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PhD Thesis

Marianthi G. Basalekou

Monitoring wine maturation with alternative chemical analytical methods. Prospects and challenges

Supervisor: Stamatina KALLITHRAKA

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Laboratory of Enology
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Παρακολούθηση της ωρίμανσης των οίνων με εναλλακτικές αναλυτικές χημικές μεθόδους. Προοπτικές και προκλήσεις.

Supervisor: Stamatina KALLITHRAKA

Advisory Committee:
Stamatina KALLITHRAKA, Laboratory of Oenology, Agricultural University of Athens
Yorgos KOTSERIDIS, Laboratory of Oenology, Agricultural University of Athens
Christos PAPPAS, Laboratory of General Chemistry, Agricultural University of Athens

Members of the Examination Board:
Petros TARANTILIS, Laboratory of General Chemistry, Agricultural University of Athens
Apostolos SPYROS, Department of Chemistry, University of Crete
Anastasios ECONOMOY, Department of Chemistry, National and Kapodistrian University of Athens
Ioannis PARASKEVOPOULOS, Department of Oenology and Beverage Technology, University of West Attica

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Abstract

Η ωρίμανση του οίνου είναι μια πολύπλοκη διαδικασία που περιλαμβάνει πολυάριθμες χημικές αντιδράσεις οι οποίες τελικά επηρεάζουν την ποιότητά του. Οι αντιδράσεις αυτές σχετίζονται άμεσα με τον τύπο του μέσου που έρχεται σε επαφή με τον οίνο, αλλά και με τη διάρκεια της ωρίμανσης. Καθώς δεν υπάρχει κάποια χημική ανάλυση που να παρέχει απαντήσεις σχετικά με τον βέλτιστο χρόνο ωρίμανσης, η απόφαση αυτή βασίζεται κυρίως στο βαθμό αύξησης της στυπτικότητας του οίνου, ενώ η διάρκεια ωρίμανσης, το είδος και τα χαρακτηριστικά του περιέκτη ωρίμανσης σε πολλές χώρες ρυθμίζεται από τη νομοθεσία.

Ο σκοπός αυτής της διατριβής ήταν να αξιολογηθεί η πιθανότητα χρήσης της φασματοσκοπίας υπερύθρου με μετασχηματισμό Fourier (FT-IR) στον έλεγχο αυθεντικότητας οίνων ανάλογα με το είδος του περιέκτη και το χρόνου ωρίμανσης, και παράλληλα να γίνει περισσότερο κατανοητός ο τρόπος με τον οποίο το είδος του περιέκτη επηρεάζει την αίσθηση στυπτικότητας.

Η φασματοσκοπία υπερύθρου με μετασχηματισμό Fourier έχει αποδείξει ότι αποτελεί σημαντικό εργαλείο σε μελέτες κατηγοριοποίησης, ενώ επίσης είναι απλή και οικονομική μέθοδος, γεγονός που την καθιστά πολύ ελκυστική για χρήση στην ανάλυση του οίνου. Στην παρούσα μελέτη, η FT-IR χρησιμοποιήθηκε για τη δημιουργία μοντέλων υπολογισμού με τα οποία θα μπορούσαν να προβλεφθούν σημαντικές παράμετροί του οίνου, όπως η συγκέντρωση ελλαγιταννινών ή η χημική του ηλικία. Σχετικά με την στυπτικότητα, αναπτύχθηκε ένα χρήσιμο εργαλείο το οποίο συνδέει την αίσθηση αυτή με τους διάφορους τύπους μονομερών που απαρτίζουν τις ταννικές αλυσίδες, ενώ ο μέσος Βαθμός Πολυμερισμού (mDP) λευκών οίνων υπολογίστηκε για πρώτη φορά.

Συμπερασματικά, η FT-IR μπορεί να είναι ένα πολύτιμο εργαλείο καθώς μπορεί να παρακολουθήσει την εξέλιξη του οίνου κατά την ωρίμανση με έναν πιο ολοκληρωμένο τρόπο.

Επιστημονικό πεδίο: Οινολογία, Χημεία

Λέξεις κλειδιά: FT-IR, οίνος, στατιστική, ταννίνες, ωρίμανση, βαρέλι,

Monitoring wine maturation with alternative chemical analytical methods. Prospects and challenges

Abstract

Wine maturation is a complex procedure that involves numerous chemical reactions, which ultimately have an effect on wine quality. These reactions are gravely influenced by the type of medium in contact with the wine, but also by the time of contact. As there is no chemical analysis to provide answers on the optimum time of maturation, this decision is based mostly on the increase of astringency of the wine, while in some countries there are strict regulations regarding the time of maturation, the types of wood allowed in wine maturation as well as the use of oak chips.

The objective of this study was to evaluate the feasibility of Fourier Transform Infrared Spectroscopy (FT-IR) in wine authentication during maturation in terms of type of container and time of contact and to deepen our knowledge on the way the type of container affects the astringency sensation.

FT-IR has been highlighted as a valuable tool for classification purposes, and its simple and cost-effective nature makes it appealing for use in wine analysis. During this work, it was used to build estimation models for the prediction of key wine parameters, such as its ellagitannin content and its “chemical age”. Concerning astringency, a helpful tool was developed that associates this sensation with the types of monomers comprising tannin chains, while the mean Degree of Polymerization (mDP) of white wine tannins was estimated for the first time.

In conclusion, FT-IR can be a valuable asset during wine maturation as it can monitor wine evolution in a more integrated manner.

Scientific area: Oenology, Chemistry

Keywords: FT-IR, wine, statistical analysis, tannins, maturation, barrel,

To my father for his faith in me,
To my mother and sister for their endless support,
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Published research

I. Publications resulting from this thesis

1. **Basalekou M., Kyraleou M., Pappas C., Tarantilis P., Kotseridis Y., Kallithraka S.**
   Proanthocyanidin content as an astringency estimation tool and maturation index in red and white winemaking technology. Food Chemistry, 299 (2019): 125135

2. **Basalekou M., Kallithraka S., Tarantilis P.A., Kotseridis Y., Pappas C.**
   Ellagitannins in wines: Future prospects in methods of analysis using FT-IR spectroscopy LWT-Food Science and. Technology, 101 (2019): 48-53.

3. **Basalekou M., Pappas C., Tarantilis P., Kotseridis Y., Kallithraka S.**
   Wine authentication with Fourier Transform Infrared Spectroscopy: a feasibility study on variety, type of barrel wood and ageing time classification (2017) International Journal of Food Science and Technology, 52 (6), pp. 1307-1313.

4. **Basalekou M., Pappas C., Kotseridis Y., Tarantilis P. A., Kontaxakis E., Kallithraka S.**
   Red Wine Age Estimation by the Alteration of Its Color Parameters: Fourier Transform Infrared Spectroscopy as a Tool to Monitor Wine Maturation Time. Journal of analytical methods in chemistry, vol. 2017, Article ID 5767613, 9 pages, 2017.

5. **Basalekou M., Strataridaki A., Pappas C., Tarantilis P.A., Kotseridis Y., Kallithraka S.**
   Authenticity determination of greek-cretan mono-varietal white and red wines based on their phenolic content using attenuated total reflectance fourier transform infrared spectroscopy and chemometrics (2016) Current Research in Nutrition and Food Science, 4 (Special Issue 2), pp. 54-62.

II. Other publications

1. **Philippidis A., Poulakis E., Basalekou M., Strataridaki A., Kallithraka S., Velegrakis M.**
   Characterization of Greek Wine by Ultraviolet-Visible Absorption Spectrometry and Statistical Multivariate Methods
   (2017) Analytical Letters, 50(12), pp. 1950-1963

2. **Loupassaki S., Abouzer M., Basalekou M., Fyssarakis I., Makris D.P.**
   Evolution pattern of wood-related volatiles during traditional and artificial ageing of commercial red and white wines: Association with sensory analysis
   (2016) International Food Research Journal, 23 (4), pp. 1459-1465.

III. Communications within the frames of international congresses and conferences

III.A Oral communications

1. **Basalekou M., Pappas C., Kotseridis Y., Strataridaki A., Geniatakis E., Tarantilis P.A., Kallithraka S.**
   38th World Congress of Vine and Wine, July 5-12, 2015, Mainz, Germany,
   Monitoring wine aging with Fourier Transform Infrared spectroscopy (FT-IR)

2. **Basalekou M., Pappas C., Kotseridis Y., Strataridaki A., Tarantilis P., Kallithraka S.**
   1st International Multidisciplinary Conference on Nutraceuticals and Functional Foods, Kalamata Greece, July 7-9, 2016
   Differentiation of wines based on grape variety by Fourier Transform Infrared Spectroscopy

3. **Basalekou M., Pappas C., Tarantilis P.A., Kyraleou M., Cotea V., Kallithraka S.**
   42nd World Congress of Vine and Wine, 15-19 July 2019, Geneva, Switzerland
   Astringency estimation of wines maturing in different types of containers

III.B. Posters

1. **Basalekou M., Pappas C., Kotseridis Y., Fysarakis I., Geniatakis E., Tarantilis P.A., Kallithraka S.**
   37th World Congress of Vine and Wine, November 9-14, 2014, Mendoza Argentina,
   Differentiation of wines aged in French and American oak barrels based on their FT-IR spectra

2. **Basalekou M., Kontaxakis E., Strataridaki A., Lydakis D., Kallithraka S.**
   Oeno 2015, 10th International Symposium of Enology of Bordeaux, 29 June – 01 July, Bordeaux, France
   Colour evolution of wines aged in different containers by CIELAb: discrimination between type of container and contact time.

3. **Basalekou M., Pappas C., Tarantilis P., Kotseridis Y., Lydakis D., Kallithraka S.**
   Macrowine 2016, June 27-30, Changins (Nyon), Switzerland
   Estimation of Chemical Age of red wines with the use of Fourier Transform Infrared Spectroscopy (FT-IR) and chemometrics

4. **Basalekou M., Konstantinou E., Proxenia N., Kotseridis Y., Kallithraka S., Tarantilis P.A., Pappas C.**
   1st International Multidisciplinary Conference on Nutraceuticals and Functional Foods, Kalamata Greece, July 7-9, 2016
   Evaluation of a Raman spectroscopic method and the conventional distillation method for the determination of alcohol content in Greek spirit Tsipouro

5. **Basalekou M., Pappas C., Tarantilis P., Kotseridis Y., Mandrakis T., Dermentzoglou S., Kallithraka S.**
   39th World Congress of Vine and Wine, 24-28 October 2016, Bento Goncalves, Brazil
   Estimation of total tannin concentration of red wine with the use of Fourier Transform Infrared Spectroscopy (FT-IR) and chemometrics

6. **Basalekou M., Pappas C., Tarantilis P., Kotseridis Y., Nikoli N., Chira K., Teissedre P.L., Kallithraka S.***

   40th World Congress of Vine and Wine, 29 May – 02 June 2017, Sofia, Bulgaria
   Effect of variety, type of aging and type of container on the Mean Degree of Polymerization in red and white wines

7. **Basalekou M., Pappas C., Tarantilis P., Kotseridis Y., Mandrakis T., Dermentzoglou S., Kallithraka S. ***
   10th In Vino Analytica Scientia Symposium, Salamanca, Spain, July 17-20, 2017
   Application of Fourier Transform Infrared Spectroscopy (FT-IR) and chemometrics for the evaluation of the tannic profile of red wines

8. **Basalekou M., Pappas C., Tarantilis P.A., Kotseridis Y., Kallithraka S.**
   Macrowine 2018, 28-31 May, Zaragoza, Spain.
   Ellagitannin concentration estimation with the use of FT-IR spectroscopy

9. **Basalekou M., Pappas C., Tarantilis P.A., Kotseridis Y., Kallithraka S.**
   41st World Congress of Vine and Wine, 19-23 November 2018, Punta del Este, Uruguay
   In situ monitoring of barrel ellagitannin extraction during wine aging with the use of FT-IR spectroscopy

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Abbreviations

HRM	High Resolution Melting
ATR	Attenuated Total Reflection
FT-IR	Fourier Transform Infrared Spectroscopy
DTGS	Deuterated Triglycine Sulfate
ZnSe	Zinc Selenide
LDA	Linear Discriminant analysis
SPE	Solid-Phase Extraction
LPP	Long Polymeric Pigments
SPP	Short Polymeric Pigments
PLS	Partial Least Squares Regression Analysis
TA	Total Anthocyanins
ID	Degree of ionisation of anthocyanins
CD	Color density
CDS	Color density corrected for SO_2
SRP	SO_2 resistant pigments
CI	Color Intensity
h	Hue
PCA	Principal Component Analysis
ANOVA	Analysis of Variance
RMSEC	Root-Mean-Square Error of Calibration
RMSEP	Root-Mean-Square Error of Prediction
RMSECV	Root Mean Square Error of Cross Validation
PRESS	Predicted Residual Error Sum of Squares
HPLC	High Pressure Liquid Chromatography
C	(+)-catechin
EC	(-)-epicatechin
GC	(+)-gallocatechin
EGC	(-)-epigallocatechin
ECG	(-)-epicatechin gallate
EGCG	(-)-epigallocatechin gallate
DP	Degree of Polymerization

mDP	Mean Degree of Polymerization
%G	Percentage of Galloylation
%P	Percentage of prodelphinidins

I. General Introduction

General Introduction

1. Wine

Wine is the result of Vitis Vinifera grapes fermentation and has been produced over thousands of years in many different parts of the world. Its production is a relatively simple process that could be carried out even without human intervention. The only requirement would be the presence of grapes and yeast, and in favorable conditions a spontaneous fermentation may occur converting the sugar in the grapes to alcohol, carbon dioxide and heat. Producing a high quality wine however, is no easy task. For this reason, the concern of winemakers around the world over the years is to control the fermentation process but also to find ways of imparting unique characteristics to their wines in order to make them distinctive. Both are ongoing processes.

The main categorizations of wines have to do with the color of the resulting wine and the way it is produced, i.e. there are different categories according to the amount of CO_2 and/or sugar left unfermented in the finished product. Given the fact that the world total surface area planted with Vitis Vinifera grape varieties for 2017 is estimated to be 7.6mha (OIV, 2018), and that the grape is among a group of crops that are closely related to the notion of terroir -which includes how a particular region's climate, soils and farming practices affect the taste of the final product-, there are also geographical classifications with such significance for the market that can even alter a wine’s final price.

Even though there are many different ways to produce wine according to various factors, such as the market’s requirements, the winemaker’s taste or tradition, there is a basic process to follow, depending on the color of the resulting wine. White winemaking is the simplest procedure and consists of five steps: harvesting, crushing and pressing, settling, fermenting and bottling. The key in white winemaking is pressing right away and settling, as the most important feature of these types of wines is their color, which can be greatly affected by oxygen exposure, mainly before fermentation, and by foreign matters in the must. Red winemaking consists of four steps -crushing, macerating and fermenting, pressing, and bottling-, although there is a fifth -barrel maturation- that can be considered necessary by many. The key in red wine production is extraction during the maceration phase, as the red color is extracted by grape skins to the juice.

Red and white winemaking are the basis of all winemaking procedures, for example rose wines are produced by red grapes following the steps of white winemaking in order to minimize color extraction from the skins. In both procedures barrel aging can be an extra step and has commonly been used in the ageing of wine because of its positive effects on the product; however, it is considered to improve more the quality of red wines. The reason behind this assumption lies in the different composition of each type of wine.

1.1.Phenolic composition

Wine is a complex mixture of several hundred compounds, many of which play an important role in its quality even though they are present in low concentrations (Markoski, Garavaglia, Oliveira, Olivaes, & Marcadenti, 2016). Generally, regardless of its color, wine consists in average of 86% water, 12% ethanol, glycerol, polysaccharides and other trace elements 1%, acids 0.5% and volatile compounds 0.5%. The difference between white wine and red wine is that the latter is found to contain tenfold more phenolic compounds due to the way it is produced (Lingua, Fabani, Wunderlin, & Baroni, 2016).

The phenols found in wine belong to a large group of compounds with a hydroxyl group bonded directly to an aromatic hydrocarbon group. Although their concentration is relatively low, around 2567mg/L in gallic acid equivalents in red wine and 239mg/L in gallic acid equivalents in white wine (Markoski et al., 2016) their presence greatly affects the taste, color and mouthfeel of wine. Indeed, when describing a wine's properties, the most useful information apart from its aroma come from its alcoholic content, its acidity, the presence or not of residual sugars and its phenolic content (Schuster, 2005). Phenolic compounds exhibit a wide diversity in their structure but can be divided into two large groups, flavonoids and non-flavonoids.

Flavonoids are molecules with two phenolic groups joined by an oxygen-containing ring structure and contain a large number of compounds that differ in the number and position of hydroxyl (-OH) and mehoxy (-OCH_3) groups. For wine, the most important between these compounds regarding chemical reactions and sensory properties are the anthocyanins (e.g. malvidin-3-O-glucoside) and the flavanols (e.g. catechin and epicatechin), followed by flavonols (yellow pigments) (Fig.1). Non-flavonoids contain phenolic acids and their esters and are differentiated by substitution of their benzene ring. From their degradation products, one of the most important is gallic acid (Oberholster, 2003).

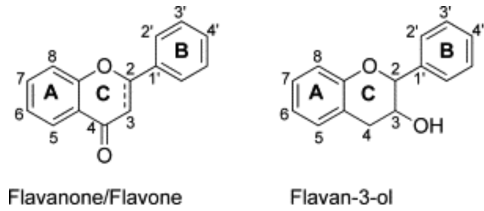
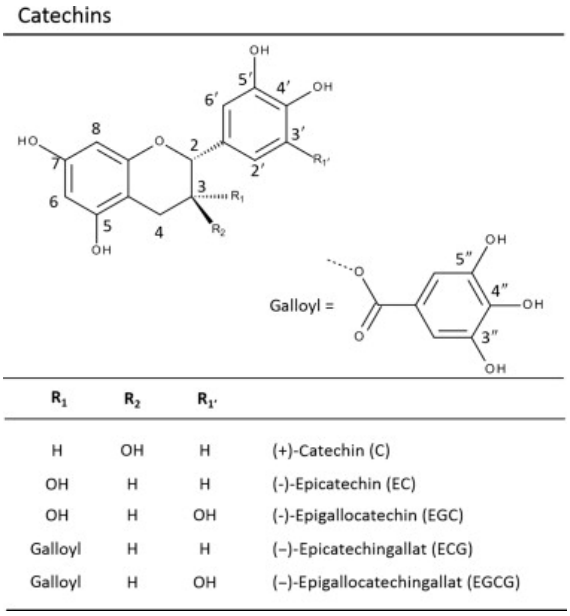


Figure 1. Structure of flavan skeleton (Mewett et al., 2009).

From the group of flavonoids, (+)-catechin and (-)-epicatechin can form dimers, trimers and higher oligomers called procyanidins through interflavan linkages (Fig.2). In the procyanidin group belongs one of the key compounds in wine, tannins.



R_1	R_2	$R_{1'}$	
H	OH	H	(+)-Catechin (C)
OH	H	H	(-)-Epicatechin (EC)
OH	H	OH	(-)-Epigallocatechin (EGC)
Galloyl	H	H	(–)-Epicatechingallat (ECG)
Galloyl	H	OH	(–)-Epigallocatechingallat (EGCG)

Figure 2. Catechin structures (Esselen & Barth, 2014).

Even though tannins are one of the most well studied groups of compounds they present such polymorphism that they still remain a challenge in terms of obtaining detailed information on their composition (Absalon, Fabre, Tarascou, Fouquet, & Pianet, 2011). The same applies for phenolic compounds in general, as their structure for example varies a great deal when wine ages in barrel or in the bottle, although these modifications, which include polymerizations, have not yet been fully explained (Ribéreau-Gayon, Glories, Maujean, & Dubourdieu, 2006).

Phenolic compounds are considered important because they are responsible for the differences between red and white wines –in terms of color and flavor- and also due to their unique properties: they are bacteriosides, antioxidants and have vitamin properties as well. For these reasons, they are strongly linked to wine quality.

1.2.Evaluating wine quality

High quality wines are a combination of many factors. They are considered a result of a complex set of interactions that include climate and soil variables, viticultural and of course winemaking decisions (Reynolds, 2010). As such a complex notion, describing quality itself, presents difficulties mainly because it is a partially subjective and strongly influenced by extrinsic factors. Many efforts have been made in order to correlate quality with chemical analyses, but without the contribution of tasting from wine connoisseurs it can only be partially successful (Jackson, 2017). While defining quality can be trivial to the consumer, since ultimately personal taste is most important when making a purchase, it is of outmost importance for the producer, who wants to be able to make better choices regarding the procedures they use.

Despite the diversity in terms of geographical origin, or the big number of grape varieties, winemakers have also tried various winemaking protocols through the years in order to be able to differentiate their wines. One of the most favorable additions to the basic procedure is aging in different types of containers.

