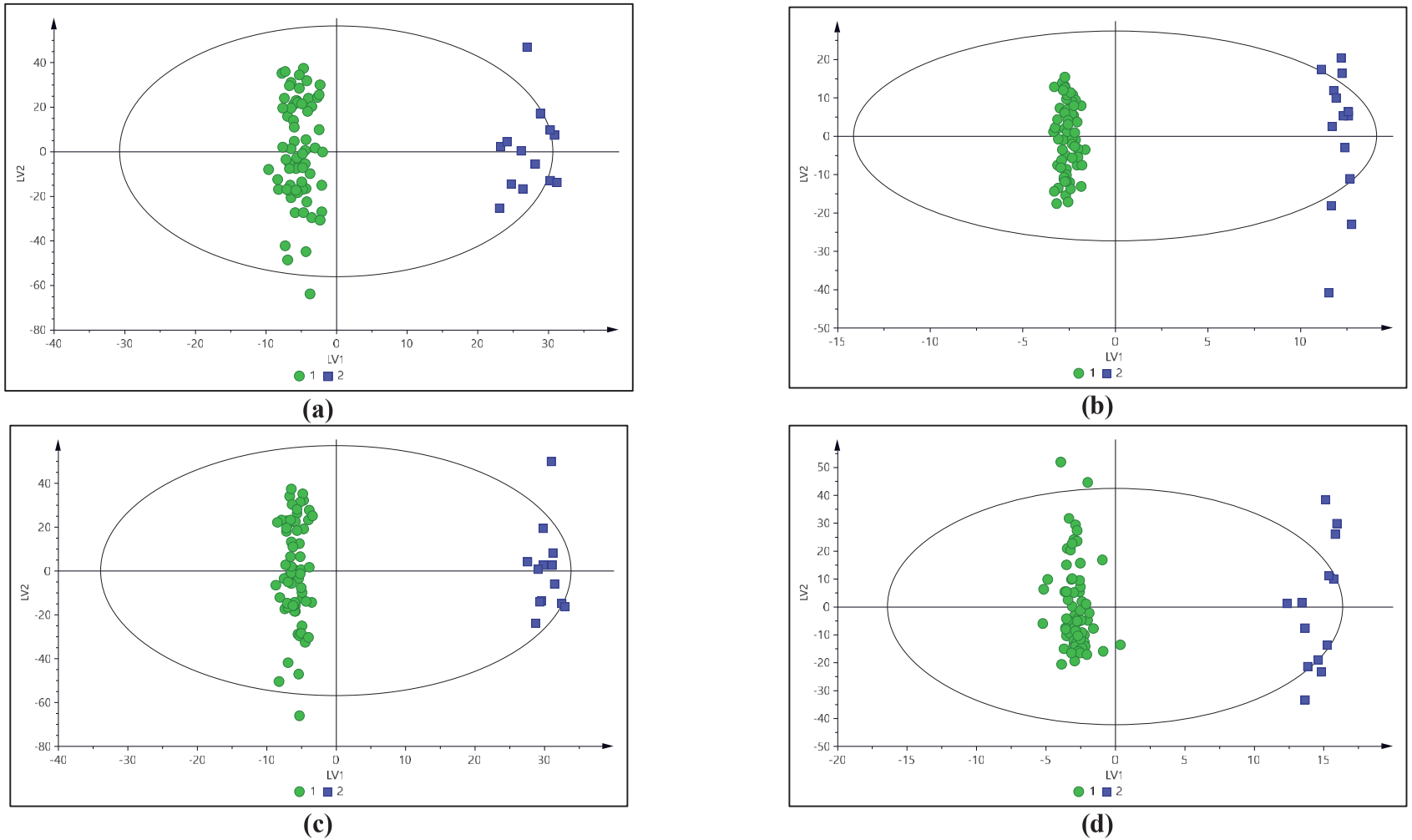


Figure 6.2. Score plots of OPLS-DA models built with (a) FT-IR, (b) UV–vis, (c) FT-IR + UV–vis, and (d) fluorescence spectroscopy for discrimination of adulterated (●1) and fresh olive oil samples (■2).