2. Wine maturation

The containers mostly used for maturation purposes early on in winemaking history, were wooden barrels, and in fact served as storage containers. The wood used was not limited to oak: chestnut, cherry, and whichever other species were abundant in the region were the wine was produced could also be used. Experimentation showed that storing wines in wooden barrels had positive results and oak in specific prevailed since it complemented the wine flavor best, as opposed to chestnut wood for example which was a little bitter and not so elegant (Brostrom & Brostrom, 2009). This observation, in the start of the century, led the countries with well-established wine traditions in a search for quality, whereas countries where winemaking was a new industry, such as Argentina and Chile, did not aim for high quality but rather for low prices and preservation of existing stocks (Campbell & Guibert, 2007). Either way, winemakers became acquainted with the advantages of barrel aging or maturation as it is mostly called. Nowadays, a wide range of wood types but also sizes and shapes are available for barrel making. In each case, the desirable effects on the final product include improvement of color stability and clarification, but also a more complex aroma. Today, nearly all the high quality red wines of the world but also many premium white wines mature or even ferment in barrels, and the reason for that is that barrels provide an ideal slow maturation for wine (Brostrom & Brostrom, 2009).

2.1.Phenomena occurring during maturation

Maturation is generally called the period after fermentation and before bottling and refers to changes in wine taking place during that time. The changes that occur in the wine matrix during maturation are numerous, they are greatly influenced by the type of container, and ultimately affect the wine's color, clarity and taste.

Stabilization and changes in color

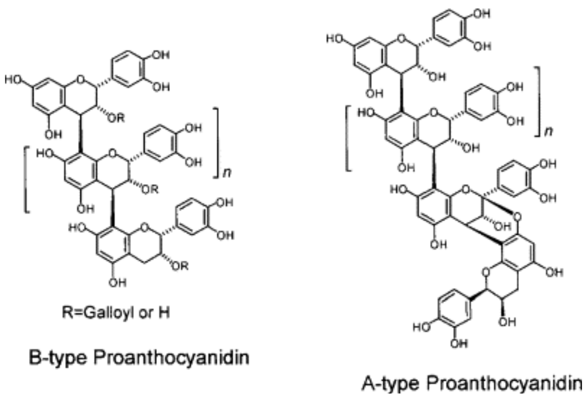
First of all, the carbon dioxide which was produced during fermentation is removed, along with excessive tartrate acid, which precipitates. If the wine is white and is exposed to oxygen its color becomes darker as several phenolic compounds are involved in oxidative reactions (R. B. Boulton, Singleton, Bisson, & Kunkee, 1999). In the case of red wines, the monomeric anthocyanins that had been extracted by the grape skin during fermentation are progressively replaced by the polymeric form, which is more stable. In the presence of oxygen there can be condensation reactions between anthocyanins and tannins, which results again in the formation of stable pigments. The polymerization of pigments may also occur anaerobically. The reactions between tannins and anthocyanins but also others that involve acetaldehyde, intensify color (Ribéreau-Gayon et al., 2006).

Mouthfeel and Aroma

Another important change is the taste and mouth feel of a wine, which becomes mellower and smoother. This happens through polymerization of phenolic compounds and reduction of acidity. Bitterness and astringency are mostly linked to flavonoid phenols, monomeric flavonoids in particular are more bitter than astringent. As they polymerize, they become less bitter but more astringent, however during maturation they precipitate after becoming large, resulting in a less astringent wine. Controlled oxidation leads to a decrease in astringency and an increase in the color stability of red wines, due to the combination between anthocyanin and tannin molecules but also to the presence of acetaldehyde that is produced by ethanol oxidation (Zoecklein, Fugelsang, Gump, & Nury, 1999).

Apart from taste, wine aroma is altered during maturation as well. Yeasty aromas give their place to new ones, while esters formed during fermentation are hydrolyzed, and the fresh and fruity aroma is lost. Moreover, in the presence of wood in the form of barrel or wooden pieces immersed in an inert container, volatile compounds -extracted by the wood- may be incorporated in the wine. Wine aroma may also deteriorate during maturation, for example due to oxidation, or because of odors formed due to spoilage by yeast or bacteria (Jackson, 2008). In the case that a wooden barrel is used as the container during maturation, then that period may last from 3 to 24 months, according to the desired effect on the wine. Maturation in contact with wood enhances the complexity of phenomena occurring during this period (R. B. Boulton et al., 1999). First, the wood -especially if it is in the form of a new barrel- absorbs some of the wine, while at the same time water and ethanol evaporate through its pores. This loss is not negligible and is affected by the prevailing conditions of relative humidity and temperature in the cellar. Up to 2-5% of the wine volume may be lost per year, adding to that 5 or 6 liters per wine for each 225 liter barrel, that are absorbed by its staves (Jackson, 2008). Then, the wood releases several volatile and nonvolatile substances.

Between the wood extracted polyphenols the most important are ellagitannins but other components are extracted as well such as lignins, lignans, trirterpenes, coumarins, and also molecules produced by the transformation of ellagitannins such as gallic acid (Ribéreau-Gayon et al., 2006). These compounds can influence the sensation of astringency and alter the mouthfeel of the wine (Zamora, 2019). Actually they may enhance astringency for some time, until the condensed tannins will soften again due to the various polymers (Ribéreau-Gayon et al., 2006). Astringency can also be reduced through reactions between anthocyanins (in the case of red wines) and proanthocyanidins, favored by the oxygen that passes through the pores of the barrel, which also help stabilize the color. Proanthocyanidins (Fig. 3) are composed of flavan-3-ols that are linked through single C-C bonds and can be divided in B-type (C4 → C6' or C4 → C8' bonds) or A-type (doubly linked with an additional bond at C2 → O → C7') (Yoshida, Hatano, & Ito, 2005). Ellagitannins in particular have been found to be highly reactive with other flavonoids. They can react with condensed tannins and form flavano-ellagitannin products and with anthocyanins to stabilize wine color (Watrelot, Badet-Murat, & Waterhouse, 2018). In some cases, there may also be a release of polysaccharides mainly consisting of hemicellulose (Ribéreau-Gayon et al., 2006). The final amount in the wine, depends on the contact time between wine and wood and of course on the wine composition (Cerdán & Ancín-Azpilicueta, 2006). The type of the polyphenols that will be extracted into wine, depend on the type of wood used during maturation in terms of geographical origin and species, seasoning, toasting but also on the flavoring process, as many winemakers choose not to use wooden barrels but to immerse wooden pieces in the form of chips or sticks into an inert container in order to minimize the time of extraction but also maturation costs. Finally, maturation in barrel also allows natural sedimentation of unstable colloidal matter (Zamora, 2019).



B-type Proanthocyanidin

A-type Proanthocyanidin

Figure 3. Proanthocyanidin structures, A and B-type (Yoshida et al., 2005).

In terms of flavor, phenolic compounds extracted from wood have an effect on the wine's final organoleptic profile. Indeed, phenolic acids such as gallic acid have an acid taste as well as coumarins which are also harsher in character in the case of coumarin aglycones and very bitter in the case of coumarin glycosides. Ellagitannins on the other hand are astringent compared to gallotannins which are perceived as more bitter and acidic. Procyanidin B3 in particular, is both astringent and bitter (Ribéreau-Gayon et al., 2006). Rapid hydrolysis of galloyl esters in wine and low degradation of complex tannins resulting from wood may not significantly impact wine flavor, however levels of these types of tannins already decrease during seasoning and toasting (Zoecklein et al., 1999). Of course, the timing of maturation is important, as it is generally believed that wood is better integrated into wine if the wine is put into barrels right after fermentation completion along with is lees. This way the wooden aromatic character is less pronounced, as a result of yeast and enzyme activity (Zoecklein et al., 1999). As for the mouthfeel, almost one third of tannins extracted by wood are absorbed by yeast cell walls, and some also combine with the colloids in the wine producing this way the effects of a natural fining (Zoecklein et al., 1999). Condensed tannins (flavan-3-ol oligomers and polymers; proanthocyanidins) are a major contributor to astringency. Their reactivity towards human salivary proteins has been better described by the mean Degree of Polymerization, which is the average number of constitutive units: high mDP means large tannins, and large tannins tend to precipitate when combined with salivary proteins, leading to a decrease in the astringency sensation, while monomers not only enhance astringency but also bitterness (Ma, Watrelot, Addison, & Waterhouse, 2018). Reactions of tannins with anthocyanins, yield tannin-anthocyanin adducts that also decrease astringency, which is ultimately influenced by many factors, such as tannin concentration but also wine matrix components, such as alcohol, pH, polysaccharides etc. (Ma et al., 2018; Weber, Greve, Durner, Fischer, & Winterhalter, 2013). The diffusion of oxygen in the barrel may intensify the sweetness of barrel matured wines, due to the formation of esters and acetals (Espitia-López, Escalona-Buendía, Luna, & Verde-Calvo, 2015). Acidity on the other hand can decrease due to precipitation. The sensation of astringency presents a challenge, as it can be enhanced indirectly without formation of new compounds but due to the ability of some wine components to alter the salivary flow even when presenting separate taste qualities, but can also decrease due to precipitation after tannin polymerization produces long enough tannin chains (Oberholster, 2000). Recent research showed that high SO_2 levels during maturation lead to low protein-binding tannin levels, low mean Degree of Polymerization and low tannin activity in general, which means that even SO_2 can change tannin composition during maturation, influencing this way their astringency (Ma et al., 2018).

2.2.Parameters affecting maturation

Impact of the wine matrix

The extent to which a wine will benefit or not by barrel maturation depends greatly on the wine itself. White wines for example with low concentrations of phenols, cannot withstand barrel maturation for long time periods, as the phenols extracted from the barrel overpower the wine, making it appear flat and oaky (Ribéreau-Gayon et al., 2006). Moreover, wines with high acidity are considered age worthy, as acidity tends to decrease during maturation due to spontaneous precipitation, but also influences the solubility of many wood aromatic compounds such as vanillin, syringaldehyde and their respective acids (R. B. Boulton et al., 1999; J.-L. Puech, 1987). Finally, the amount of wood compounds to be extracted from the barrel seems to be mostly influenced by its ethanol content. It is well known that spirits extract barrel compounds faster than wine and this has been attributed to their high ethanol concentration (van Jaarsveld, Hattingh, & Minnaar, 2017). However, contradictory reports exist in wine, most of which point to higher ethanol contents being correlated with higher concentrations of extractable compounds, especially volatile ones (Cerdán, Goñi, & Azpilicueta, 2004). Finally, the variety of grape used during vinification has been found to have an influence on the extraction of compounds from the wood to the wine (Ortega-Heras, González-Huerta, Herrera, & González-sanjosé, 2004).

Impact of the type of container

A wine container should ideally be inert, impervious, durable, economical, easy to maintain and should not impart flavors on the wine, except if those flavors are desirable (R. B. Boulton et al., 1999). Most of these conditions are met by the use of stainless-steel containers, however containers made from clay, concrete, wood and more recently plastic, have also been used. All have certain advantages but also disadvantages; for instance, even though plastic and concrete are impermeable to oxygen, some plastic tanks are permeable to light which can deteriorate wine quality. Concrete tanks need to be coated in order to be impervious and wooden barrels impart favorable elements to the wine, but they may be nearly exhausted of flavor contribution after three or four cycles of use, raising this way the cost of use. Based on these qualities, over the last decades stainless steel and oak barrels have presented a continuous rise in use especially in commercial wineries, even though today there are many wineries experimenting again with the use of clay amphoras, concrete tanks of different shapes and barrels made from wood other than oak. The reason stainless steel tanks remained so popular is because they are practically the most inert type of container: they only need to be filled to capacity or have the headspace blanketed with an inert gas and the only effect on the wine will be time, and the continuation of any types of reactions already in progress (R. B. Boulton et al., 1999). Oak barrels on the other hand, are so popular because even though they are not inert (oxygen passes to the wine through the pores of wood), they impart favorable flavors to the wine, through the extraction of phenols and volatile compounds. The infusion of oxygen into the wine allows a better tannin and color development over time and the addition of the extracted compounds improve the wine's structure, complexity and roundness (Brostrom & Brostrom, 2009).

Wineries from the so-called New World countries (referring to countries that are relatively new to winemaking, outside of the traditional winegrowing areas of Europe, such as the USA, Australia, New Zealand etc.) have first experimented with alternatives to barrels, as a technique to give their wines the benefits of barrel maturation but in a much lower cost. These techniques include wooden staves suspended inside stainless steel tanks, and wooden chips in many sizes and shapes, again added to wines in tanks. The wood used for the manufacturing of staves and chips would mostly be oak, either French or American, as it was in high demand but also easier to find in terms of production (Brostrom & Brostrom, 2009). Barrel alternatives were met with a lot of skepticism from the Old World countries, and as a result, many of them considered macerating wine with wooden pieces adulteration, resulting in making it illegal to use these products. This prohibition is still active in Europe, however not for all wines, but for some quality wines of specific appelations (Brostrom & Brostrom, 2009).

Impact of wood geographical origin and species

Many differences derive from the geographical origin of the wood. In the case of oak which has been extensively researched, there are even differences between oaks coming from the same country (France) but from different forests. Indeed, oaks coming from the Limousin and Bourgogne region (*Quercus robur* or *Quercus pedunculata*), have a high extractable content and low concentrations of volatile compounds, while oaks coming from the Centre and Vosges regions (*Quercus petraea* or *Quercus sessilis*) have low levels of extractable ellagitannins but a high aromatic potential. Moreover, wood from *Quercus robur* from the Limousin region, is less porous than that of *Quercus petraea* coming from the Vosges region (Ribéreau-Gayon et al., 2006). The quality of oak wood also depends on the age of the tree, even from the direction in which it was facing. Regarding the species, the most prominent differences are found between French and American oak (*Quercus alba*), as interestingly, most European oaks such as those coming from Central Europe (Russia, Hungary etc.) present similarities to French oak. *Quercus alba*, or white oak, has a low phenol content but a high aromatic potential compared to French oak (Ribéreau-Gayon et al., 2006). Most differences between French and American oak are apparent during the first fill, and progressively are less marked, especially in the extraction of nonflavonoid compounds as their extraction does not drop as fast as that of total phenolics, they rather extract proportionally with each fill (Jackson, 2008).

Impact of wood seasoning

Seasoning or drying is the procedure during which a wood’s moisture content is reduced before its use. When done naturally it can take several years, but generally it takes between 24 and 36 months according to the thickness of the staves (the narrow planks of wood that form the sides of the barrel). Seasoning is highly heterogeneous, and also involves enzymic reactions depending on the presence of fungal microflora on the wood. During seasoning, the wood becomes less astringent, and the extractable ellagitannins decrease due to hydrolysis by enzymes, especially if they are water soluble monomers and oligomers. Polymerized forms take more than three years to decrease. Moreover, glycosylated coumarins which are bitter, are also hydrolyzed to aglycones that have neutral to acidic flavors.

Impact of wood toasting

Toasting is the phase during which the wood is subjected to heating. This way the plasticity of lignin is affected, and the bending of staves is facilitated. Again, this is a procedure of high heterogenicity as each cooperages' toasting conditions vary due to the human element but also to the type and intensity of the heat source. Depending on the time of heating and the intensity of the heat source, there are three levels of toasting: light, medium and heavy. In each case, as a result of the heat, cellulose, hemicellulose and lignin that are present in the wood, give rise to different decomposition products, while ellagitannins break down (mostly after medium toasting) and produce gallic and ellagic acid (Ribéreau-Gayon et al., 2006). Products of hemicellulose resulting from heat treatment include furfural, maltol, cyclotene and ethoxylactone (Zoecklein et al., 1999). Toasting gives rise to a lot of aromatic compounds, through the formation of volatile compounds of several origins, or through thermal degradation of lignin and polyols but also of some lipids or fatty acids, with maximum quantities being form when the wood is medium toasted. In general, when red wines mature in high-temperature toasted barrels, they contain lower concentrations of ellagitannins, higher mass condensed tannins, and pigmented tannins in higher percentage than wine matured in low-temperature toasted barrels (Watrelot et al., 2018). According to Francis *et al.*, (1992), heat is the most important barrel production parameter regarding influence on the sensory characteristics of wine.

Impact of the wine flavoring process

As mentioned earlier, a winemaker may choose to mature a wine into a wooden barrel or immerse wooden pieces into the wine, in order to minimize costs and time as extraction is generally rapid and intense due to the large contact surface. The different forms of wooden fragments that have been used in wineries include staves, splints, shavings, chips or even powder. They are cut into dices, dominoes or even square pieces referred to as blocks or segments. Except for tanks, these fragments can also be used in old barrels depleted by their extractable compounds, in which case they are called oak chains, sticks or barrel inserts. The majority of these fragments are produced by different kinds of oak wood, such as French, American, Hungarian and Pyrenean, and can be subjected to different levels of toasting (Hernández-Orte et al., 2014). Wines maturing in contact with oak chips have been found to undergo a quicker maturation, quicker loss of anthocyanins and to suffer a higher number of polymerisations than the wine maturing in barrels (Del Alamo Sanza, Escudero, & De Castro Torío, 2004).

Although oxygen can be incorporated into wine through many ways such as racking or aeration, maceration of wooden pieces in wine may very well be a procedure completed in oxygen absence. However, oxygen is necessary in order to bring out the wooden character through oxidation reactions. Apart from the alterations in the aromatic character, these techniques of wood maceration give a certain flavor to the wine as well, however it cannot be compared to that of real barrel aging, maybe due to the fact that most of the time, wine treated that way is not of high quality. In general, results are better the thicker is a wooden piece, meaning that macerating with shavings produces the less satisfactory results (Brostrom & Brostrom, 2009; Ribéreau-Gayon et al., 2006). It has been proved that chips particles with a diameter of 3-5mm favor the extraction process (Giménez Martínez et al., 2001).

Barrel alternatives cannot replace barrel maturation, as the result of a barrel aged wine is more elegant, complex and balanced, while wines produced using barrel alternatives come off as more strong and invasive (Brostrom & Brostrom, 2009; Ribéreau-Gayon et al., 2006). Another issue is that macerating wooden pieces is not legally authorized everywhere. Regarding the amount of ellagitannins extracted to the wine, those depend on the type of wood used for the production of the wooden pieces. When ellagitannins are present in large quantities, they may be also accompanied by bitter coumarins.

Another technique of wine flavoring is using barrels of different sizes and shapes, although 225 liters is the most popular barrel style. Lately there has been a trend for large barrels (between 400 and 700 liters), in order to lower the impact of wooden flavor in the wines, by minimizing the contact surface. Polyphenols extracted from these barrels, will be as expected, lower than those from a classic style barrel of 225 liters (Brostrom & Brostrom, 2009).

Impact of alternative wood sources

Many types of woods were used earlier in winemaking history such as Chestnut (Castanea sativa), Acacia (Robinia pseudoacacia), Cherry (Prunus avium), Ash (Fraxinus excelsior), Beech (Fagus sylvatica) or even canary pine (Pinus canariensis) (Zamora, 2019). Today, especially Acacia, Chestnut but also Cherry and Mulberry seem to be making a comeback (Rosso, Panighel, Vedova, Stella, & Flamini, 2009). The reason is that they impart different characteristics to the wine, the most important of which come from their phenolic group, volatile and nonvolatile.

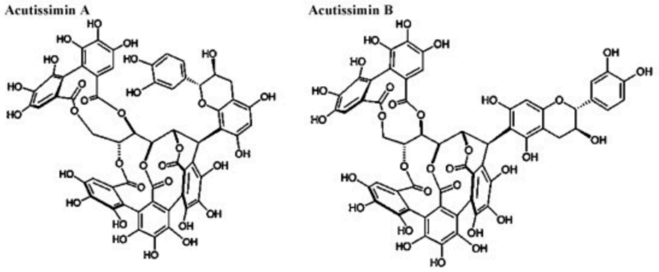
As mentioned earlier, phenolic compounds take part in copigmentation processes, influence color stability, protect against oxidation and help decrease the astringency of the wines (Darias-Martín, Carrillo, Díaz, & Boulton, 2001; Viriot, Scalbert, Lapierre, & Moutounet, 1993; Vivas

& Glories, 1996). The primacy of oak is based on the specific properties it possesses. Its wood is straight-grained, exhibits strength and resilience and structurally it is free of faults that could make the cooperage leaky. It also does not have odors that could be deemed undesirable (Jackson, 2008). Moreover, it is rich in ellagitannins, such as castalagin, vescalagin, roburin E and grandinin and it has been found to contain flavanols and oligomeric proanthocyanidins (Pallenbach, Scholz, König, Rimpler, & Hunkler, 2007). The flavan-3-ols (+)-catechin and (-)-epicatechin are very commonly present in bark, probably as precursors of condensed tannins, or even as byproducts of their biosynthesis (Hon & Shiraishi, 2000). It also contains low molecular weight phenolics ellagic and gallic acid and lignin derivatives, but no other kind of phenols such as flavonoids (Bŕigida Ferńandez De Simón et al., 2014). The phenolic and furanic aldehydes oak wood adds to the wine, can react with flavanols with the contribution of acetaldehyde in particular and produce a condensation product composed of two flavanols. Such condensation products have been thought to intervene in red wine color and astringency evolution (Nonier, Pianet, Laguerre, Vivas, & Vivas De Gaulejac, 2006).