6.1.4. Prediction Studies

Quantification of adulterant level (0-50% v/v) in fresh olive oil samples was conducted by applying PLS algorithm to the calibration and external validation data sets from each spectroscopic technique. Statistical results of each spectroscopic method as well as the combination of FTIR and UV-vis are provided in Table 6.2. Different pre-processing techniques and appropriate combinations were used in model development, and it was found out that OSC: WDTs provided better results compared with the rest of the transformations (Table 6.2). OSC was also reported as a more successful pre-processing technique compared to the other methods in the literature (Cen and He 2007). Therefore, models developed by the OSC in combination with WDTs will be explained in more detail. Prediction performance of the models were evaluated by some critical internal and external as well as cross validation parameters such as regression coefficients (R^2) and error values (RMSE) (Table 6.2). A model must have high R^2 values and low RMSE values to have high predictive ability (Gurdeniz and Ozen 2009).

First approach was using FTIR data set to quantify adulteration level. The model was constructed using 9 LVs with relatively high R^2 values for calibration (0.96), cross validation (0.77) and prediction, (0.84) and comparably low error values of 3.45% for calibration, 10.19% for cross validation, and 7.01% for prediction as well as robust RPD value of 2.5 were also obtained for this model (Table 6.2). There is only one preliminary study in the literature predicting limited number of lower quality olive oil (lampante) in fresh olive oil by FTIR spectroscopy successfully with R^2 of 0.999 and error values lower than 1% (Hirri et al. 2015). Results of the present study have lower performance due to higher prediction error compared to the previous study. In the former study, smaller number of samples (n=45) were used, and the old olive oil samples were in a more degraded condition as lampante virgin oil with free fatty acidity of 3.28% compared to the samples having an average 0.92% of free fatty acid value in the present study.

PLS model of UV-vis spectral data have moderate prediction power including 6 LVs along with acceptable $R^2 \geq 0.80$ and close error values with approximate RPD value of 2.2 (levels of RPD are defined in section 3.4.1) (Table 6.2). UV-vis spectroscopy had similar prediction power with FTIR spectroscopy. In the literature, there is not any study which used UV-vis spectral data in prediction of this type of adulteration. UV-vis studies

were performed for determining the level of adulteration of extra virgin olive oil with refined olive oil and refined olive-pomace oil (Torrecilla et al. 2010a) and also for the quantification of binary and ternary mixtures of monovarietal extra virgin olive oils (Aroca-Santos et al. 2016).

FTIR+UV-vis data are quite successful in the prediction of varying levels of old olive oil samples in fresh ones with robust statistical parameters (R^2_{cal}=0.94, R^2_{pred}=0.91, RMSEC=4.22%, RMSEP=5.20%, and RPD=3.2) (Table 6.2). For better visualization of the prediction model, PLS regression plot is presented in Figure 6.3a. It is clear that the data fusion approach is more successful in the quantification of adulteration compared to individual methods (FTIR or UV-vis) (Table 6.2). In a recent study, it was also reached to a similar conclusion about the prominent improvement in the model prediction power for the quantification of rapeseed oil in olive oil blends by near infrared (NIR) and mid infrared (MIR) spectroscopy (Li, Xiong, and Min 2019).

PLS regression plot of fluorescence spectroscopic data for the prediction model built with 9 LVs was presented in Figure 6.3b. High R^2 values for both calibration (0.98) and external validation (0.97) sets as well as lower error values for the same data sets (2.68% and 2.82%, respectively) showed that fluorescence spectroscopy is a promising tool in the detection of old olive oils mixed with fresh olive oils (Table 6.2). Results of the present study is in accordance with two very recent study, both of which used laser diode induced excitation. These studies were able to detect expired extra virgin olive oil with error values around 1.5% and lower than 10% by using different statistical approaches of intelligent non-linear model based on a supervised artificial neural network (Torreblanca-Zanca et al. 2019) and a linear model relying on chaotic parameters (Lastra-Mejías et al. 2019), respectively.