Comparing French and American oak, American oak wood has lower levels of phenols and higher levels of aromatic flavourants (Cerdán, Rodríguez Mozaz, & Ancín Azpilicueta, 2002; Du Toit, 2004). Research mainly suggests that maturation in different types of oak affects primarily the quantitative than the qualitative composition of wood extracted compounds (Jindra & Gallander, 1987). Moreover, wines matured in woods that have high tannin potential extract more ellagitannins and have condensed tannins with lower mass (Watrelot et al., 2018).

In Europe, Chestnut (*Castanea sativa*) and Acacia (*Robinia pseudoacacia*) used to be employed for the construction of large storage containers (Jackson, 2008) but now they are in high demand for barrel making. However, only the use of Chestnut is approved by the International Enological Codex of the International Organisation of Vine and Wine for cooperage [OENO 4/2005].

Chestnut was found to be suitable for maturation even though it is more porous than oak (R. Boulton, 2009). High porosity means that more oxygen will be diffused faster into the wine, which makes the risk of oxidation higher. However, the high concentrations of low molecular weight polyphenols it contains make its use appealing for the production of wines rich in antioxidants. One drawback is that Chestnut wood is particularly rich in gallic acid, which is known to be particularly astringent and bitter (Hufnagel & Hofmann, 2008; Sanz et al., 2010). It is also rich in ellagitannins, compared to oak species (Alañón et al., 2011). Both gallic and ellagic acids are released into wines maturing in Chestnut barrels in higher quantities after six months of maturation. Regarding the concentrations of flavan-3-ols they seem to remain constant during maturation even though they have been known to fall during storage of model wine (Alañón, Schumacher, Castro-Vázquez, et al., 2013). Moreover, it has been found to contain ellagic acid derivatives, such as ellagic acid deoxyhexose, ellagic acid dimer dehydrated and valoneic acid dilactone, with the latter being qualified by research for use as a chemical marker for wine maturation (Bŕigida Ferńandez De Simón et al., 2014; Sanz et al., 2010). In conclusion, compared to oak, only chestnut contains protocatechuic acid and protocatechuic aldehyde, presents higher concentrations of gallic acid than oak, and except for vescalagin and castalagin which are the main ellagitannins, chestnut has been found to also contain acutissimin (Sanz et al., 2010) (Fig.4).


Acutissimin A

Acutissimin B

Figure 4. Structures of Acutissimin A and B (De Vasconcelos et al., 2010).

Acacia on the other hand, is also rich in phenolic compounds but generally poor in extractable tannins and polyphenols (Tavares, Jordão, & Ricardo-da-silva, 2017). It contains flavonoid and nonflavonoid compounds, which have been shown to be affected by the procedure of seasoning: more flavonoids and less nonflavonoids are found in seasoned acacia wood. The most abundant flavonoids are dihydrorobinetin and robinetin, which are characteristic compounds of Acacia as they are not detected in any other wood. Another difference is that Acacia barrels contain only a small amount of condensed tannins, and no ellagitannins (Sanz, Fernández de Simón, et al., 2012) even though ellagitannins have been identified in chips made from Acacia wood (Kyraleou, Kallithraka, Chira, et al., 2015). Acacia has been promoted as a wood that can enhance the sensory profile of the white wines and can provide structure and mouthfeel in wines without the tannin flavours that are associated with oak.

Impact of time of contact

The time a wine spends in contact with wood depends on the content of volatile and nonvolatile phenols that the winemaker wants to extract into the wine. Of course, the type of maturation plays a great role in this decision, as wooden pieces take a lot less time to extract the favorable compounds. For instance, it is estimated that oak chips with a diameter smaller than 1mm, 90% of the extractable compounds are removed within a week. Volatile compounds take longer to appear due to their slower formation. In the case of wooden pieces, the extraction of ellagitannins is also facilitated (Jackson, 2008). The duration of contact also influences the type of flavors extracted, for instance shorter duration extracts higher proportion of woody flavors, while longer durations are required for more appreciated flavors (Jackson, 2008).

2.3.Choosing the optimum type of maturation

Choosing the optimum type of maturation is a demanding task as it depends on many factors. First of all, the winemaker must choose the right type of wood, the right type of barrel size, shape, or the type of barrel alternative (chips, sticks etc.), the level of toasting, whether to use new or already used barrels, and then decide how much additional flavor each wine can hold up without being overpowered by the wood. This is crucial especially in the case of white wines, which do not have the phenolic content of the reds, so each phenolic addition to the wine is easier to be perceived. The right type of barrel must enable wines to develop their full character while remaining in balance. Experimentation has shown that natural seasoning is better for red wines than artificially seasoned wood, while toasting eliminates undesirable green aromas by imparting more pleasant spicy and vanilla notes. Regarding oak, it has been found that coarse grained oak releases more amounts of phenols than fine grained oak. The same happens with high density wood, and for this reason in cases of wines with a rich tannic structure but without body and roundness, lower density woods may prove helpful in reinforcing those wines' astringency. As for choosing between new and used barrels, a rule of thumb is that aging in new barrels is advisable for wines with a robust structure which can resist being overpowered by an excessive woody character. On the other hand, used barrels provide a more discreet and subdued woody character (Ribéreau-Gayon et al., 2006). Regarding shape and size, they influence the surface to wine ratio. A sphere has the smallest surface and for this reason there are egg shaped concrete tanks that emerge in today's market, since the small surface per liter prolongs maturation (R. Boulton, 2009). If size increases, the surface decreases as well. This means that to achieve the same results of a three year maturation in a 200 liter barrel, one would need one and a half years in ten 20 liter barrels (R. Boulton, 2009). The characteristics of wine matured in 220-liter and 1000-liter barrels, also depend on whether the barrel is new or used. In new barrels, larger barrel volumes translate in lower concentrations of compounds related to oaky aromas (L. J. Pérez-Prieto, López-Roca, Martínez-Cutillas, Pardo Mínguez, & Gómez-Plaza, 2002).

2.4.The case of Stainless-steel containers

Inox containers are mostly inert; they do not impart flavors to the wine either favorable or not. However, wines that mature in inox containers do present changes in their composition, as for example, precipitation of compounds still occurs. In general, these types of containers can be used as control containers, as they present all the changes imparted on wines by time. These include decrease in anthocyanic content of red wines, decrease in phenolic acid content - however less pronounced compared to wines maturing in contact with wood-, and stable contents of flavan-3-ols (Beer et al., 2008).

3. Monitoring wine maturation

As a complex phenomenon involving many reactions and dependent on various factors, wine maturation is a difficult phenomenon to monitor. Most studies dealing with maturation have been based either on the changes in volatile or nonvolatile groups, however even after that division, the multitude of compounds makes selecting specific compounds of interest imperative. When studying the nonvolatile fraction of a wine, the focus is mostly on the phenolic compounds deriving either from the wine or the container it matured in or even specifically on anthocyanins, as color is a very important aspect especially for red wines. However, this is a complicated task because tannins and anthocyanins tend to react leading to the copigmentation phenomenon, in which colored forms of the anthocyanins along with non colored organic compounds form complexes (R. B. Boulton, 2001). Moreover, studies trying to evaluate woods different than oak according to their enological potential, focus on the identification and characterization of as more as possible compounds, some of which could be potentially be used as markers when examining in what wood matured a certain wine (Alañón, Schumacher, Castro-vázquez, & Díaz-maroto, 2013; Alañón, Schumacher, Castro-Vázquez, et al., 2013; Castro-Vázquez et al., 2011).

Analyses during maturation target compounds that influence the organoleptic properties of the wine such as color, aroma and mouthfeel, or that are influenced by the type of maturation, such as volatile compounds and phenolic compounds in general. The major methods for the identification of grape procyanidins are electrospray ionisation mass spectrometry (ESI-MS) and ^{1}H NMR and ^{13}C NMR spectrometry (Oberholster, 2000).

3.1.Color

In order to evaluate the way a wine ages, the first parameter to monitor is its color. As mentioned earlier, the color of white wines changes during maturation, acquiring more brownish hues depending on the level of oxidation, while the color of red wines gradually changes from cherry red to deep red and then brick red (Ribéreau-Gayon et al., 2006). Many methods exist that evaluate color, the most used between which due to their simplicity are measurements of Color intensity, Hue, Color composition, Brilliance of red wines. All of these methods describe color after measuring the wine's optical density measurements at 420, 520 and 620nm with a spectrophotometer, where yellow, red and blue colors absorb. The results give only an indication of the wine color and do not reflect its overall visual perception, however more improved methods such as the CIELAB universal color appreciation system are not commonly used, as their results are difficult for winemakers to interpret (Ribéreau-Gayon et al., 2006).

More specialized methods, especially for red wines as white wines do not contain pigments that contribute to their color rather than compounds that change color when oxidized, analyze their anthocyanic content in terms of concentration but also in terms of quality. Such measurements are the Ionization and PVPP indexes, which define the percentage of anthocyanins in their colored form, and the phenolic polymers (pigmented or not) respectively (Oberholster, 2000; Ribéreau-Gayon et al., 2006).

Somers and Evans, (1977) also proposed spectrophotometric analyses that dealt with the anthocyanin equilibria in a wine. They introduced analysis of the Degree of Ionization of Anthocyanins after abolishing SO_2 effect upon wine color, but also the Chemical age Indexes i and ii. Chemical age measurements are the first color measurements that actually deal with the matter of aging, as they express the extent to which polymeric forms have displaced the monomers in the course of ageing reactions.

Sophisticated methods target specific compounds, such as the total free anthocyanic content but also more complex compounds such as pyranoanthocyanins, new pigments derived from anthocyanins and appear in wine during aging and are performed using instrumentation such as High Pressure Liquid Chromatography and Mass Spectrometry (J Bakker, Preston, & Timberlake, 1986; Stamatina Kallithraka, Aliaj, Makris, & Kefalas, 2009; Marquez, Serratosa, & Merida, 2013).

3.2.Aroma

The evolution of aroma during wine maturation is normally performed by identifying aromas extracted from the barrel. Most analyses on wine aroma are made after in bottle aging in order to evaluate the loss of fermentation and varietal aromas and the formation of the "bouquet", aromas produced after chemical reactions among wine compounds due to aging, and that can also include aromas extracted due to exposure to oak etc. (Dubourdieu & Tominaga, 2004; Ribéreau-Gayon et al., 2006; Rodríguez-Rodríguez & Gómez-Plaza, 2012). The effect of wood on the maturing wine is evaluated measuring volatile compounds extracted from the barrel, such as furfural and 5-methylfurfural, guaiacol, 4-methylguaiacol, cis- and trans-oak lactone, and vanillin and are measured using sophisticated instrumentation such as Gas Chromatography coupled to Mass Spectrometry(De Rosso, Cancian, Panighel, Dalla Vedova, & Flamini, 2009; Luis J. Pérez-Prieto, López-Roca, Martínez-Cutillas, Pardo-Mínguez, & Gómez-Plaza, 2003). Some of these compounds are specific wood components, while others such as vanillin can also be found in wine in traces, however their concentration increases significantly with the wood contribution (Cheynier, Schneider, Salmon, & Fulcrand, 2010).In most cases, sensory analysis is also performed in order to profile the extracted aromas, using descriptive analysis techniques (Spillman, Sefton, & Gawel, 2010). Sensory characterization of barrel aged wines, uses word descriptors such as “toasted aroma”, “vanillin”, “wood aroma” etc. and “astringency” and “persistence” when evaluating mouthfeel (De Coninck, Jordão, Ricardo-Da-Silva, & Laureano, 2006).

3.3.Phenolic load

The most important part when studying the nonvolatile fraction of a barrel aged wine, is the estimation and identification of the phenolic compounds it extracted from the wood it came in contact with. Between the extractable phenols, ellagitannins are the most important group (Ribéreau-Gayon et al., 2006). Flavonoid and nonflavonoid phenols are also measured, however the barrel effect on them is often not evident, probably due to short contact times (De Coninck et al., 2006). Barrel maturation is connected to the extraction of various types of phenolic compounds, among which benzoic and cinnamic compounds (vanillin, vanillinic acid, syringaldehyde, syringic acid, coniferaldehyde, sinapaldehyde), gallic acid, ellagic acid and cummarines (scopoletine, umbelliferone) as already mentioned (Del Alamo Sanza, Nevares Domínguez, Cárcel Cárcel, & Navas Gracia, 2004). These compounds play an important role, as not only do some of them contribute to the organoleptic profile of the wine, they also act as antioxidants promoting the health-giving properties of red wine as well in particular (V.L. Singleton, 1992). Indeed, they have been found to influence mouthfeel, color but also clarity of wines. For example benzoic and cinnamic acids but also aldehydes (which can be oxidized to the correspondent phenolic acids) have been found to alter wine’s sensory qualities by contributing to bitterness and harshness even though they are present in very low amounts in wines (V.L. Singleton & Noble, 1976). Cinnamic acids are also known to take part in copigmentation processes with anthocyanins affecting this way their color (R. B. Boulton, 2001; Brouillard & Dangles, 1994). For this reason, low molecular weight phenolic compounds are also quantified (Brígida Fernández De Simón, Cadahía, & Jalocha, 2003). However, analyses of phenols have been proven useful mostly in producing chemical markers for wines maturing in a certain type of wood (Castro-Vázquez et al., 2011; De Siḿon, Esteruelas, Muñoz, Cadah́ia, & Sanz, 2009; Bŕigida Ferńandez De Simón et al., 2014; Sanz, Fernández de Simón, et al., 2012). The evolution of phenolic compounds' concentrations cannot be directly related to maturation or contact time, due to the numerous reactions they take part in, such as oxidation, copigmentation and polymerization reactions and the fact that they are prone to precipitation but also possible to be absorbed in some level by the barrel material through sorption mechanisms (Cheynier et al., 2010; Kammerer & Carle, 2009).

Phenols are commonly quantified in the winery through optical density measurements, such as OD280 which is based on the characteristic absorption of the phenolic benzene cycles at 280nm. It is not as accurate as Folin-Ciocalteau method which is normally used for an average estimation of the phenolic load; however, it is used for its simplicity and speed. A number of tannin assays exist as well, that calculate the tannin concentration based on the observation that they are polymerized flavanols, which heated in the acid medium will convert to anthocyanins (LA method) or on their several properties including instability in a concentrated acid medium (HCL index), ability to pass through a dialysis membrane with speed correlating to their structure and charge (Dialysis index) but most of the methods are based on the fact that tannins react with proteins forming stable combinations (Gelatin index, BSA assay etc.) (Ribéreau-Gayon et al., 2006). All other measurements targeting specific phenolic compounds require instrumentation such as HPLC or HPLC-MS, including the estimation of the mean Degree of Polymerization, an analysis which gives information on the proanthocyanidin content of the wines and the average degree of tannin polymerization. The mean Degree of Polymerization is mostly determined after acid catalysis with phloroglucinol, or phloroglucinolysis as described in Figure 5. After phloroglucinolysis, a segmentation of flavan-3-ol interflavanoid bonds is observed; the terminal unit is released as a flavan-3-ol monomer and C4 phloroglucinol adducts of the extension units are formed. This is one of the most informative regarding phenolic analyses as it can give information on the concentration of proanthocyanidins, on their structure but it can also be correlated with the astringency and bitterness as these sensations are influenced by the length of the tannin chain (Chira, Jourdes, & Teissedre, 2012).

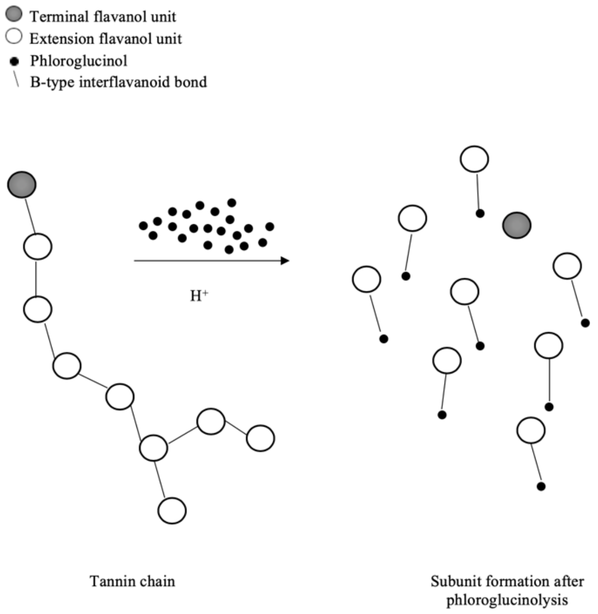


Figure 5. Phloroglucinolysis mechanism for the estimation of the mean Degree of Polymerization.

3.4.Astringency

Maturation for wines with high tannic concentrations is almost necessary, as it is a natural way of softening their palate. Indeed, the extraction of wood phenols have been shown to alter the astringency and bitterness of wines, mostly indirectly through reactions between wood and wine tannins, which produce less astringent compounds (Ribéreau-Gayon et al., 2006). To evaluate mouthfeel, apart from the examination of phenolic compounds, sensory analysis with a trained panel is necessary. In analytical level, the Gelatin index seems to be linked to astringency, while PVPP index (tannic strength) to both astringency and bitterness. There is difficulty in establishing correlations between the extent to which proteins precipitate (an attribute of tannins to which they also owe their name and has been helpful in defining the sensation of astringency) and the quantity of flavanols precipitating, which is a problem related to many other astringency related compounds (Oberholster, 2000).

4. Fourier Transform Infrared Spectroscopy

During the last years the assessment of wine authenticity and traceability has become a critical issue given the need for consumer protection in an expanding and globalized wine market but also the great economic costs for producers due to counterfeit wines. For this reason, stable isotope analysis has been implemented already from the 1990s, in order to detect addition of sugar into wine (chaptalization), prohibited in many winemaking areas (Christoph, Hermann,

& Wachter, 2015). Until today, it has been used to detect illegal addition of ethanol, organic acids, glycerol and carbon dioxide, as well as to confirm geographic origin, a concept implemented in many agricultural areas for products more than wine. Geographical indications add value to the finished product as they represent a quality assurance for the product, which is expected to maintain standards by being subject to precise regulations regarding its production. More methods have been used to tackle authenticity issues, such as the use of multidimensional fluorescence and chemometrics (Ríos-Reina et al., 2017), NIR spectroscopy and chemometrics (Ríos-Reina, García-González, Callejón, & Amigo, 2018), High Resolution Melting (HRM) (Pereira et al., 2017), DNA based techniques (Barrias, Fernandes, Eiras-Dias, Brazão, & Martins-Lopes, 2019), as well as chromatography based methods (Stupak, Goodall, Tomaniova, Pulkrabova, & Hajslova, 2018), however Fourier Transform Infrared Spectroscopy has gained a lot of attention for its quick and nondestructive nature (Anjos, Santos, Estevinho, & Caldeira, 2016; Kasprzyk, Depciuch, Grabek-Lejko, & Parlinska-Wojtan, 2018; Ríos-Reina et al., 2018). Moreover, it has shown great potential in predicting key wine compounds such as phenolic content (Sen, Ozturk, Tokatli, & Ozen, 2016; Silva, Feliciano, Boas, & Bronze, 2014; Teixeira dos Santos, Páscoa, Porto, Cerdeira, & Lopes, 2016; Versari, Paola, Scazzina, & Del, 2010). One of the most interesting characteristics of FT-IR spectroscopy is its ability to produce databases while it is also considered to be advantageous compared to other methods as it is considered to be a reproducible technology not influenced by changes in sensitivity over time (Donarski, Camin, Fauhl-Hassek, Posey, & Sudnik, 2019).

4.1. FT-IR principle

FT-IR spectroscopy is typically based on the Michelson Interferometer Experimental Setup, which consists of a beam splitter, a fixed mirror and a moving mirror. Mid-IR deals with the infrared region of the electromagnetic spectrum (4000-400cm^{-1}) which has a longer wavelength and a lower frequency than visible light. The basis of this analysis is that bonds between different elements absorb light at different frequencies. When IR radiation is passed through a sample, some radiation is absorbed by it and some is passed through (is transmitted). The resulting signal at the detector which is called an Interferogram, is converted by Fourier Transform to an interpretable spectrum representing the sample (Subramanian & Rodriguez-Saona, 2009) (Fig.6).

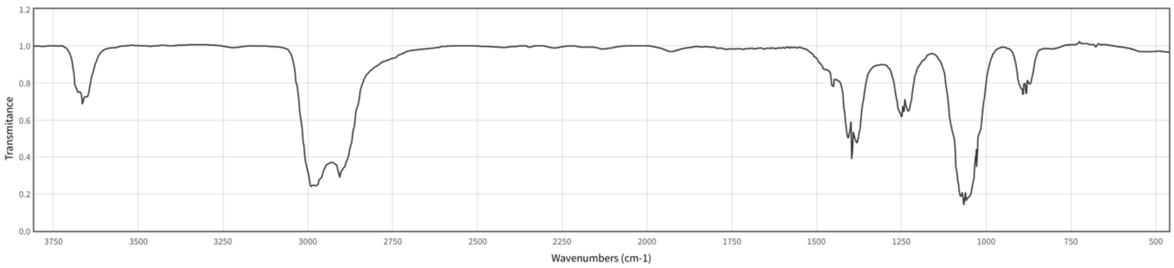


Figure 6. Ethanol Infrared spectrum (NIST Chemistry WebBook).