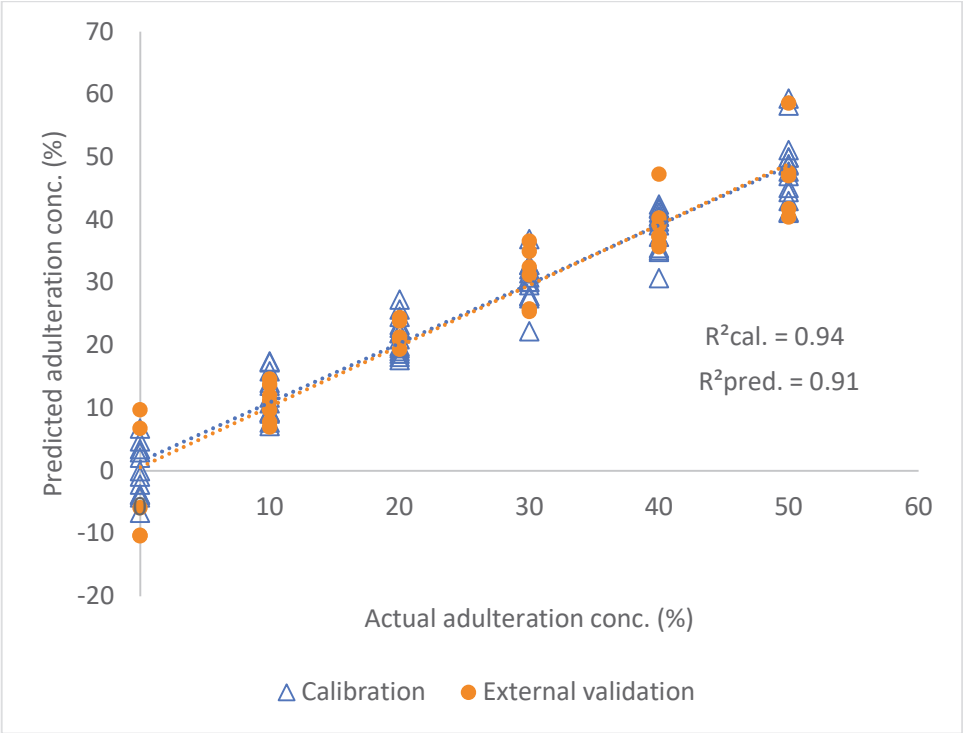
In summary, fluorescence and combination of FTIR and UV-vis spectroscopic data provided better results in the quantification of adulteration than two other individual spectroscopic data. Therefore, it is recommended to use combined data rather than individual UV-vis and FTIR methods alone to determine this type of adulteration. In addition, fluorescence spectroscopic data also resulted in robust prediction models with similar statistical parameters as fused data. Detection errors for both techniques were lower than 10%. Moreover, fluorescence spectroscopy performed slightly better than combined spectroscopy in terms of determination limit as well as other statistical parameters. It was found that 10% detection limit is satisfactory for this type of

adulteration since fraudsters could make little profit lower than that ratio as also indicated in a different type of adulteration study (Li et al. 2015)