The spectra that FT-IR generates, have patterns that provide structural insights. These patterns in spectra help identify or quantify the material, and represent the molecular fingerprint of the sample ("Introduction to Fourier Transform Infrared Spectroscopy," 2013). A particular compound can be identified through the light that it transmits or absorbs, but also via the vibrations (bending, stretching, rocking, scissoring and wagging) of its chemical bonds. These vibrations are elicited by the IR energy, which is different depending on the frequency, and are determined by many factors associated with the nature of the atom (shape, strength of bonds between the atoms, mass of the atoms etc.) (Blanco & Villarroya, 2002). The vibrations originating in spectra obtained in the IR region of the spectrum are related to the stretching, bending and rotations of chemical bonds.

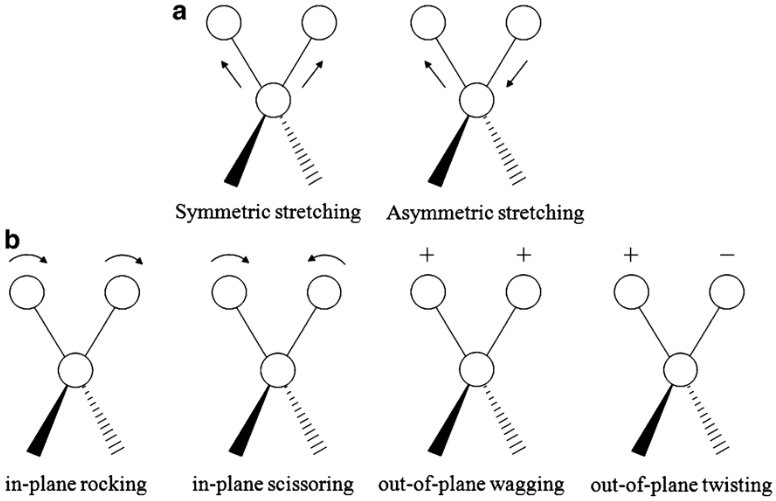


Figure 7. Types of molecular vibrations (Ojeda & Dittrich, 2012).

An IR spectrum is basically a plot of energy versus intensity (of transmittance or absorbance). The presence of peaks at specific stretching frequencies gives information on what functional groups are present in a molecule. Differences on the intensity of the peaks gives information on the relative polarity of the bonds. In order to interpret the IR spectrum, all the characteristic peaks need to be determined, but also the regions of the spectrum on which they exist. To each region (the spectrum can be divided in four regions ranging from 4000 to 400cm^{-1}) different functional groups can be assigned.

There are four major sampling techniques in FT-IR, namely Transmission, Attenuated Total Reflection (ATR), Specular Reflection and Diffuse Reflectance, each one of which is used for specific samples. For liquid samples, Transmission and ATR are recommended, however Diffuse Reflectance can also be used if the sample is first subjected to lyophilization.

The advantages of FT-IR instruments are speed, as all of the frequencies are measured simultaneously in a matter of seconds, mechanical simplicity, sensitivity, and are self-calibrating (they employ an Helium-Neon reference laser as an internal wavelength calibration standard) ("Introduction to Fourier Transform Infrared Spectroscopy," 2013).

4.2. The fingerprint region

An infrared spectrum presents absorption peaks which correspond to the frequencies of vibrations between the bonds of the atoms that make up the material (Fig.6). Each material is a unique combination of atoms; thus, no two compounds can produce the same IR spectrum. The region that contains most of the information, usually from about 1500 to 500 cm^{-1} is called the fingerprint region. It usually contains a very complicated series of absorptions, that are due to bending vibrations within the molecule. For this reason, it is frequently used for authentication and classification purposes (Arianna Ricci, Parpinello, Olejar, Kilmartin, & Versari, 2015).

4.3. Chemometrics

Chemometrics or multivariate analysis, helped revolutionize FT-IR spectroscopy by allowing not only identification but also quantitation of chemical compounds, through their ability to handle large data matrices -such as the ones resulting from an IR spectrum- by considering multiple variables simultaneously. The most commonly used multivariate analysis techniques include Principal Component Analysis (PCA), Discriminant Analysis (DA), and regarding modelling and calibration Linear and Multiple Linear Regression and Partial Least Squares (PLS) regression analysis ("Near infrared spectroscopy in the Australian grape and wine sector," 2009). However, whichever multivariate technique is to be used, particular attention needs to be paid on the selection of the optimum number of variables in order to avoid over or under-fitting of the model. For this reason, FT-IR spectroscopy's accuracy is based on the correct selection of the variables to be used, and on the number of the samples to be used in order to develop the model which lineary correlates with the accuracy of the developed model's predictions.

4.4. FT-IR potential in wine analysis

FT-IR has already been used with success in wine analysis either to quantify some of its key components or to differentiate wines made from different grape varieties, while different measurement techniques have been evaluated (Banc, Loghin, Miere, Fetea, & Socaciu, 2014; Friedel, Patz, & Dietrich, 2013; Tarantilis, Troianou, Pappas, Kotseridis, & Polissiou, 2008). Regarding its potential in authenticity, it could be used to protect the consumer from fraud, but it could also be a tool for audit or regulatory institutions. Wine is subject to many regulations around the world, some of them are related to additions such as the addition of sugar, tartrate acid or even oak chips, and some of them are related to grape varieties or geographical origin of the grapes that are used in vinification of certain wines. Moreover, FT-IR is already used as rapid technique to assess the quality of grapes and spirits. It has gained popularity as it offers a more objective evaluation of quality as opposed to other types of measurements which due to bias are insufficient as quality indicators such as tasting (Fischer, Boulton, & Noble, 1994),or are deemed incomplete such as unilateral measurements of chemical compounds.

II. HYPOTHESIS AND OBJECTIVES

Hypothesis and Objectives

During the last years, there has been a shift in preference regarding the type of barrel used during wine maturation. New types of wood such as Acacia, Chestnut but also different forms of maturation such as the use of wooden pieces are emerging in order to satisfy the consumer. Winemakers have always been experimenting with wood, mainly oak, in order to enhance the quality of their wines and give them personality and finesse. Even though not all wines benefit from maturation, the woody character imparted to wines during maturation has been used in some cases in order to give “ordinary” wines a different prospect, or else, turn them into “quality” wines. This leads to wines overpowered by oak, that have lost their fruity character in favor of toasty and vanilla notes imparted by the barrel and possibly harsh and astringent due to the wood phenols extracted during maturation.

There are no rules or guidelines in deciding how much time to mature a wine, or in what type of barrel wood. Moreover, until recently there had been little research on woods used in cooperage other than oak. Regarding analyses performed in order to evaluate the enological potential of each wood, they are laborious and highly sophisticated, as they have to deal with characterization of many compounds, some of which are identified for the first time. Most of the research on the enological potential of a certain wood, is divided in two parts: studies on the volatile and studies on the nonvolatile compounds.

The group of nonvolatile compounds can produce information on the way a wine ages, but it can also help characterize a wine based on its mouthfeel, an important feature of its organoleptic properties. Astringency is one of the most decisive parameters of wine quality, and its intensity plays a major role in consumers’ appreciation of a wine. However, there is no way to predict how much this sensation will be enhanced after a wine matures in a certain type of barrel wood or for a certain amount of time.

Greek wines, among these wines produced in Crete by autochthonous Cretan varieties, present a high variability in terms of tannin composition and concentration, astringency and general polyphenolic load, however most of these observations are empirical as research on native Greek varieties is almost scarce. Research data on their aging potential, recommended times of maturation and preferable types of wood to be used during maturation are non existent as well.

On the other hand, today the matter of wine authentication is of great interest. Many wines are marketed under the umbrella of Protected origin indications, which have strict regulations in terms of production, maturation, even regarding viticultural practices. Moreover, these regulations indicate even acceptable and non-acceptable types of maturation, that are difficult to control with the given analytical tools. Fourier Transform Infrared Spectroscopy is emerging as a very useful tool in terms of authentication, while it also can help characterize wines with the help of chemometrics. Apart from discriminatory tasks, it can also be useful to quantify or estimate the levels of certain chemical compounds in wine.

In view of the above, the main objective of this study was to “monitor’’the wine maturation process based on its phenolic composition by employing both conventional analysis and Fourier transform spectroscopy and examine whether the use of FT-IR can facilitate maturation analyses and authentication needs.

In order to examine whether FT-IR has the ability to record differences imparted to wines by the type of container used during maturation, it was of interest to evaluate discrimination results regarding not only the type of container but also the length of maturation. A successful discrimination would provide a useful tool for authentication purposes. Moreover, it would be of interest to examine whether results from analyses performed during maturation could be estimated with the use of FT-IR by statistically processing the analytical data using chemometrics. This way mathematical models could be obtained that would help overcome laborious and cost-effective analyses which are necessary for the chemical characterization of the wines during maturation by estimating the content of specific chemical compounds such as ellagitannins, or other wine parameters such as its chemical age.

Finally, since the evolution of astringency is critical for the determination of the wine maturation length, it was of interest to study key structural characteristics, such as proanthocyanidins, that have been linked with this sensation focusing on any possible relations between chemical and sensory data as well. The results could be possibly used as a guide by winemakers when deciding for the optimum maturation length for a wine.

III. EXPERIMENTAL OUTLINE

Experimental Outline

In this study, grapes from autochthonous varieties from the island of Crete, Greece, were used for the production of two red and two white wines. The grape varieties used for the production of the white wines were Vilana and Dafni, and for the production of the red wines were Kotsifali and Mandilari. These varieties were chosen based on the basis of their popularity (Vilana, Mandilari), their unique sensory character (Dafni) and their phenolic content (low in the case of Kotsifali, high in the case of Mandilari) (EDOAO, 2019; Stavrakakis, 2010). Vinifications were carried out during the vintages of 2012 and 2013.

The containers chosen for maturation purposes were Inox tanks, Inox tanks with oak sticks and barrels made from French and American oak, Acacia and Chestnut. Chestnut was selected because it is the main wood from which barrels for home winemaking is mostly made of, but it was used only in the case of the red wines due to the notoriously harsh character it is said to impart to wines (white wines' sensory profile would be overpowered by chestnuts' tannins which would mask any other aroma and flavor). To examine the effect of the container on maturation, wines in Inox tank were used as control wines, for comparison purposes. Oak sticks were used in order to study the effect of different maturing techniques, while French and American oak barrels were chosen due to their popularity. The sticks that were selected, Oenostick V18 are of cylindrical shape, and their dimensions have been chosen to optimize perpendicular contact with the wood. The recommended contact time by the producer is 6 to 8 months, however wineries tend to reuse sticks, given their larger than chips surface which is easier to "clean". To mimic the conditions of in barrel aging, 7 grams of oenosticks per liter of wine were added in each tank. Acacia was studied as it is an emerging trend in winemaking because of its promising enological potential.

The four unique different types of wines resulting from each variety, as well as the selected types of containers that would be used for their maturation were used to examine whether there can be a satisfactory discrimination in terms of variety and type of container with the use of FT-IR. In order to evaluate the capability of FT-IR to discriminate between wines maturing for different periods of time, samples from all containers were taken every 3 months for a period of 9 months for the 2012 vintage and 12 months for the 2013 vintage. All wines were stored in a winery cellar, with controlled humidity and temperature conditions. Barrels were topped using wine from the inox tank (2 tonnes total volume). To monitor the process of ellagitannin extraction and in order to evaluate FT-IR's feasibility in estimating its levels in matured wines by creating a predictive model, samples needed to reflect common practices in terms of time of contact, which affects ellagitannin levels. Representative samples would be used to calibrate the model. Since many wineries reuse their barrels, and the number of times a barrel has been used affects the concentration of phenolic compounds that will be extracted into the wine, it was decided that the same barrels should be used for the wines vinified in 2013. For comparison reasons but also to replicate common practice, the same oenosticks were also used.

To examine the capability of FT-IR to monitor maturation, predictive models were decided to be built based on the chemical analyses that provide the most information. These analyses were Total anthocyanin concentration and the Chemical age indexes i and ii, however most parameters evaluating wine color such as Degree of ionisation of anthocyanins, Color density, Color density corrected for SO2, SO2 resistant pigments, Color Intensity and Hue were also estimated in order to examine their potential for use complimentary to FT-IR.

Finally, to examine wine astringency, a sensory panel consisting of trained wine professionals assessed the wines every 3 months, during each vintage. Between the chemical analyses evaluating tannin concentrations of the wines, mean Degree of Polymerization of proanthocyanidins was selected to estimate the tannic potential as it has the closest correlation to the astringency sensation. The protocols of analysis for all methods mentioned herein, as well as information on the vinification procedure or the types of barrels used during the experiment, are described in detail in the following chapters.

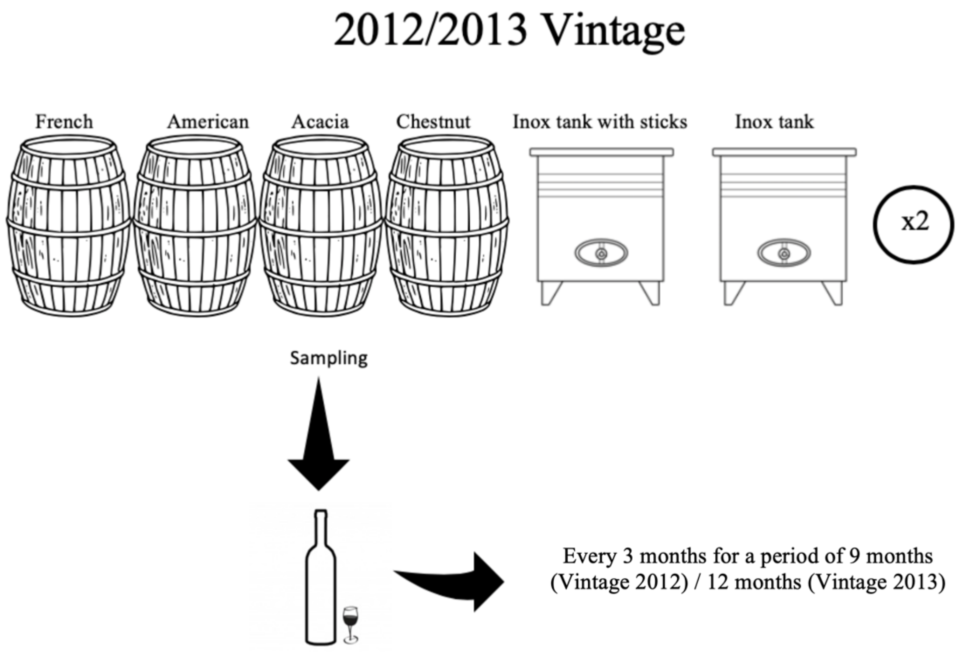


Figure 8. Experimental visualization

IV. RESULTS

i. Wine authentication with Fourier Transform Infrared Spectroscopy (FT-IR): A feasibility study on variety, type of barrel wood and ageing time classification

Introduction

Aging wine in barrels is one of the most common practices in winemaking. The contact with wood results in the extraction of specific compounds such as polysaccharides, volatile compounds and wood tannins (elagitannins) which alter not only the sensory profile of the wine but –indirectly- also its price due to the high cost of storage (Garde-cerdán et al., 2008). The type and quantity of the extractable compounds depend mainly on the geographical origin and the type of wood used (Garde-cerdán et al., 2008) the age of the barrel, the contact time between wine and wood (Cerdán & Ancín-Azpilicueta, 2006), but also on the aging technology, the wood botanical species (Frangipane, Santis, & Ceccarelli, 2007; Marco, Artajona, Larrechi, & Rius, 1994), the toasting level (Chira & Teissedre, 2013a) and the barrel size (De Simon, Cadahía, Del Álamo, & Nevares, 2010).

Regarding the wood extractable compounds, ellagitannins are one of the most important groups since they may occur in concentrations up to 10% in dry weight of oak heartwood. They are basically constituted of gallic acid or at least one hexahyrdoxydiphenyl acid moiety and its dilactone (ellagic acid) which is formed by oxidative coupling between two gallic acid units (Chira & Teissedre, 2013a). The ellagitannins, also called hydrolysable tannins, mostly occur in woods and woody parts of plants.

Traditionally, oak wood species are most often used in cooperage, specifically *Quercus alba* (American white oak), and the French *Quercus robur L.* (pedunculate oak). Wood from other species as false acacia (*Robinia pseudoacacia*) and chestnut (*Castanea sativa*), are also being considered as possible sources of wood for wine aging, since recent studies on barrels made from Acacia and Chestnut woods showed good potential for use in not only red but also white winemaking (Kozlovic, Jeromel, Maslov, Pollnitz, & Orlić, 2010; Rosso et al., 2009). However, according to the International Organization of Vine and Wine (OIV), only the use of oak and chestnut woods is approved in wine technology (Kyraleou, Kallithraka, Chira, et al., 2015).

French and American oak differ on their ellagic tannin content and aromatic potential (Chatonnet & Dubourdieu, 1998) but also produce barrels of different prices as the anatomy of the French oak results in 80 per cent wastage and double material cost (Grainger, 2009; Vernon L Singleton, 1995). Chestnut, is a quite hard, long-lasting wood, very rich in hydrolysable tannins and gallic acid (Salagoity-Auguste, Tricard, & Sudraud, 1987). Its high phenolic content coupled with the bigger porosity of the wood, makes it more suitable for shorter aging. On the contrary, Acacia adds less wood character to the wines while making them suppler and finer textured, an attribute that makes it more appealable for use with white wines (Kozlovic et al., 2010).

Most of the methods implemented to differentiate wines aged in different types of barrels focus on the polyphenolic or volatile profile of the wines (Castro-Vázquez et al., 2011; Bŕigida Ferńandez De Simón et al., 2014). Recent research using the non-anthocyanic profile of wines aged in different types of barrels (Bŕigida Ferńandez De Simón et al., 2014) showed that valoneic acid dilactone and dihydrorobinetin (among others) can be used as phenolic markers of aging wine with Chestnut and Acacia woods, respectively. However, in the case of French and American oak early research has showed their chemical profiles to be mainly quantitatively different (Vernon L Singleton, 1995). For this reason, recent studies focus on genetic markers (Guichoux & Petit, 2014) which due to the technology used are costly although precise (Scotti-Saintagne et al., 2004).

Infrared spectroscopy based methods are increasingly used in wine because of their versatility and efficiency, their cost effectiveness, and their fast and non –invasive nature (Herrera, Guesalaga, & Agosin, 2003; Picque, Lefier, Grappin, & Corrieu, 1993). Combining spectroscopic techniques with multivariate data analysis is proving to be very promising for the discrimination of wines of different varietal origin, wines from different countries or with different sugar contents, but also for the determination of oak volatile compounds and ethylphenols in aged red wines (Garde-Cerdán, Lorenzo, Alonso, & Rosario Salinas, 2010; Liu et al., 2008; Riovanto, Cynkar, Berzaghi, & Cozzolino, 2011; Tarantilis et al., 2008; Y. Zhang et al., 2010). The discrimination is based on the samples' (solid, liquid or gaseous) 'fingerprint', the unique spectrum that results after it absorbs some of the infrared radiation at specific wavelengths (Cozzolino, 2014). However, as far as the authors are aware, this technique has not been employed so far to differentiate wines aged in barrels according to the container type or the time of contact.

The investigation presented herein was undertaken in order to explore the suitability of Fourier transform infrared spectroscopy (FT-IR), coupled with multivariate analysis, as a rapid, simple and economical method for the discrimination between grape varieties and between wines aged in different types of containers and for different time periods.

Materials and Methods

- **Wines samples and container types**

Four mono-varietal wines made from indigenous *Vitis Vinifera* varieties of Crete, two white (Vilana and Dafni) and two red (Kotsifali and Mandilari) were analyzed in this study. Regarding the barrels, different types of woods were used, based on current market data and emerging trends. French (*Quercus robur*) and American (*Quercus alba*) standard barrels (225 L) were purchased from 'Tonnellerie du Monde World Cooperage', Acacia standard barrels were purchased from Tonellerie du sud ouest, and Chestnut standard barrels were purchased from Tesias Metsovo. Chestnut barrels were used only in the case of red wines. All barrel woods received medium toasting process. Oak chips (French oak) were purchased from Seguin Moreau, (Oenostick®, V14) and were used for accelerated maturation (7 g/L oak chips). All wines completed fermentation in stainless steel tanks and were then transferred to the different types of containers. Control wines remained in stainless steel tanks for comparison reasons. Samples from each container were bottled and analyzed after 3, 6, 9 and 12 months of contact (12 months of contact only for the 2013 vintage), resulting in 22 red and white wine samples for each vintage's trimester.

- **Sample pretreatment and spectroscopic analysis**

Triplicate spectra of 84 samples for red varieties and 70 samples for white varieties (154 in total) were collected with a Thermo Nicolet 6700 FT-IR spectrophotometer (Thermo Electron Corporation, MA, USA) equipped with a deuterated triglycine sulfate (DTGS) detector according to the method described by Basalekou et al. (2015). The spectra were collected with a 4 cm^{-1} resolution (100 scans in total), using a standard ZnSe window against a ZnSe background. Each spectrum was smoothed using the Savitsky-Golay algorithm (5-point moving, second-degree polynomial) and the baseline was corrected (20 iterations, second-degree polynomial). The average spectrum of each sample was calculated by means of the corresponding function of the software accompanying the spectrometer (OMNIC 7.3, Thermo Fisher Scientific Inc.).

- **Chemometrics**

Discriminant analysis was performed using the JMP Statistical Discovery software, version 11. Spectral data (4000-500 cm^{-1}) were exported to JMP, and discriminant analysis (linear method) was performed using selected parts of the spectrum to discriminate between the variety, the type of container and time of contact. In the case of container discrimination, the second derivative of the spectra was used for analysis.

Results and Discussion

- **Spectroscopic analysis**

A typical spectrum for each variety of the wine samples analyzed can be seen in Figure 9.

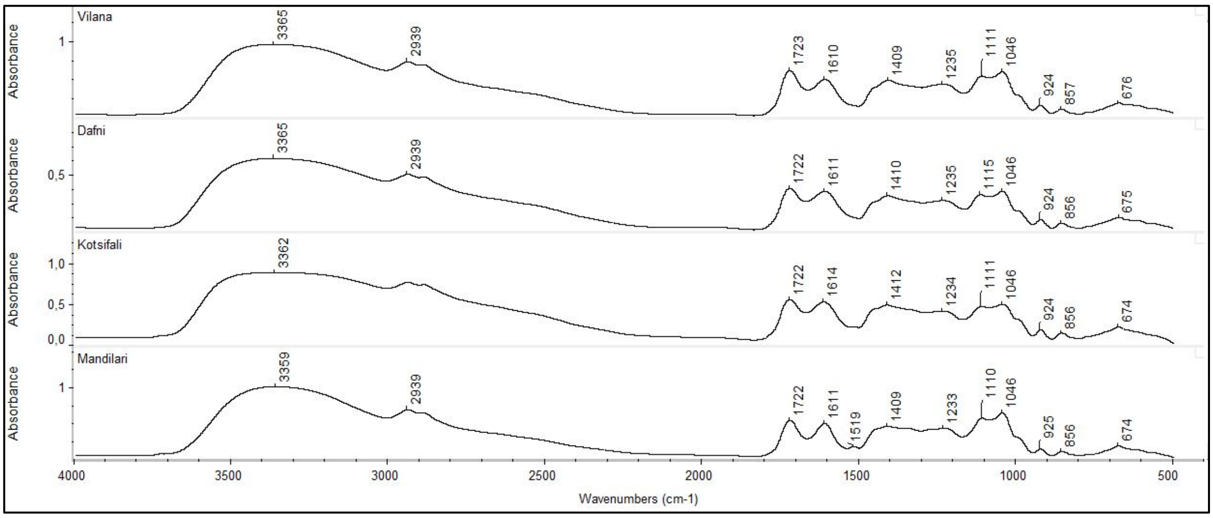


Figure 9. Typical FT-IR spectra of Vilana (first), Dafni (second), Kotsifali (third) and Mandilari (fourth) samples

All spectra were characterized by a similar profile. The band in the 3700-3012 cm^{-1} region of the spectra originates from compounds having an –OH group in their molecules (Y. Zhang et al., 2010), while the band in the 3010-2800 cm^{-1} region is where the stretching vibrations of C-H appear (Arianna Ricci, Olejar, Parpinello, Kilmartin, & Versari, 2015). Various spectral bands occur in the region from 1800-900 cm^{-1}, which is part of the fingerprint region, such as those corresponding to the vibration of the C-O, C-C, C-H and C-N bonds (Movasaghi, Rehman, & Rehman, 2007). The peak at 1722-1723 cm^{-1} is due to the stretching of carbonyl group (C=O). In this region the C-O stretching of esters of hydrolysable tannins, especially derivatives of gallic acid can be seen (Grasel, Ferrão, & Wolf, 2016) and the C=C stretching, typical of flavanoid-based compounds in wine (Arianna Ricci, Olejar, et al., 2015). The absorbance at 1610-1614 cm^{-1} is due to C=C stretching (typical for aromatic molecules) (Edelmann, Diewok, Schuster, & Lendl, 2001). The absorbance at 1409-1412 cm^{-1} corresponds to the antisymmetric stretching vibration of the COO^- (Agatonovic-Kustrin, 2013; Y. Zhang et al., 2010). The absorbance at 1110-1115 cm^{-1} corresponds to the stretching vibration of C-O, while the signal at 674-676 cm^{-1} can be attributed to aromatic C-H out of plane bending vibration (Edelmann et al., 2001).

- **Discriminant analysis**

The discrimination of the samples based on either the grape variety or the container type and the storage time was performed with Linear Discriminant analysis (LDA). LDA is based on the measurement of the distance between each point in the data set and the multivariate mean of each group. The Mahalanobis distance, which takes into account the variances and covariances between the variables was used in this study for the construction of the model. The selection criterion of the latent variables was to obtain the maximum differentiation between the categories with simultaneous minimum variance within categories.

To estimate model parameters, 50% of the samples were used (training set). To assess the predictive ability of the obtained models, a validation set was used by randomly excluding 40% of the samples. The rest 10% was used to test the model's predictive ability. To avoid overfitting, the number of the variables used for discrimination was lower than the number of the samples.

The discrimination of the samples was based on the phenolic content of the wines, specifically their tannin content. Tannins can be classified into two main classes: hydrolysable tannins further divided in ellagitannins (lactones of ellagic acid) and gallotannins (polymers of gallic acid esters) which are mainly derived from the wood and condensed tannins commonly known as proanthocyanidins which are polymers of covalently bonded flavanol monomeric units and are mainly derived from the grape (B. Zhang, Cai, Duan, Reeves, & He, 2015). Regarding the IR spectra of these compounds, some differences can be found due to their typical structures. A diagnostic region for hydrolysable tannins is within 1750-1680 cm^{-1} where the stretching vibration of the C=O group of the carbonyl moiety occurs, giving a strong peak (Arianna Ricci, Olejar, et al., 2015; Arianna Ricci, Parpinello, et al., 2015).This band is usually quite weak in condensed tannins, except if an oxidation and rearrangement of –OH group occurs as a consequence of extraction process. Regarding condensed tannins, there are several peaks used to characterize them. The asymmetric stretching vibration of the ethereal group results in a peak at around 1288 to 1280 cm^{-1}. The spectral region from 1250 to 900 cm^{-1} provides information about hydroxylation patterns, so that it could be used to discriminate between hydrolysable and condensed tannins according to (Arianna Ricci, Parpinello, et al., 2015). Therefore, two spectral regions were selected for further statistical analysis for discrimination purposes in this study: 1800 to 1500 cm^{-1} and 1300 to 900 cm^{-1} for hydrolysable and condensed tannins respectively.

- **Variety discrimination**

For the differentiation of the wines based on grape variety, LDA was performed on the part of the spectra where the grape derived condensed tannins occur (1300-900 cm-1). Figure 10 demonstrates the discrimination according to the grape variety (100% discrimination, 82% validation, Table 1). The first two canonical variables account for 99% of the variability. The two white varieties (Vilana and Dafni) are situated on the lower left part of the diagram quite close to each other whereas the two red, Kotsifali and Mandilari, are found on the upper left and right part of the graph respectively.

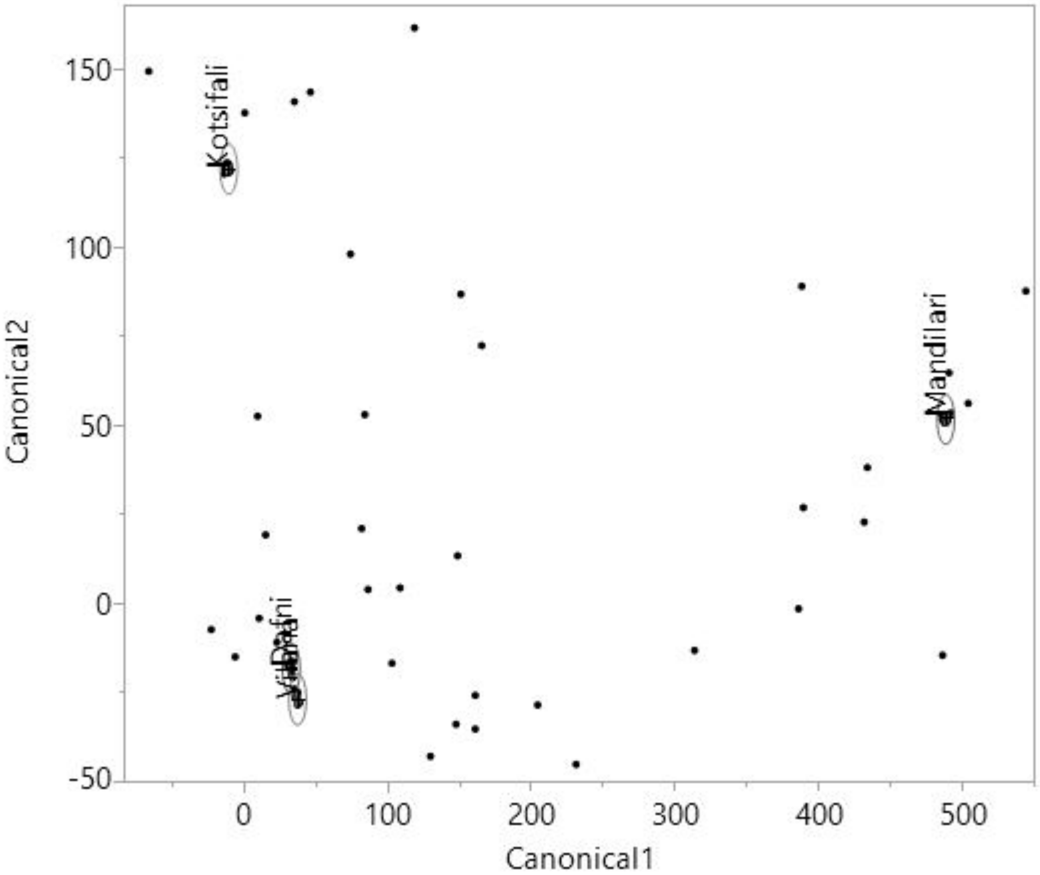


Figure 10. Representation of discrimination results of wine FT-IR data based on the variety (Vilana: 35 samples, Dafni: 35 samples, Kotsifali: 42 samples, Mandilari: 42 samples)

The discriminant values obtained after the statistical analysis for all discrimination analyses performed (regarding variety, container type and contact time) are shown in Table 2 and include the number and percentage of the samples that were misclassified, the validation and test set results and the negative log-likelihood, which is the negative sum of natural logs of the observed probabilities (small values of this function indicate better fit).

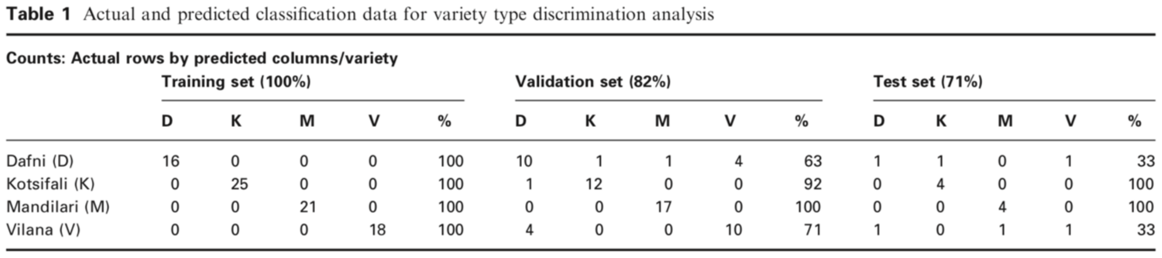


Table 1 Actual and predicted classification data for variety type discrimination analysis

Counts: Actual rows by predicted columns/variety	Training set (100%)					Validation set (82%)					Test set (71%)				
	D	K	M	V	%	D	K	M	V	%	D	K	M	V	%
Dafni (D)	16	0	0	0	100	10	1	1	4	63	1	1	0	1	33
Kotsifali (K)	0	25	0	0	100	1	12	0	0	92	0	4	0	0	100
Mandilari (M)	0	0	21	0	100	0	0	17	0	100	0	0	4	0	100
Vilana (V)	0	0	0	18	100	4	0	0	10	71	1	0	1	1	33

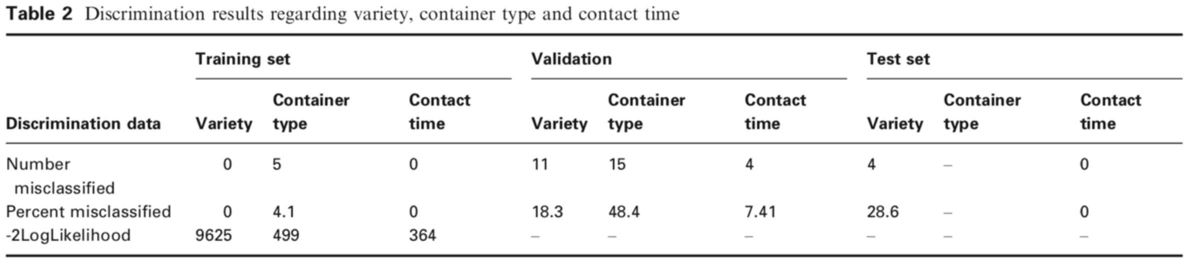


Table 2 Discrimination results regarding variety, container type and contact time

	Training set			Validation			Test set		
Discrimination data	Variety	Container type	Contact time	Variety	Container type	Contact time	Variety	Container type	Contact time
Number misclassified	0	5	0	11	15	4	4	–	0
Percent misclassified	0	4.1	0	18.3	48.4	7.41	28.6	–	0
-2LogLikelihood	9625	499	364	–	–	–	–	–	–

These results suggest that although the phenolic composition may be influenced by vinification, maturation and aging, the differences in the overall phenolic fingerprints might still be characteristic for each cultivar since the discrimination using specific spectral characteristics associated with chemical characteristics was possible.

To our knowledge, this is the first study to show a complete discrimination between wines from different varieties, using also samples aged in different types of containers. Recent literature highlights the use of emerging technologies such as Fourier-transform ion cyclotron resonance (Alañón, Pérez-coello, & Marina, 2015), which are able to correctly discriminate wines, although only when samples are analyzed immediately after alcoholic fermentation. Moreover, mass spectrometry coupled with multivariate statistics resulted in a complete discrimination between Chilean wines, although the method validation results were lower than of those presented herein (Villagra, Santos, Vaz, Eberlin, & Felipe Laurie, 2012). Using wine FT-IR spectroscopy, Tarantilis et al. (2008) differentiated three Greek red wines (Xinomavro, Agiorgitioko and Merlot) based on the grape variety. The separation was based on small differences between the spectra of their phenolic extracts. The spectroscopic area of 1800 to 900 cm^{-1} was chosen for the discrimination. Moreover, Edelmann et al. (2001) using the spectral region of 1640 to 950 cm^{-1} discriminated six red wines based on their cultivars. However, both studies used phenolic extracts obtained after solid-phase extraction (SPE) with

C-18 columns and elution with methanol. In the present study, the SPE step was omitted and the separation was based on the spectral differences attributed mainly on their condensed tannin content.

- **Container type discrimination**

For the discrimination based on wood type and time of contact, LDA was performed on the 1800-1500 cm^{-1} region of the spectrum, which contains signals mainly due to hydrolyzable tannins (Arianna Ricci, Parpinello, et al., 2015). For the container type discrimination, the second derivative of the spectra was used, to help enhance the separation of overlapping peaks. Figure 11 shows the plot of samples on the plane defined by the first two canonical discriminant variables, which accounted for 73,6% of the variability, after performing LDA. Samples were correctly classified according to the type of container used for maturation by 96%, although for the validation set only 52% of the samples were correctly classified (Tables 1, 3 (a) and 3 (b)). This may be attributed to the high variability of the tannic composition of the samples. Specifically, the chemistry of hydrolysable tannins can be affected by their solubility in water and their reaction with oxygen (A Ricci et al., 2016). Figure 11 demonstrates that there is a clear separation between the samples that matured in Acacia and Chestnut barrels as expected, given their unique tannin profiles. Indeed, validation results for Chestnut and Acacia barrels were 100% and 82% respectively (Table 3 (b)).

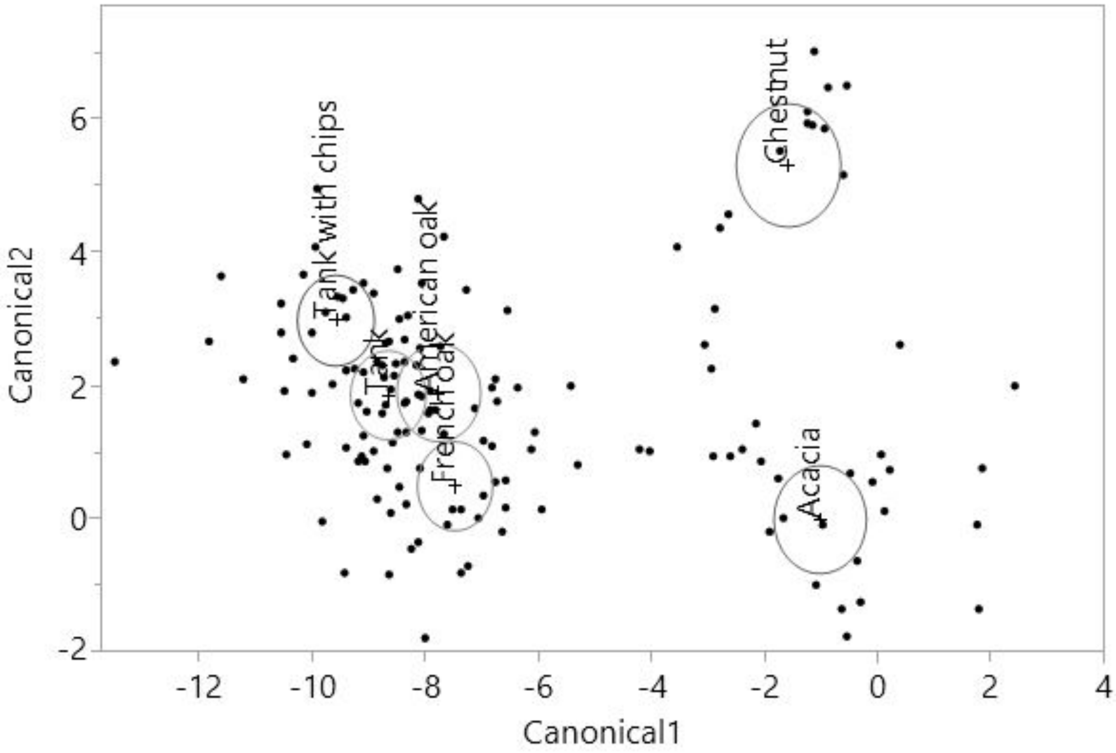


Figure 11. Representation of discrimination results of wine FT-IR data based on the container type (Acacia barrel: 28 samples, French and American oak barrels: 28 samples each, Chestnut barrel: 14 samples, Tank with oak chips addition: 28 samples, Tank: 28 samples)

Wine samples matured in chestnut barrels are situated on the right upper part of the graph while those aged in acacia on the right lower part, well separated from each other by the second canonical variable. Sanz et al., (2012a) reported that acacia wood contained only a small amount of condensed tannins, prorobinetin type, whereas oak and chestnut woods were abundant in hyrdolyzable tannins (mainly ellagitannins). Low molecular weight phenolic composition of acacia wood also differed from that found in other woods, since acacia included compounds with a β-resorcylic structure, some gallic and hydroxycinnamic related compounds, protocatechuic aldehyde and only a small quantity of gallic and ellagic acid (Sanz, De Simón, et al., 2012; Sanz, Fernández de Simón, et al., 2012). Moreover, chestnut heartwood shows the most similar polyphenolic profile to oak, although its low molecular weight phenolic and tannic contents are higher, highlighting the presence of gallotannins and the high levels of gallic acid (Bŕigida Ferńandez De Simón et al., 2014). Few published reports exist on differentiation of chestnut wood from other species. However the samples that were discriminated according to their IR spectra were solutions of commercial tannin extract and not wine samples (Grasel et al., 2016; Arianna Ricci, Olejar, et al., 2015). Regarding the samples aged in contact with oak wood, they appear to be situated close to each other in the left part of the graph probably due to similarities of their tannin contents. The oak wood contains mainly monomeric ellagitannins (vescalagin and castalagin) which represent 40-60% of the total amount. In addition dimmers (roburin A and roburin D) and lyxose/xylose derivatives (grandinin, roburin B, roburin C and roburin E) have also been identified in oak woods (B. Zhang et al., 2015).