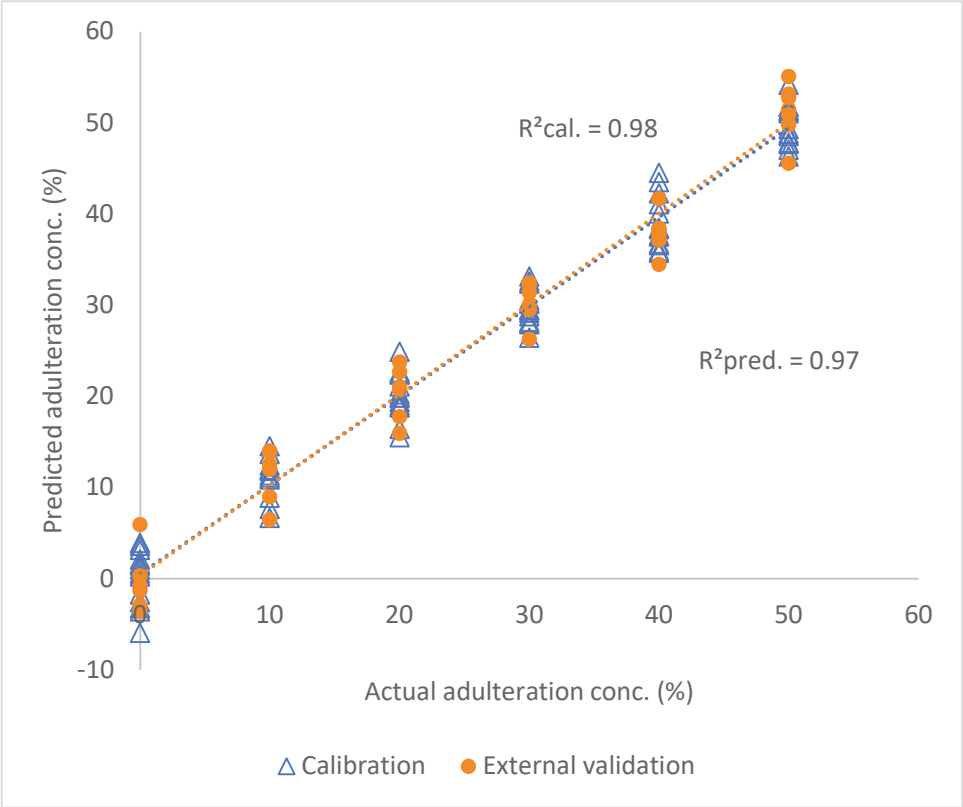
Table 6.2. Statistical parameters of PLS regression models for prediction of adulteration by different spectroscopic methods

Method	Pre-treatment[a]	LVs	R^2_{cal}	R^2_{cv}	R^2_{pred}	RMSEC	RMSECV	RMSEP	RPD	Slope
FTIR	FD	3	0.90	0.58	0.54	5.44	12.02	11.53	1.5	0.90
	S-G:MSC	4	0.67	0.45	0.46	10.14	12.62	13.34	1.3	0.67
	FD:S-G:MSC	4	0.97	0.48	0.57	2.97	13.95	11.64	1.5	0.97
	WDTs:OSC	9	0.96	0.77	0.84	3.45	10.19	7.01	2.5	0.96
UV-vis	FD	6	0.73	0.61	0.66	9.35	10.56	10.06	1.7	0.73
	S-G:MSC	5	0.69	0.59	0.60	9.93	10.89	11.03	1.5	0.69
	FD:S-G:MSC	4	0.72	0.62	0.57	9.30	10.36	11.44	1.6	0.72
	WDTs:OSC	6	0.86	0.80	0.80	6.78	8.02	7.76	2.2	0.86
FTIR+UV-vis	FD	3	0.92	0.63	0.62	5.10	11.33	10.56	1.6	0.92
	S-G:MSC	5	0.77	0.49	0.64	8.57	12.45	10.29	1.7	0.77
	FD:S-G:MSC	6	0.99	0.70	0.61	1.49	10.91	10.70	1.6	0.99
	WDTs:OSC	5	0.94	0.85	0.91	4.22	6.96	5.20	3.2	0.94
Fluorescence	FD	4	0.73	0.32	0.37	9.15	16.61	13.61	1.3	0.73
	S-G:MSC	10	0.79	0.44	0.49	8.31	13.07	12.28	1.4	0.79
	FD:S-G:MSC	5	0.72	0.40	0.29	9.43	13.82	15.05	1.1	0.72
	WDTs:OSC	9	0.98	0.95	0.97	2.68	6.52	2.82	6.2	0.98

[a]FD first derivative, S-G:MSC combination of Savitzky-Golay and multiplicative scatter correction, FD:S-G:MSC combination of first derivative with Savitzky-Golay and multiplicative scatter correction, WDTs:OSC combination of wavelet denoising techniques and orthogonal signal correction