Table 3 (a): Actual and predicted classification data for container type discrimination analysis (training set)

Container (Training set)

Counts*	**Acacia**	**American oak**	**Chestnut**	**French oak**	**Tank**	**Tank with chips**
Acacia	17	0	0	0	0	0
American oak	0	18	0	0	1	1
Chestnut	0	0	13	0	0	0
French oak	0	0	0	25	0	0
Tank	0	0	0	1	23	1
Tank with chips	0	0	0	0	1	22

*Counts: Actual Rows by Predicted Columns

Table 3 (b): Actual and predicted classification data for container type discrimination analysis (validation set)

Container (Validation set)

Counts*	**Acacia**	**American oak**	**Chestnut**	**French oak**	**Tank**	**Tank with chips**	**% classification per container**
Acacia	9	0	0	1	0	1	82
American oak	1	2	1	2	0	2	25
Chestnut	0	0	1	0	0	0	100
French oak	0	1	0	1	0	1	33
Tank	0	0	1	0	2	0	67
Tank with chips	0	1	0	2	1	1	20

*Counts: Actual Rows by Predicted Columns

- **Time of contact discrimination**

Regarding the time of contact, Figure 12 shows a clear separation between samples (100% discrimination, 93% validation, Table 4). The first two discriminant functions accounted for 87% of the variability. The wines aged for twelve and nine months in contact with the wood were separated by the first canonical variable and are situated on the lower left and lower right part of the graph respectively. The samples that spent three and six months in the containers are found in the upper right part of the graph and were separated by the second canonical variable.

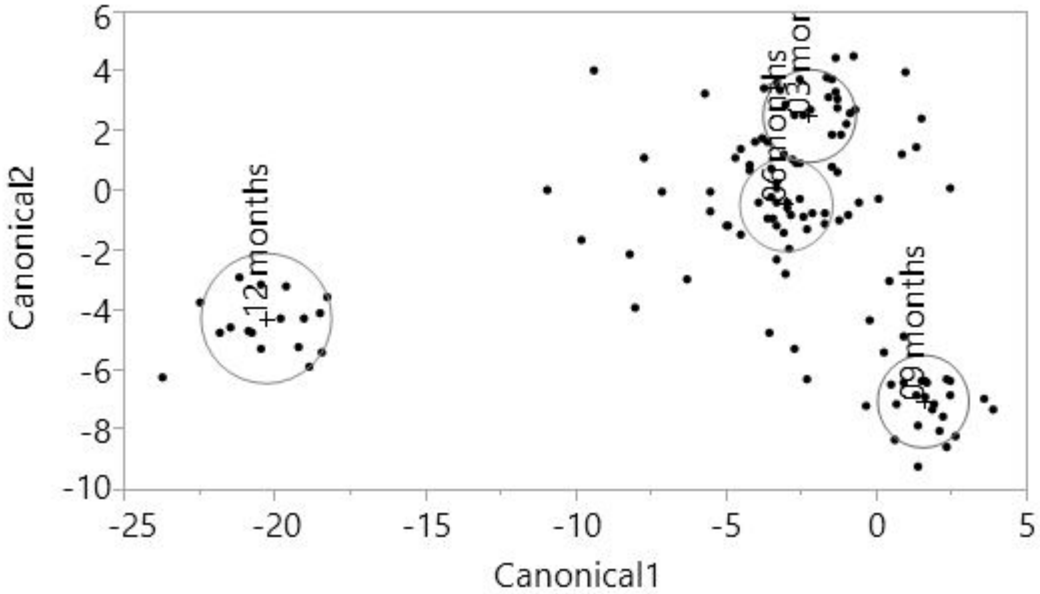


Figure 12. Representation of discrimination results of wine FT-IR data based on different contact times, i.e. months of maturation (three months: 44 samples, six months: 44 samples, nine months: 44 samples and twelve months: 22 samples)

Contact time has a definitive effect on wine chemical composition due to the greater resulting extraction of wood originated compounds. Gordillo et al. (2012) and Kyraleou et al. (2016) discriminated wine samples having aged in contact with wood for different time periods employing analytical methods to measure wine color parameters, polyphenolic profile and volatile composition which are expensive, time consuming and require sample pretreatment steps and use of chemical reagents. Kyraleou et al. (2016) reported that the release of hydrolysable tannins from American and French oak chips reached a maximum after two months. During the first two months, wine extracts hydrolysable tannins at a faster rate (Chira & Teissedre, 2013a). Thereafter, when most of the hydrolysable tannins have been extracted from the first millimetre of the wood, the wine needs to go deeper in the wood to extract more, consequently, at a slower rate. Moreover, the differences in porosity of the particular woods may alter the extraction rates, affecting thus the hydrolysable tannin content of the wines. Kyraleou et al. (2016) reported that the hydrolysable tannin content of the wines having aged in contact with acacia chips reached a maximum after the first month and since then it remained unaffected indicating a faster extraction rate. It is thus possible that the different initial hydrolysable content and the porosity of particular woods resulted in quantitative differences in the hydrolysable tannin content of the wines examined in this study. These differences were adequate to discriminate the FT-IR spectra of the samples according to the time spent in contact with the specific woods.

Table 4: Actual and predicted classification data for time discrimination analysis

Counts: Actual Rows by Predicted Columns / Time

	Training set (100%)					Validation set (93%)					Test set (100%)				
	3	6	9	12	%	3	6	9	12	%	3	6	9	12	%
03 months	24	0	0	0	100	15	1	1	0	88	3	0	0	0	100
06 months	0	24	0	0	100	1	15	0	0	94	0	4	0	0	100
09 months	0	0	24	0	100	0	1	14	0	93	0	0	5	0	100
12 months	0	0	0	12	100	0	0	0	6	100	0	0	0	4	100

- **Conclusion**

FT-IR spectroscopy could be used for the discrimination for authentication purposes of mono-varietal wines aged in different containers for different time periods. The spectroscopic analysis combined with the linear discriminant analysis of specific spectral areas of the samples using the JMP statistical software resulted in complete discrimination of the grape variety and the time of contact, although container discrimination models showed poor validation results due to high sample variability. The proposed method is promising since it is simple, rapid and economical without the requirement of chemical reagents and sample pretreatment steps.

ii. Red wine age estimation by the alteration of its color parameters: Fourier Transform Infrared Spectroscopy (FT-IR) as a tool to monitor wine maturation time

Introduction

Wine age is a parameter of great importance as it is linked with wine quality. However, there is no direct way to measure it. Most information related to age is derived from the evolution of the wine's organoleptic characteristics, such as color, mouth feel and aroma which depend on the phenolic and volatile content of the wine.

Color in particular, is an indicator of the interactions between phenolic compounds, such as the replacement of monomer forms by polymers which are time dependant reactions. Young wines' color depends on the concentration of free anthocyanins, which on the wine's pH are on their colored form (positively charged flavylium). As monomers, they are highly susceptible to changes in the medium's environment, such as pH and SO_2 changes (Mercurio, Dambergs, Herderich, & Smith, 2007). During aging, while monomers are replaced by polymers, color changes and stabilization occurs. For example, color density is reduced as a result of oxidation but also due to changes in the structure of anthocyanin-tannin polymers and precipitation of some of the polymers formed. Moreover, the purple hue of young red wines is replaced by brickish red to tawny red hues. Those changes in color can be observed with the naked eye, but also easily using a spectrophotometer by measuring color parameters such as intensity and hue (Jackson, 2017). The transformation of monomeric pigments to more stable polymeric forms is well documented in research (Del Alamo Sanza *et al.*, 2004; Gómez-Plaza and Cano-López, 2011). The evolution of the physicochemical characteristics of wines occurs primarily during wine maturation and ageing, as phenolic compounds participate in numerous chemical reactions such as copigmentation and polymerization. One of them is the gradual formation of condensed pigments between free anthocyanins and colorless phenols present in grapes. These compounds can be formed either in the presence or in the absence of oxygen. When aging is oxidative i.e. barrel aging or vat aging with high levels of aeration, the anthocyanins and tannins react also with acetaldehyde which is formed as a result of ethanol oxidation, to form pigments of various structures. These reactions are catalysed by ellagitannins, phenols that are extracted from the barrel (Ribéreau-Gayon et al., 2006). The polymeric pigments can be further classified into two categories: long polymeric pigments (LPP) and short polymeric (SPP) pigments. LPP have a polymer length greater than three and can be precipitated with protein while SPP are shorter polymers that do not precipitate with proteins (Harbertson *et al.*, 2003). Copigmentation refers to the self-association of anthocyanins or their association with certain phenolic monomers and results in color stabilization due to hyperchromic and bathochromic shifts in the visible absorbance (Boulton, 2001).

Monitoring the evolution of anthocyanins can be a demanding task in terms of analytical methods. For a time-efficient and in-depth analysis of the anthocyanin forms over time, without the use of specified instruments, Somers introduced in 1977 the concept of "chemical age". Chemical age is defined as the extent to which monomeric anthocyanins have been displaced by polymeric pigment forms (Somers & Evans, 1977). Indices of chemical age are the indexes "chemical age i" and "chemical age ii", which are derived from two spectral ratios. Both refer to the extent to which pigments have become less susceptible to the changes of pH and to SO_2 bleaching, changes to which free anthocyanins are more susceptible as less stable. The sample absorbance at 520 nm is due to the free forms of anthocyanins, copigmented anthocyanins and polymeric pigments. Absorbances are measured after pH adjustment or the addition of bisulfite solution or acetaldehyde to separate total anthocyanins and polymeric pigments. Lowering the pH of the wine allows the determination of free anthocyanins and polymeric pigments; acedaldehyde removes the bleaching effect of any SO_2 present in the wine and the addition of bisulfite reveals the degree to which the color is due to the polymeric anthocyanin forms (Somers and Verette, 1988). This UV-vis based method commonly used in the wineries requires multiple step sample processing and measurements.

Given the importance of wine color, an indirect method able of determining the color parameters in red wines with reasonable accuracy and ease of use would be of great value to wine industry. Infrared spectroscopic methods have the advantage of being nondestructive, fast and require small sample quantities. When combined with multivariate data analysis, such as partial least squares regression analysis (PLS) they are suitable for correlating the spectral response of a sample to its chemical composition and represents (Versari *et al*., 2012). The application of both near- and mid- infrared spectroscopic methods to grape and wine analysis has become a valuable alternative to the slow and destructive analytical procedures which are routinely used such as the classic UV-Vis and chromatographic techniques (Fernandez and Agosin, 2007; Fragoso *et al*., 2011; Regmi *et al*., 2012; Silva *et al*., 2014; Cozzolino, 2015; Kyraleou *et al*., 2015;) or even for monitoring wine ageing in barrels (Basalekou et al., 2015). The first applications of FT-IR for the quantification of total anthocyanins in wine were carried out by Versari *et al*., (2006). They also determined LPP, SPP, total wine color and copigmented anthocyanins. The best results were obtained using vector normalization and no-preprocessing of spectral data. Later studies using direct orthogonal signal correction pre-processing have improved the prediction of polymeric pigments in red wines by FT-IR spectroscopy compared to the row data (Laghi *et al*., 2011). Picque *et al*., (2010) also used FT-IR spectroscopy to build a predictive model that was able to quantify anthocyanins in red grape extracts. Anthocyanin prediction was improved if a separate calibration model was calculated for each geographical region. The best prediction for monitoring anthocyanins in red grapes using FT-IR was presented by Fragoso *et al*., (2011). They obtained a valid regression model for prediction of total anthocyanins working in the region of 979-2989 cm^{-1}.

Individual anthocyanin concentration can also be predicted using FT-IR (Soriano *et al*., 2007) in young red wines. Recently, Rasines-Perea *et al.,* (2015) determined twelve individual anthocyanins in red grape musts using FT-IR and PLS. However, in both the above studies there was a need to employ correction factors to improve the prediction due to systematic errors.

There exists a number of important studies concerning the changes in phenolic content and color parameters of red wines during ageing (Del Alamo Sanza, Escudero, et al., 2004; Gambuti, Capuano, Lisanti, Strollo, & Moio, 2010; Guadalupe & Ayestarán, 2008). However, despite the importance of visual appearance to the final wine quality, there is not much published data concerning the correlation of color parameters with wine age. It was thus of interest to determine the alteration of selected color parameters of red wines during short maturation periods and to evaluate the overall changes with respect to their age.

Moreover, it was of interest to explore the suitability of FT-IR spectroscopy as a simple, less time consuming and more economical technique to monitor wine chemical age which is of high technological importance.

Materials and methods

- **Wines and Containers**

The wines used were made of two major red grape varieties of Crete, Kotsifali and Mandilari, which differ greatly in their anthocyanic and tannic content. Kotsifali produces wines low in color, relatively high in alcohol with smooth tannins, whereas Mandilari produces age worthy wines with deep red color. All wines were vinified following the protocol for classical red winemaking and received the same SO2 additions. No tartaric acid additions were made as pH values for all wines were satisfactory (pH 3.4 -3.5). For the maturation process, different types of containers were used (tank, tank with French oak chips and barrels made of French oak, American oak, Acacia and Chestnut) and samples were taken from each container every three months over the period of nine months for two consecutive vintages (2012 and 2013) (12 months of contact only for the 2013 vintage), resulting in 12 red wine samples for each vintage's trimester.

Table 5. Samples and containers per vintage (V1: 2012, V2: 2013)

		SAMPLES			
Grape variety	**Container**	**3 months**	**6 months**	**9 months**	**12 months**
Kotsifali/Mandilari	Inox	V1/V2	V1/V2	V1/V2	V1/-
	Inox with oak chips	V1/V2	V1/V2	V1/V2	V1/-
	French oak	V1/V2	V1/V2	V1/V2	V1/-
	American oak	V1/V2	V1/V2	V1/V2	V1/-
	Acacia	V1/V2	V1/V2	V1/V2	V1/-
	Chestnut	V1/V2	V1/V2	V1/V2	V1/-
Sum of samples for both varieties		12/12	12/12	12/12	12/-

- **Spectrophotometric Analyses**

Total Anthocyanins (TA), Degree of ionisation of anthocyanins (ID), Color density (CD), Color density corrected for SO_2 (CDS), SO_2 resistant pigments (SRP) and of Chemical ages i and ii were determined according to the modified Somers assay as described by Mercurio et al., (2007). According to the method, prior to analysis pH and alcohol content of all wine samples were standardized to 3.4 and 12% v/v respectively with the use of a buffer solution. After that, the wines are treated with excess SO_2, excess acetaldehyde and hydrochloric acid. The absorbance of the samples is read in four steps: first, the absorbance of wine in its original state is read at 420 and 520nm ($A_{420\ buffer}$, $A_{520\ buffer}$), then the second reading is after the addition of excess SO2 ($A_{520\ sulfite}$). This reading allows the measurement of color resulting from SO2-resistant pigments. Subsequently, the absorbance of wine samples treated with excess acetaldehyde is read ($A_{520\ acetal}$), to estimate the anthocyanins which are colored at wine pH. Finally, the absorbance of wine diluted with hydrochloric acid is read ($A_{520\ HCl}$). This treatment lowers the pH of wine and this way all anthocyanins are converted into their colored forms.

Calculations:

Total Anthocyanins (mg/L): $20 \text{ x } [(50 \text{ x } A_{520\ HCl}) - (1.6667 \text{ x } (10 \text{ x } A_{520\ sulfite})])$.

Degree of ionisation of anthocyanins (%):

$\{[(10\text{x}A_{520buffer}) - (10\text{x}A_{520sulfite})]/[(50\text{x}A_{520HCL}) - (1.6667\text{x}(10\text{x}A_{520sulfite}))]\text{x}100$

Color Density (au): $(A_{420\ buffer} + A_{520\ buffer}) \text{ x } 10$

Color density corrected for SO_2 (au): $(A_{420\ acetal} + A_{520\ acetal}) \text{ x } 10$

SO_2 resistant pigments (au): $A_{520\ sulfite} \text{ x } 10$

Chemical Age i (no units): $A_{520\ sulfite}/A_{520\ acetal}$

Chemical Age ii (no units): $A_{520\ sulfite}/(5 \text{ x } A_{520\ HCl})$

Both indexes are close to zero in wines right after fermentation is completed (Somers & Evans, 1977).

Color Intensity (CI) and Hue (h) were determined according to the method proposed by Glories, (1984) and were calculated after measuring the absorbances at 420, 520 and 620nm as follows: CI= $A_{420}+A_{520}+A_{620}$ and h= A_{420}/A_{520}.

All absorbance measurements were performed on a Hitachi U-2000 spectrophotometer.

- **FT-IR**

Mid-IR spectra of all samples were collected in triplicate by a Fourier Transform Infrared Spectrophotometer (Thermo Nicolet 6700 FT-IR by Thermo Electron Corporation, MA, USA) equipped with a deuterated triglycine sulfate (DTGS) detector, following the procedure described by Basalekou et al., (2015). All samples' spectra were recorded in the region 4000-500 cm^{-1}.

- **Statistical analysis**

Principal Component Analysis (PCA) and Analysis of Variance (ANOVA) were performed to plot differences between samples and to examine each variable's (vintage, time, container) effect on wine color compounds. The statistical program used for these analyses was JMP v.11. Determination models were also developed using Partial Least Squares (PLS) and the FT-IR's build in software TQ Analyst. PLS is a quantitative analysis technique. The PLS algorithm examines the specified region of the calibration spectra to determine areas that vary statistically as a function of component concentration. The calibration model is then developed using spectral and concentration information. The spectral region considered for the statistical analysis was 1830-1500 cm^{-1}.

Results and discussion

- **Chemical analyses**

Chemical analyses highlighted the differences in the phenolic content of Kotsifali and Mandilari wines. For Kotsifali, the maximum initial average concentration of anthocyanins (i.e. after fermentation completion) was 145.4 mg/L while for Mandilari 322.6 mg/L (Vintage 2013). Mandilari wines are characterized as rich in anthocyanins and tannins, presenting a deep red color and a quite astringent palate. Their high tannic and anthocyanin concentration favours the formation of more anthocyanin-tannin polymers.

Table 6. Changes in average values of color parameters and chemical age indexes (CI: Color Intensity, h: hue, CA1: Chemical Age i, CA2: Chemical Age ii, ID: Degree of Ionisation of

anthocyanins, CD: Color Density, CDS: Color Density corrected for SO_2, TA: Total Anthocyanin concentration, SRP: SO_2 Resistant Pigments) during maturation, for Kotsifali and Mandilari wines.

		2012 vintage			2013 vintage		
		3 months	6 months	9 months	3 months	6 months	9 months
Kotsifali	CI	2.8	3.4	3.9	5.5	5.1	6.1
	h	0.9	0.9	1.0	0.7	0.8	0.8
	CA 1	0.2	0.4	0.5	0.3	0.4	0.5
	CA 2	0.1	0.1	0.2	0.1	0.1	0.2
	ID	18.5	15.5	28.3	23.7	23.0	24.8
	CD	2.9	4.0	3.2	4.4	4.4	4.5
	CDS	3.4	4.5	3.3	4.9	4.5	4.7
	TA	143.0	140.5	56.6	145.4	130.7	100.3
	SRP	0.4	1.1	0.9	1.0	1.1	1.3
Mandilari	CI	14.8	16.4	16.2	14.8	12.7	14.0
	h	0.5	0.5	0.5	0.5	0.5	0.6
	CA 1	0.4	0.6	0.7	0.3	0.4	0.5
	CA 2	0.2	0.3	0.4	0.1	0.2	0.2
	ID	32.8	34.4	57.4	28.1	26.8	27.7
	CD	11.2	13.1	11.6	10.6	10.1	9.9
	CDS	12.1	13.3	11.9	11.1	10.2	10.3
	TA	285.9	187.8	92.4	322.6	278.4	221.5
	SRP	3.2	5.2	4.9	2.6	2.9	3.3

The anthocyanin concentration of Kotsifali and Mandilari wines decreased by a threefold over the nine-month period of maturation for both vintages and in all containers (Table 6). Indeed, anthocyanins are unstable molecules and they are incorporated into the tannin structure, forming pigmented polymers. The decrease in anthocyanin concentrations is also associated with the formation of more stable pigments such as pyranoanthocyanins as well as the degradation of anthocyanins (McRae, Kassara, Kennedy, Waters, & Smith, 2013). These results are in agreement with the results obtained by Kallithraka *et al.*, 2015. The method used for anthocyanin determination is based on the effect of pH on anthocyanin structure. Considering that oligomeric and polymeric pigments are more resistant to pH changes than the monomeric ones (Johanna Bakker & Timberlake, 1997) anthocyanin determination is based mainly on free monomeric anthocyanins. Therefore, the decrease observed in total anthocyanin content during maturation is consistent with the participation of monomeric anthocyanins in numerous condensation reactions as well as in hydrolytic and other degradation reactions (Santos-Buelga, Francia-Aricha, De Pascual-Teresa, & Rivas-Gonzalo, 1999) to a minor extent.