(a)



(b)

Figure 6.3. Actual versus predicted percentages of old olive oil adulteration (0% to 50% v/v) determined by (a) FTIR + UV–vis and (b) fluorescence spectroscopy

6.2. Conclusions

In the present part, it was aimed to develop reliable analytical tools to detect and quantify adulteration made with mixing fresh olive oils with old olive oil samples. Different spectroscopic approaches individually and as a combination are compared with each other using multivariate statistical techniques. The results indicated that both fluorescence and combination of FTIR and UV-vis spectral data are better than FTIR and UV-vis spectroscopy alone in the determination of adulteration due to their lower error values for prediction (2.82% and 5.20%, respectively) as well as their higher regression coefficients of prediction (0.97 and 0.91, orderly). Both UV-vis and FTIR are rapid methods; however, collecting and analyzing the data statistically would require a longer time. However, even in this condition, using combined spectroscopy would have advantages over wet chemical analysis methods due to its minimal waste generating, no sample preparation and easy to use nature. Differentiation of adulterated samples are due to the presence of oxidation products and change in the pigment concentration of the oils. These methods could be used as reliable, fast, non-destructive, and environmentally friendly tools in both detection and quantification of adulteration as well as screening of olive oil quality, simultaneously.

CHAPTER 7

CONCLUSIONS

Redrafted, modified, and extended from:

Uncu, Oguz, Banu Ozen, and Figen Tokatli. 2019. “Use of FTIR and UV–Visible spectroscopy in determination of chemical characteristics of olive oils.” *Talanta* 201: 65–73. https://doi.org/10.1016/j.talanta.2019.03.116.

Uncu, Oguz, and Banu Ozen. 2019. "A comparative study of mid-infrared, UV–Visible and fluorescence spectroscopy in combination with chemometrics for the detection of adulteration of fresh olive oils with old olive oils." *Food Control* 105: 209-218. https://doi.org/10.1016/j.foodcont.2019.06.013.

Uncu, Oguz, Banu Ozen, and Figen Tokatli. 2020. “Authentication of Turkish Olive Oils by using detailed pigment profile and spectroscopic techniques.” *Journal of the Science of Food and Agriculture* 100 (5): 2153–65. https://doi.org/10.1002/jsfa.10239.

Uncu, Oguz, and Banu Ozen. 2021. "Fatty acid alkyl ester and wax compositions of olive oils as varietal authentication indicators." *Journal of Food Measurement and Characterization* (in press). https://doi.org/10.1007/s11694-021-01184-2.

Olive oil samples obtained from different cultivars and various parts of Aegean Region had different fatty acid profiles and two of these varieties had similar quality parameters. According to orthogonal partial least squares discriminant analysis (OPLS-DA) use of individual fatty acid alkyl esters (FAAE) profile resulted in 80% correct classification rate while waxes alone was 67% successful in classifying the olive oils according to variety. It was found that alkyl esters in combination with waxes were more

effective in discrimination of olive oils with respect to cultivar compared to their individual forms and the correct classification rate for the generated model is 92%. Since FAAEs along with waxes have effect on cultivar differentiation, they could have a potential as authentication tools for olive oil besides their known quality characteristics. It was also found that use of detailed pigment profiles is quite promising in authentication of olive oils. However, UV–visible and Fourier transform infrared (FTIR) spectroscopic techniques could be reliable alternatives for the same purposes. All of the studied techniques have potentials in identification of 'protected designation of origin' certification of the products.

Due to the importance of these chemical measures for olive oil, they were tried to be predicted from spectroscopic data for their rapid and simultaneous determination. Prediction models were constructed by using partial least squares regression with cross and external validation. Fatty acid ethyl esters (FAEEs) were estimated best with FTIR + UV–Vis spectroscopy (R^2_{cv}=0.84, R^2_{pred}=0.90, and RPD=3.0). An average PLS model (R^2_{cv}=0.79, R^2_{pred}=0.71, and RPD=1.9) was obtained for the estimation of 1,2 DAG using FTIR spectral data. Major pigments, lutein, pheophytin *a* and their derivatives and total xanthophylls were quantified successfully by FTIR + UV–Vis (a range of R^2_{cv} of 0.71–0.85, R^2_{pred} of 0.70–0.84, and RPD=1.5–2.5 values) but the prediction of the rest of the pigments were poor (R^2cv=0.60–0.76, R^2_{pred}:0.42–0.62, and RPD=1.2–1.5). Combination of two spectral data resulted in average prediction of wax content of oils (R^2_{cal}=0.95, R^2_{pred}=0.75, and RPD=1.9). FTIR and UV–vis spectroscopic techniques in combination with PLS regression provided promising results for the prediction of several chemical parameters of olive oils; therefore, they could be alternatives to traditional analysis methods.

Three spectroscopic methods were tested to investigate their ability in detecting old olive oils in fresh oils. After the application of various pre-treatment methods, all of the OPLS-DA classification models generated for every spectroscopic technique successfully differentiated adulterated and non-adulterated oils with over 90% correct classification rate. FT-IR + UV–vis and fluorescence spectral data were also successfully used to predict adulteration levels with high coefficient of determinations for both calibration (0.94 and 0.98) and prediction (0.91 and 0.97) models and low error values for calibration (4.22% and 2.68%), and prediction (5.20% and 2.82%), compared to individual FT-IR and UV–vis spectroscopy. Therefore, FT-IR + UV–vis and fluorescence

spectroscopy as being fast and environmentally friendly tools have great potential for both classification and quantification of adulteration practices involving old olive oil.

Although FAAEs and pigment profile have potential in discrimination of olive oils, rapid spectroscopic methods have several advantages over wet chemical methods. They not only provide differentiation of olive oils with respect to olive variety but also allow prediction of different chemical measurements. They can also detect and quantify mixtures of old and fresh olive oils.

As a future study, these chemical parameters could be measured for olive oils obtained from other regions of Turkey. In this way, a larger database in terms of these parameters for Turkish olive oils could be obtained. As a result, some of these potential parameters for future legislations could be considered for stricter regulations.