In general, all color parameters were characterized by higher values for Mandilari wines, given their higher anthocyanin content. Chemical age indexes i and ii of Mandilari wines were also higher than the corresponding values measured in Kotsifali, indicating that in Mandilari wines more polymeric pigments are present at wine pH, and that those polymeric forms are resistant to SO_2 bleaching (Almela, Javaloy, Fernández-López, & Lôpez-Roca, 1995).

- **Statistical analysis**

To examine internal structure and patterns of the wine data set Principal Components Analysis was employed. The variables used, namely Total Anthocyanins, Degree of ionisation of anthocyanins, Color density, Color density corrected for SO2, SO2 resistant pigments, Chemical ages i and ii, Color Intensity and Hue, produced the PCA plot shown in Figure 13.

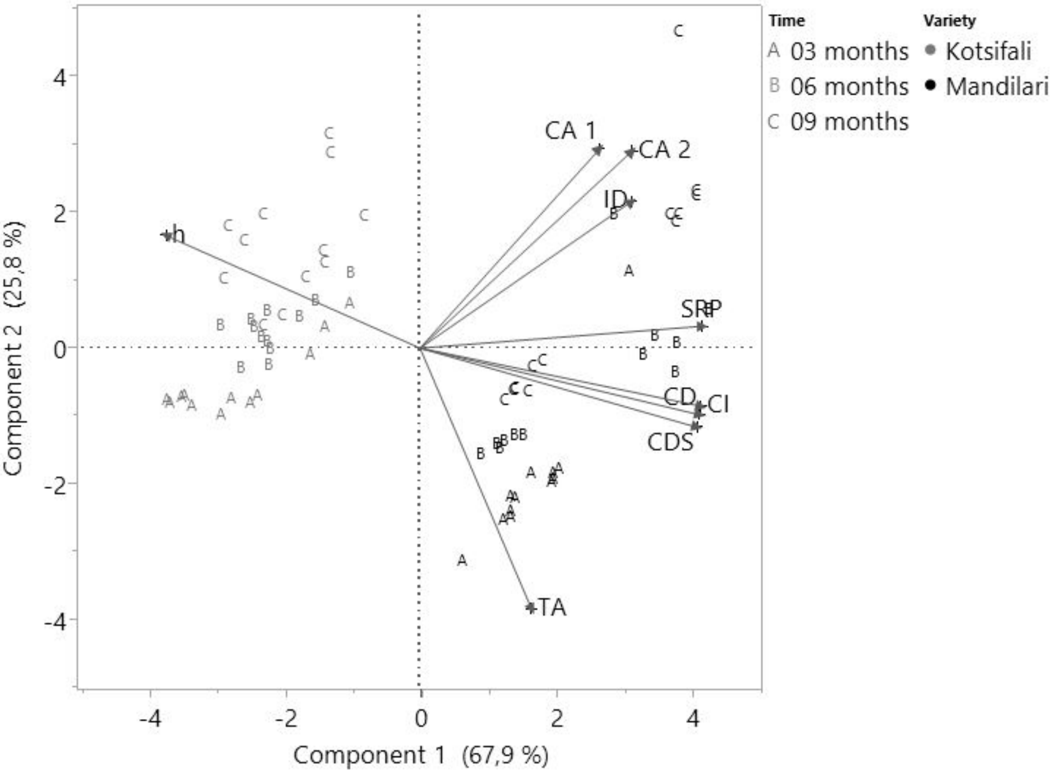


Figure 13. Biplot of principal components 1 and 2 for mean scores of Kotsifali and Mandilri color parameters

The first two principal components explain 93.7% of the variance. As can be seen in Figure 13, the PCA plot resulted in a clear separation of the two varieties, based on the first principal component. However, a discrimination trend according to maturation time can also be observed, based on the second principal component. Most of the samples that have been aged for 3 months are situated lower than the rest samples that have been matured for 6 and 9 months respectively. Moreover, samples that have been matured for 9 months are mostly found on the top of the diagram. As expected, this trend is more obvious between samples that aged for three monthσ (lower part of the plot) and samples that aged for nine months (higher part of the plot) since it requires more than three months to observe differences in wine chemical parameters.

Given the strong influence of the variety on phenolic content and wine color which is evident in the PCA plot, analysis of variance was also performed to examine if any of the variables were independent to variety, vintage and container effects and were only influenced by time. The results showed that Total Anthocyanins, Hue, Pigments resistant to SO2 and Chemical ages i and ii were influenced by time, however, variety and vintage had a stronger effect in all cases except for Chemical Age i (Table 7) were time had the most definitive effect.

Table 7. Test results for Fixed Effects of Hue, Chemical Age indexes i and ii, Total Anthocyanins and SO2 Resistand Pigments for Kotsifali and Mandilari wine samples.

Hue	**Nparm**	**DF**	**Sum of Squares**	**F Ratio**	**Prob > F**
Variety	1	1	2.081079	617.9852	<.0001*
Vintage	1	1	0.0592846	17.6048	<.0001*
Time	2	2	0.1038602	**15.4209**	<.0001*
Container	5	5	0.0467252	2.775	0.0252*
Chemical Age i	**Nparm**	**DF**	**Sum of Squares**	**F Ratio**	**Prob > F**
Variety	1	1	0.15791902	30.0835	<.0001*
Vintage	1	1	0.04734925	9.02	0.0038*
Time	2	2	0.64369316	**61.3116**	<.0001*
Container	5	5	0.04057336	1.5458	0.1888
Chemical Age ii	**Nparm**	**DF**	**Sum of Squares**	**F Ratio**	**Prob > F**
Variety	1	1	0.13732412	40.686	<.0001*
Vintage	1	1	0.05456823	16.1673	0.0002*
Time	2	2	0.20179451	**29.8935**	<.0001*
Container	5	5	0.02202906	1.3053	0.2734
Total Anthocyanins	**Nparm**	**DF**	**Sum of Squares**	**F Ratio**	**Prob > F**
Variety	1	1	225840.64	121.065	<.0001*
Vintage	1	1	42884.5	22.9888	<.0001*
Time	2	2	139047.49	**37.2692**	<.0001*
Container	5	5	8431.81	0.904	0.4843

SO_2 Resistant Pigments	**Nparm**	**DF**	**Sum of Squares**	**F Ratio**	**Prob > F**
Variety	1	1	135.1368	317.3832	<.0001*
Vintage	1	1	6.22457	14.6191	0.0003*
Time	2	2	10.32205	**12.1212**	<.0001*
Container	5	5	0.35078	0.1648	0.9745

Table 7 presents the effect tests' results. The Nparm value indicates the number of parameters associated with the effect, DF is the degrees of freedom, Sum of Squares gives the sum of squares for the hypothesis that the effect is zero, F Ratio indicates whether the model differs significantly from a model where all predicted values are the response mean, while the Prob > F value measures the probability of obtaining an F Ratio as large as what is observed. According to Table 7, only the type of container does not have a statistically significant effect on the parameters measured, while the variety exhibits the strongest influence. Variety exhibits the strongest influence on Hue (F ratio 617.9) followed by SO2 Resistant Pigments (F ratio 317.4) and Total Anthocyanin Concentration (F ratio 121.1). Vintage seems to have a lesser effect, although in some cases more important than time. The only case where time is less dependent to variety and vintage is in Chemical age index i (time F ratio 61.3 > variety F ratio 30.1 > vintage F ratio 9).

- **FT-IR analysis**

According to Analysis of Variance, Chemical age index i is the only parameter that is mostly time dependant and can produce significantly statistical differences in the short time period of nine months without strong variety interferences. The conventional analysis employed to measure Chemical age indexes i and ii is simple, but time consuming and needs chemical agents, making in less appealing to be implemented in the wineries as a routine protocol. When grape, must or wine is analyzed for quality control, analysis time becomes a key parameter. For this purpose, the suitability of FT-IR combined with PLS was investigated as an alternative method to provide information of such technological interest to the wineries.

For this reason, FT-IR coupled with chemometrics was used to develop calibration models. A typical FT-IR spectrum for Kotsifali and Mandilari samples is shown in Figure 14. The region that was selected for further statistical analysis was the region from 1830 to 1500 cm^{-1}.

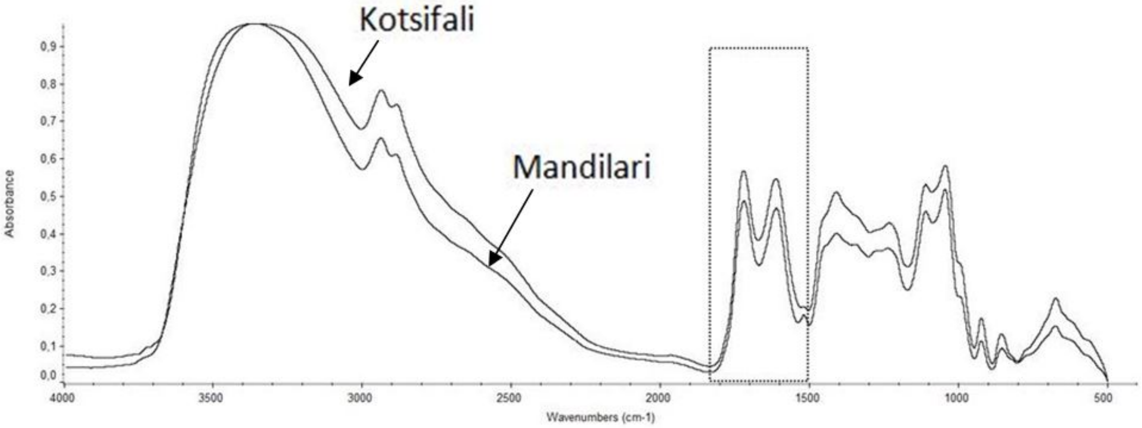


Figure 14. Typical FT-IR spectra for Kotsifali and Mandilari wines.

This area, as part of the fingerprint region contains many information such as various IR-bands, including those corresponding to the vibrations of the C-O, C-C and C-H bonds (Smith, 1999). As it is shown in Figure 14, two major peaks are included in the selected area. One centred at 1722 cm^{-1}, due to the stretching of carbonyl group (C=O), and one at 1610 cm^{-1}, due to C=C stretching (typical for aromatic molecules) (Edelmann et al., 2001). The first peak is mainly connected with the C-O stretching of the esters of hydrolysable tannins, especially derivatives of gallic acid (Grasel et al., 2016) and the C=C stretching, typical of flavanoid-based compounds in wine (Ricci et al.,2015), while the second is mainly attributed to the carboxyl ion (COO-) (symmetrical stretching) (Agatonovic-Kustrin, 2013; Y. Zhang et al., 2010). A smaller peak is also detected at 1520 cm^{-1}, due to the deformation of the aromatic ring, which can be attributed to simple catechin (Fernandez & Agosin, 2007; Arianna Ricci, Olejar, et al., 2015). The region from 1535 to 1520 cm^{-1} is affected by the structural modifications that occur during polymerization (Arianna Ricci, Olejar, et al., 2015) and for this reason it was included in the spectral region used for the statistical analysis. Overall, the selection of the spectral region was based on absorptions that are due to the most important chemical compounds that participate in color stabilization reactions, such as phenolic acids, oak tannins and flavonoids (R. B. Boulton, 2001).

Wine spectra are extremely multivariate and hence complex. It is necessary to use advanced mathematical techniques to generate the calibration equations for the individual parameters. This type of calculations is often referred as chemometrics. The multivariate regression methods have been widely used to provide a better insight into such systems and to build calibration and prediction models. Partial least squares (PLS) regression is one of the most used models (Passos, Cardoso, Barros, Silva, & Coimbra, 2010) where the regression algorithm uses the absorptions at selected frequencies or blocks of frequencies to generate an equation that best fits the reference values in a data set. In wine analysis, those models were successfully applied for the determination of various compounds such as anthocyanins and tannins (Fernandez & Agosin, 2007; Soriano, Pérez-Juan, Vicario, González, & Pérez-Coello, 2007), but also ethanol and organic acids (Coldea et al., 2013; Moreira & Santos, 2005).

In this experiment Partial Least Squares were used to develop calibration models for the chemical age indexes. The accuracy of the models is determined by their correlation coefficient (r) and the root-mean-square error of calibration (RMSEC) and prediction (RMSEP) values. For Kotsifali wines, the models' correlation coefficient (r) for chemical age (i) was 0.86 and the root-mean-square error of calibration (RMSEC) and prediction (RMSEP) were 0.066 and 0.115 respectively (Figure 15a). For Mandilari samples (Figure 15b), the correlation coefficient (r) for chemical age (i) was 0.90 (RMSEC=0.050, RMSEP=0.040). For each variety 42 samples were used to build the model, nine of which were used for validation purposes. For Kotsifali 10 factors were used for each component calculated (Root Mean Square Error of Cross Validation RMSECV=0.164), while for Mandilari six (RMSECV=0.089).

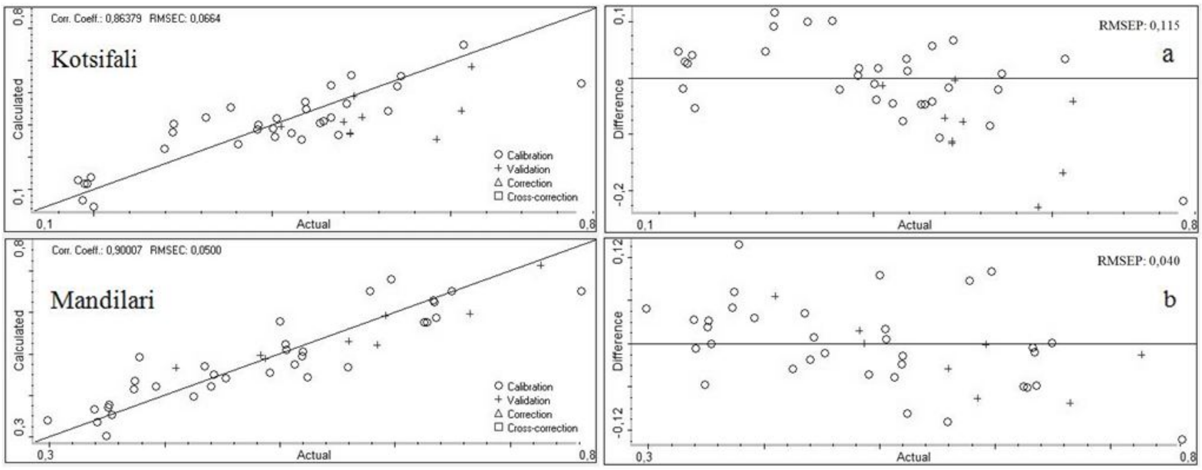


Figure 15 (a,b). Correlation between the actual chemical age i values and the values predicted by the model for Kotsifali (a) and Mandilari (b) samples.

For chemical age (ii) the correlation coefficients (r) for Kotsifali and Mandilari wine samples (Figure 16a and b) were 0.86 and 0,97 respectively (Kotsifali: RMSEC=0.044, RMSEP= 0.088 and Mandilari RMSEC= 0.024, RMSEP=0.033). For each variety 42 samples were used to build the model, 9 of which were used for validation purposes. For both Kotsifali and Mandilari 10 factors were used for each component calculated (Kotsifali RMSECV=0.214, Mandilari RMSECV=0.078). Calibration and prediction values for all models as well as Predicted

Residual Error Sum of Squares (PRESS) are given as supplementary material (Tables S1- 4 and Figures S1-4).

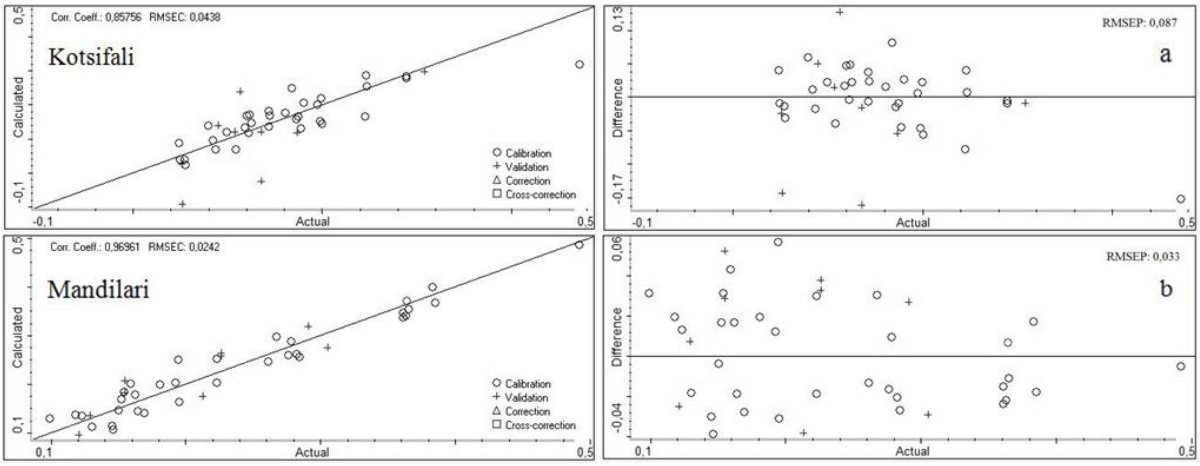


Figure 16 (a, b). Correlation between the actual chemical age ii values and the values predicted by the model for Kotsifali (a) and Mandilari (b) samples.

An interesting observation was that in all cases studied, r values of Mandilari were higher than the corresponding values of Kotsifali samples indicating a better fit of the proposed model for wines richer in phenolic compounds including anthocyanins. FT-IR is an indirect analytical method that is product sensitive. Every chemical substance has an IR ‘fingerprint’ that is a function of the molecular bonds that are present in the sample. These characteristic signals are the basis of the measurement and are used in the calibration process. Different grape varieties may contain different compounds with similar IR absorption bands that may interfere with and disturb the calibration model. Indeed, the calibration models developed by FT-IR for musts or wines richer in anthocyanins and acids were more robust compared with those obtained for samples where the concentration of the compounds of interest was lower (Rasines-Perea, Prieto-Perea, Romera-Fernández, Berrueta, & Gallo, 2015; Regmi, Palma, & Barroso, 2012)
In this study, r was greater than 0.86 in all cases (higher than 0.90 in Mandilari wines), which is a satisfactory statistically significant value. The closer this value is to "one" the more linear is the relationship between the calculated and actual values. RMSEC refers to the uncertainty of calibration while RMSEP estimates how well the method should predict concentration values for unknown samples. The calculated RMSEP values for all models were below 0.1, indicating low uncertainty values concerning the methods’ prediction ability. The low values of RMSECV also demonstrate that the validation efficiency of the model is satisfactory. These

data suggest that chemical age indexes may be estimated using FT-IR and the aforementioned models.

As monomeric anthocyanins decrease during ageing color is no longer determined by monomeric forms hence there is a lesser need to estimate them individually. In that case polymer forms are of greater importance, for the estimation of which chemical age indexes are more useful. To our knowledge this is the first time FT-IR is used to estimate the chemical age status of a wine.

- **Conclusions**

Wine ageing has a defining effect on the color of red wines. Chemical age index i values are significantly correlated with maturation time and are less dependent to the variety, the vintage or the type of container used for maturation. FT-IR combined with PLS allowed developing regression models to provide approximate quantitive values for chemical age indexes in a quick and simple way that can be easily implemented for routine control of wines. Linear relationships were found with correlation coefficients (r) 0.93 and 0.91 for the chemical age index i and 0.95 and 0.88 for index ii for Mandilari and Kotsifali samples respectively. The low RMSEC values in each model, demonstrate the robustness of the proposed method. It could be a starting point for the design of more specific models according to the requirements of the wineries.