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APPENDIX A

STANDARD CALIBRATION CURVES FOR PIGMENTS

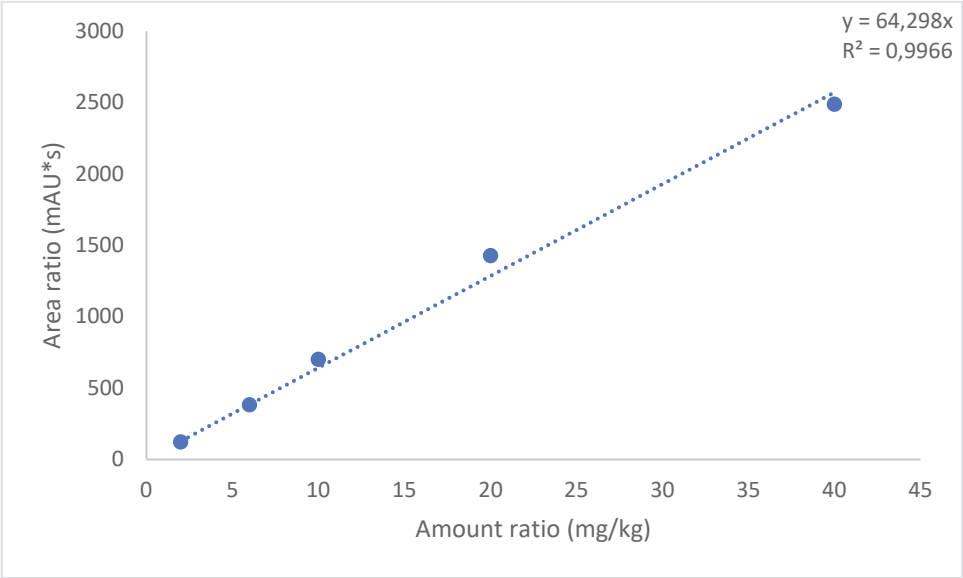

Figure A.1. Standard calibration curve for pheophytin *a* and its derivatives

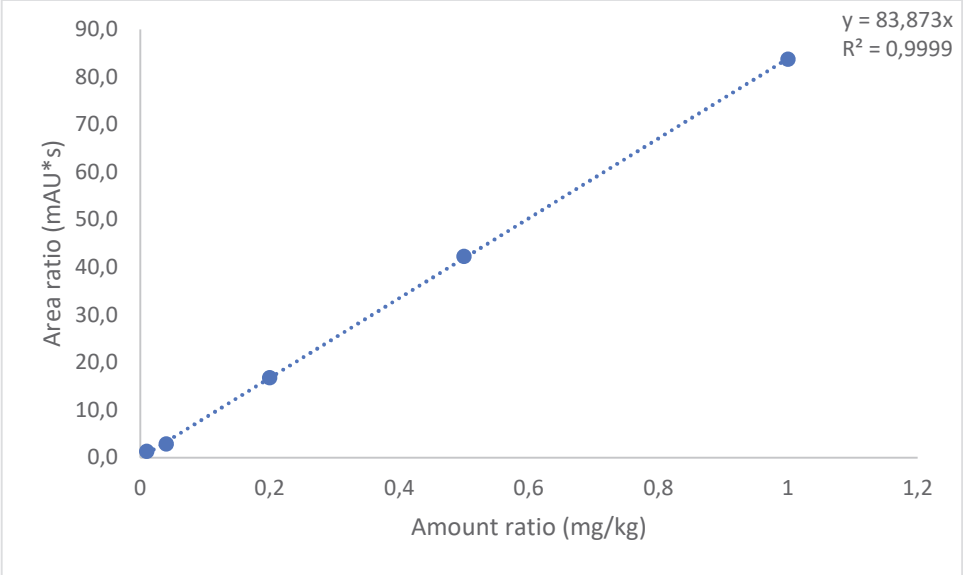

Figure A.2. Standard calibration curve for chlorophyll *a* and its derivative

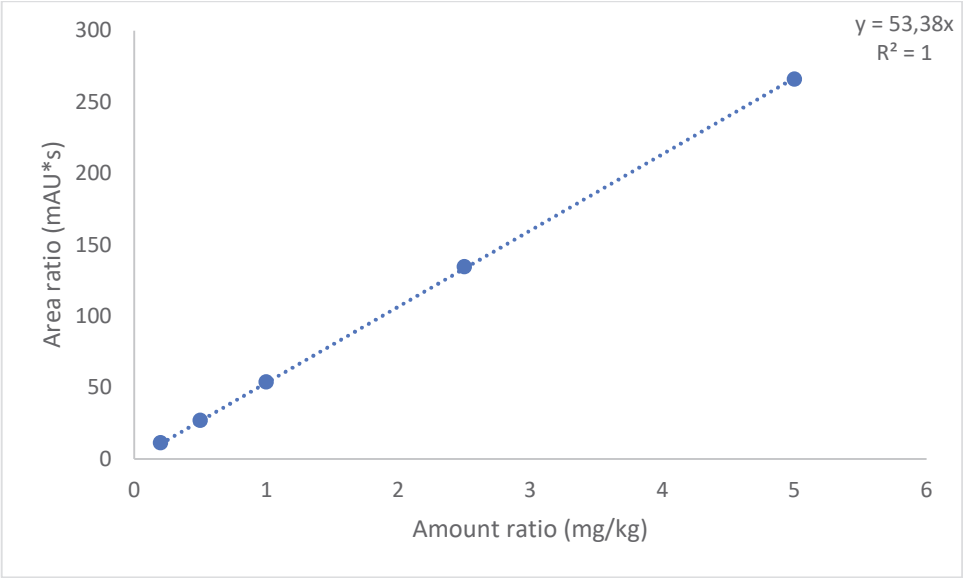

Figure A.3. Standard calibration curve for pheophytin *b* and its derivative

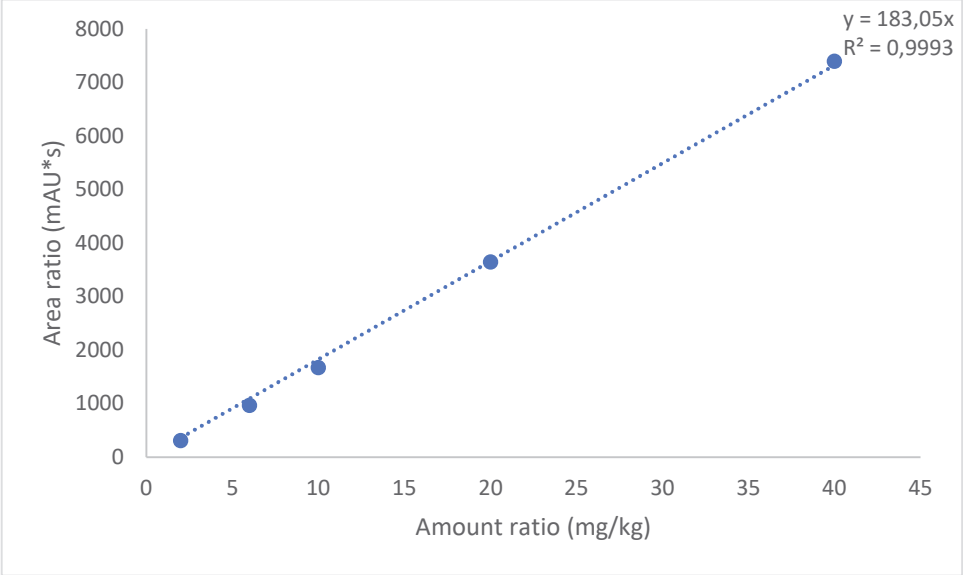

Figure A.4. Standard calibration curve for lutein and its derivatives and other xanthophylls

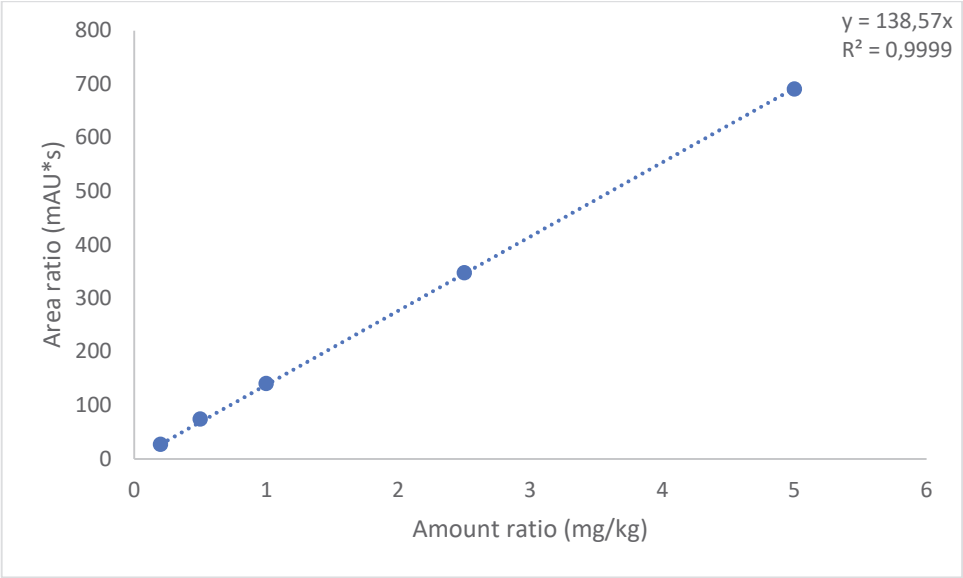

Figure A.5. Standard calibration curve for chlorophyll *b* and derivative

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