- **Supplementary Information**

Table S1. Actual and predicted values of Chemical age index (i) for Kotsifali samples

Sample ID	Usage	Actual	Calculated
1	Calibration	0,189	0,212
2	Validation	0,48	0,431
3	Calibration	0,279	0,315
4	Calibration	0,384	0,331
5	Validation	0,488	0,4
6	Calibration	0,747	0,613
7	Calibration	0,192	0,152
8	Calibration	0,474	0,443
9	Calibration	0,403	0,358
10	Validation	0,501	0,361
11	Validation	0,585	0,369
12	Validation	0,613	0,447

13	Calibration	0,2	0,154
14	Calibration	0,458	0,472
15	Calibration	0,288	0,327
16	Calibration	0,326	0,355
17	Calibration	0,405	0,456
18	Calibration	0,466	0,492
19	Calibration	0,182	0,22
20	Calibration	0,438	0,459
21	Calibration	0,383	0,363
22	Calibration	0,454	0,435
23	Calibration	0,53	0,479
24	Calibration	0,544	0,546
25	Calibration	0,197	0,265
26	Calibration	0,437	0,436
27	Calibration	0,289	0,241
28	Calibration	0,362	0,392
29	Calibration	0,422	0,423
30	Calibration	0,489	0,522
31	Calibration	0,187	0,152
32	Validation	0,41	0,415
33	Calibration	0,354	0,409
34	Calibration	0,433	0,456
35	Validation	0,488	0,384
36	Calibration	0,54	0,555
73	Calibration	0,615	0,63
74	Validation	0,624	0,556
75	Validation	0,492	0,61
76	Calibration	0,484	0,458
77	Calibration	0,466	0,466
78	Calibration	0,401	0,428

Table S2. Actual and predicted values of Chemical age index (i) for Mandilari samples

Sample ID	Usage	Actual	Calculated
37	Calibration	0,52	0,506
38	Calibration	0,762	0,651
39	Calibration	0,352	0,379
40	Calibration	0,435	0,471
41	Calibration	0,524	0,443
42	Calibration	0,634	0,625
43	Calibration	0,298	0,339
44	Calibration	0,597	0,681
45	Calibration	0,349	0,302
46	Calibration	0,453	0,442

47	Calibration	0,513	0,474
48	Validation	0,585	0,522
49	Calibration	0,378	0,493
50	Validation	0,592	0,591
51	Calibration	0,351	0,37
52	Calibration	0,425	0,396
53	Validation	0,483	0,498
54	Calibration	0,519	0,495
55	Calibration	0,374	0,434
56	Calibration	0,578	0,651
57	Calibration	0,354	0,354
58	Calibration	0,443	0,451
59	Calibration	0,491	0,455
60	Calibration	0,506	0,511
61	Calibration	0,373	0,415
62	Calibration	0,559	0,468
63	Calibration	0,341	0,336
64	Validation	0,41	0,465
65	Validation	0,487	0,487
66	Calibration	0,505	0,523
67	Calibration	0,392	0,422
68	Calibration	0,625	0,576
69	Calibration	0,339	0,367
70	Calibration	0,44	0,422
71	Calibration	0,5	0,579
72	Validation	0,56	0,531
79	Validation	0,727	0,715
80	Calibration	0,649	0,651
81	Validation	0,665	0,596
82	Calibration	0,628	0,577
83	Calibration	0,636	0,587
84	Calibration	0,633	0,629

Table S3. Actual and predicted values of Chemical age index (ii) for Kotsifali samples

Sample ID	Usage	Actual	Calculated
1	Validation	0,051	0,027
2	Calibration	0,118	0,135
3	Calibration	0,079	0,138
4	Validation	0,107	0,122
5	Calibration	0,195	0,202
6	Calibration	0,472	0,32
7	Validation	0,052	-0,091
8	Calibration	0,125	0,148

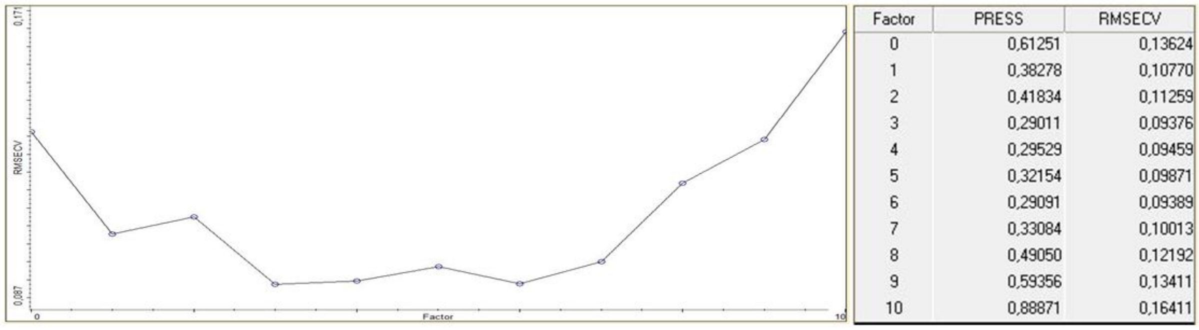
9	Calibration	0,174	0,165
10	Calibration	0,2	0,144
11	Calibration	0,245	0,167
12	Calibration	0,289	0,28
13	Calibration	0,055	0,025
14	Calibration	0,143	0,181
15	Calibration	0,084	0,095
16	Validation	0,09	0,139
17	Calibration	0,12	0,168
18	Calibration	0,168	0,25
19	Calibration	0,048	0,089
20	Calibration	0,122	0,118
21	Calibration	0,143	0,136
22	Calibration	0,161	0,177
23	Calibration	0,198	0,152
24	Calibration	0,247	0,255
25	Calibration	0,054	0,041
26	Validation	0,113	0,24
27	Calibration	0,087	0,069
28	Calibration	0,099	0,122
29	Validation	0,136	0,12
30	Calibration	0,18	0,207
31	Calibration	0,049	0,04
32	Calibration	0,108	0,069
33	Calibration	0,123	0,172
34	Calibration	0,144	0,168
35	Calibration	0,177	0,132
36	Calibration	0,246	0,286
73	Calibration	0,289	0,285
74	Validation	0,308	0,299
75	Calibration	0,199	0,221
76	Validation	0,173	0,119
77	Calibration	0,172	0,157
78	Validation	0,136	-0,025

Table S4. Actual and predicted values of Chemical age index (ii) for Mandilari samples

Sample ID	Usage	Actual	Calculated
37	Validation	0,291	0,318
38	Calibration	0,361	0,337
39	Calibration	0,123	0,136
40	Calibration	0,162	0,179
41	Calibration	0,223	0,204
42	Calibration	0,363	0,341

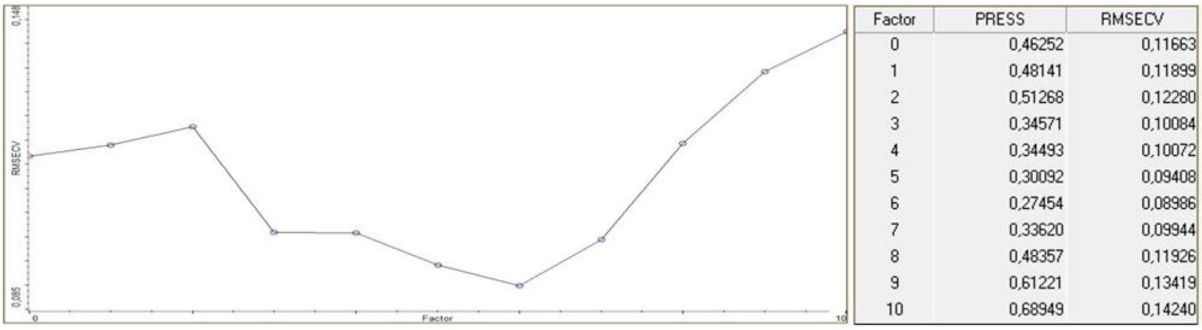
43	Calibration	0,099	0,13
44	Calibration	0,261	0,248
45	Calibration	0,145	0,115
46	Calibration	0,169	0,142
47	Validation	0,213	0,175
48	Calibration	0,278	0,288
49	Validation	0,155	0,207
50	Validation	0,305	0,276
51	Validation	0,129	0,136
52	Calibration	0,146	0,108
53	Calibration	0,181	0,201
54	Calibration	0,223	0,253
55	Calibration	0,159	0,202
56	Calibration	0,267	0,297
57	Calibration	0,13	0,112
58	Validation	0,155	0,184
59	Calibration	0,195	0,164
60	Validation	0,226	0,259
61	Calibration	0,154	0,185
62	Calibration	0,276	0,26
63	Validation	0,121	0,096
64	Calibration	0,152	0,169
65	Calibration	0,192	0,204
66	Validation	0,226	0,264
67	Calibration	0,164	0,145
68	Calibration	0,284	0,257
69	Calibration	0,118	0,137
70	Calibration	0,15	0,147
71	Calibration	0,194	0,251
72	Calibration	0,282	0,262
79	Calibration	0,492	0,487
80	Calibration	0,383	0,4
81	Calibration	0,385	0,367
82	Calibration	0,361	0,346
83	Calibration	0,365	0,354
84	Calibration	0,364	0,371

Figure S1. Predicted Residual Error Sum of Squares (PRESS) values and Root Mean Square Error of Validation (RMSECV) for each factor for Kotsifali Chemical age (i) prediction.



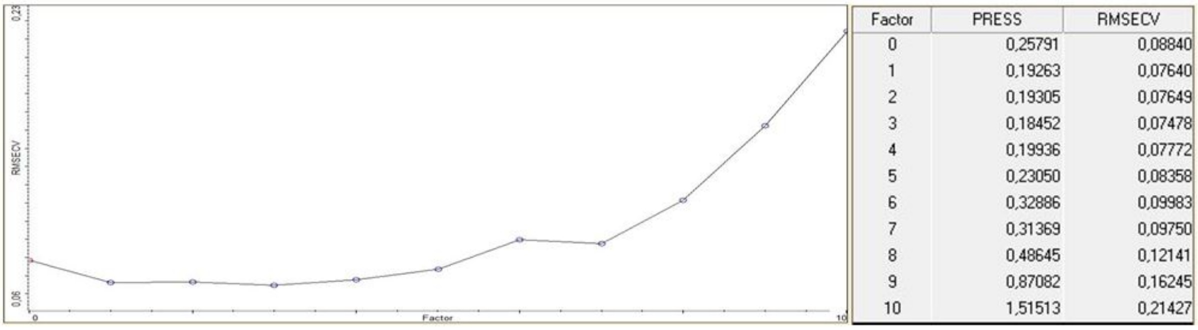
Factor	PRESS	RMSECV
0	0,61251	0,13624
1	0,38278	0,10770
2	0,41834	0,11259
3	0,29011	0,09376
4	0,29529	0,09459
5	0,32154	0,09871
6	0,29091	0,09389
7	0,33084	0,10013
8	0,49050	0,12192
9	0,59356	0,13411
10	0,88871	0,16411

Figure S2. Predicted Residual Error Sum of Squares (PRESS) values and Root Mean Square Error of Validation (RMSECV) for each factor for Mandilari Chemical age (i) prediction.



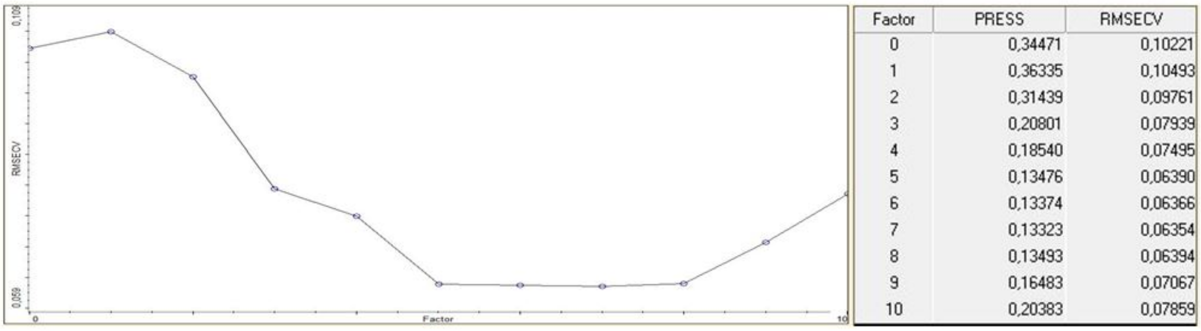
Factor	PRESS	RMSECV
0	0,46252	0,11663
1	0,48141	0,11899
2	0,51268	0,12280
3	0,34571	0,10084
4	0,34493	0,10072
5	0,30092	0,09408
6	0,27454	0,08986
7	0,33620	0,09944
8	0,48357	0,11926
9	0,61221	0,13419
10	0,68949	0,14240

Figure S3. Predicted Residual Error Sum of Squares (PRESS) values and Root Mean Square Error of Validation (RMSECV) for each factor for Kotsifali Chemical age (ii) prediction.



Factor	PRESS	RMSECV
0	0,25791	0,08840
1	0,19263	0,07640
2	0,19305	0,07649
3	0,18452	0,07478
4	0,19936	0,07772
5	0,23050	0,08358
6	0,32886	0,09983
7	0,31369	0,09750
8	0,48645	0,12141
9	0,87082	0,16245
10	1,51513	0,21427

Figure S4. Predicted Residual Error Sum of Squares (PRESS) values and Root Mean Square Error of Validation (RMSECV) for each factor for Mandilari Chemical age (ii) prediction.



Factor	PRESS	RMSECV
0	0,34471	0,10221
1	0,36335	0,10493
2	0,31439	0,09761
3	0,20801	0,07939
4	0,18540	0,07495
5	0,13476	0,06390
6	0,13374	0,06366
7	0,13323	0,06354
8	0,13493	0,06394
9	0,16483	0,07067
10	0,20383	0,07859

iii. Ellagitannins in wines: future prospects in methods of analysis using FT-IR spectroscopy

Introduction

Wine aging in wooden barrels is a common practice used for centuries from winemakers as a way to improve a wine's aroma, color and mouthfeel. This sensory modification is achieved by the extraction into wine of specific wood chemical compounds, such as volatile phenols, lignins and hydrolysable tannins (Jackson, 2008).

Hydrolysable tannins constitute a diverse group that contains gallotannins (which after acid hydrolysis yield gallic acid) and ellagitannins (which after acid hydrolysis yield ellagic acid by spontaneous lactonization of their structural component hexahydroxydiphenic acid). A great number of compounds from this group have been isolated from various plants and have been fully characterized, however big emphasis is being given to C-glucosidic ellagitannins, specifically vescalagin and its C-1 epimer castalagin. Ellagitannins have been reported to not only alter a wines' sensory features but also to take part in several chemical reactions with wine phenols (Michel et al., 2011). Apart from the monomers vescalagin and castalagin, six other ellagitannins have been isolated, namely the lyxose/xylose-bearing monomers grandinin and roburin E, the dimers roburins A and D and the lyxose/xylose-bearing dimers roburins B and C (Viriot, Scalbert, Hervé du Penhoat, & Moutounet, 1994). All six, have been first isolated from *Castanea* (chestnut) and *Quercus* (oak) woody species of the *Fagaceae* family (Okuda & Yoshida, 2008). Even though their contents in wooden barrels depend on many factors such as botanical species, the age of the tree, the toasting degree during barrel making, vescalagin and castalagin appear to be dominant in terms of concentration, representing between 40-60% of the total ellagitannins in *Quercus petraea* and *robur* heartwoods (Cadahía *et al.*, 2001; Michel *et al.*, 2011). In general, in a sample of red wine aged in oak barrels, vescalagin and castalagin contents could be around 2 and 8 mg/L respectively (Jourdes, Lefeuvre, & Quideau, 2009). They can even reach a total of 63 mg/g of dry wood in chestnut species such as *Castanea sativa* (Viriot et al., 1994). During barrel ageing, their levels in wine are mainly affected by the contact time between the wine and the barrel wood, the wine's alcoholic degree and the structure of the wood and its polyphenolic load (Taloumi & Makris, 2017).

Among oak woods, European oak is the richest in ellagitannins -specifically Q. robur- and American oak is the poorest. There are now also a few published results concerning other barrel woods such as Acacia, and even though the aspect of geographical origin has not been researched thoroughly, its ellagitannin content appears to be low (Bŕigida Ferńandez De Simón et al., 2014; Kyraleou, Kallithraka, Chira, et al., 2015; Sanz, Fernández de Simón, et al., 2012). Data on Chestnut wood showed that it contains the highest ellagitannin concentration, but also contains an interesting variety of other tannin compounds (Tanaka, Ueda, Shinohara, Nonaka, & Kouno, 1997).

Ellagitannins have the ability to interact with wine phenols forming new compounds, and after prolonged exposure to ethanolic solutions higher than 40% (v/v) such as spirits may be transformed into ethyl-based hemiketal and ketal derivatives (J. L. Puech et al., 1999). Vescalagin and castalagin also present high instability (Klumpers, Scalbert, & Janin, 1994; Viriot et al., 1994) even in aqueous solutions, so they require careful handling during analysis. For this reason, the first methods for their quantification are based in their ability to degrade to ellagic acid after acid hydrolysis. HPLC chromatography is then used for their quantification as ellagic acid equivalents (Quinn and Singleton, 1985; Peng *et al.*, 1991). Quantification of specific ellagitannins requires preparative steps and the use of Mass Spectrometry. New methods have recently been applied (Navarro et al., 2017) but they are also require the use of sophisticated instrumentation.

Infrared spectroscopy methods are increasingly used because of their efficiency, cost effectiveness, and fast and non–invasive nature. There exists a number of important studies concerning the successful quantification of numerous chemical compounds with FT-IR spectroscopy (Anjos, Campos, Ruiz, & Antunes, 2015; Duarte, Barros, Delgadillo, Almeida, & Gil, 2002; Mallah, Sherazi, Bhanger, Mahesar, & Bajeer, 2015; Pelusi et al., 2016; Teixeira dos Santos et al., 2016). FT-IR spectroscopy was also efficient in monitoring the wines' color parameters during aging as well as in the discrimination of wines based either on variety or the type of wood used for their maturation (Basalekou, Pappas, Kotseridis, et al., 2017; Basalekou, Pappas, Tarantilis, Kotseridis, & Kallithraka, 2017). However, despite the importance of the ellagitannin content to the final wine quality, there is not yet any published data concerning their quantification by FT-IR spectroscopy. It was thus of interest to explore the suitability of FT-IR spectroscopy to monitor wine ellagitannin content which is of high technological importance. The outcomes of such a study would be of practical interest to winemakers -given the user-friendly nature of spectrophotometers- since they could improve the control over the wood extraction process and thus improve the quality of the produced wine.

Materials and Methods

- **Wines and containers**

Four different monovarietal wines were aged in different types of barrel woods for different periods of time to obtain samples with varying ellagitannin levels. More specifically, two red wines (Kotsifali and Mandilari) and two white wines (Vilana and Dafni) from Crete, (Greece) were aged in inox tanks with oak sticks and barrels made of French oak, American oak, Acacia and Chestnut (Chestnut was used only for red wine samples). Vinifications were carried out in two consecutive vintages (Vintage 2012 and Vintage 2013) reusing the same barrels in order to obtain wines with ellagitannin levels close to those of the common winemaking practice. French (Quercus robur) and American (Quercus alba) barrels (225 L) with a medium toasting were purchased from 'Tonnellerie du Monde World Cooperage', Acacia barrels with a light plus (L+) toasting were purchased from Tonellerie du sud ouest, and Chestnut barrels with a medium toasting were purchased from Tesias Metsovo, Greece. The oak sticks (French oak) were from Seguin Moreau (Oenostick®, V18). Samples were taken from containers every three months for a period of nine months for analysis purposes. Oenological parameters of resulting wines are shown in Table 8.

Table 8. Oenological parameters after vinification of wine samples for the vintages of 2012 and 2013.

	Oenological parameters							
	Ethanol %(v/v)		Total acidity (g/L in tartaric acid)		pH		Volatile acidity (g/L in acetic acid)	
Vintage / Variety	2012	2013	2012	2013	2012	2013	2012	2013
Vilana	12.4±0.2	14.3±0.1	6.07±0.1	6.54±0.2	3.45±0.05	3.38±0.05	0.22±0.04	0.39±0.05
Dafni	13.1±0.2	12.8±0.2	4.50±0.2	5.30±0.3	3.73±0.06	3.44±0.04	0.24±0.06	0.30±0.05
Kotsifali	12.2±0.1	13.6±0.3	5.65±0.5	5.55±0.5	3.60±0.02	3.45±0.07	0.29±0.04	0.41±0.06
Mandilari	13.3±0.1	13.0±0.1	6.45±0.3	5.55±0.1	3.45±0.04	3.35±0.05	0.32±0.02	0.28±0.08

- **Total Ellagitannin quantification**

Total ellagitannin contents were determined using the method described by Chira and Teissedre (2013), according to which samples are subjected to acid hydrolysis prior to HPLC analysis (2 h at 100 °C, HCl 2M in Methanol). All samples were analysed in triplicate. The equipments used (AS-1555 Intelligent Sampler, PU 2089 Plus Quaternary Gradient Pump, MD-910 Multiwavelength Detector, LC-Net II/ADC) were from Jasco Corporation, Tokyo, Japan. The column was a 250 mm × 4.6 mm, 4 μm Pinnacle II C18 by Restek Corporation, Bellefonte, PA, USA. The software used for the statistical analysis of the ellagitannin levels was JMP v.11.SAS.

- **Spectroscopic analysis**

Triplicate spectra of 10 samples for red varieties and eight samples for white varieties per trimester of both vintages (108 in total) were collected with a Thermo Nicolet 6700 FT-IR spectrophotometer (Thermo Electron Corporation, MA, USA) equipped with a deuterated triglycine sulfate (DTGS) detector (Basalekou, Pappas, Kotseridis, et al., 2017). For the analysis, 200μL of each wine sample were placed on a ZnSe disc and were incubated at 40°C for 30 minutes. After incubation, spectra from all the samples were recorded. All spectra were collected with a 4 cm^{-1} resolution (100 scans in total), using a standard ZnSe window against a ZnSe background. Each spectrum was smoothed using the Savitsky-Golay algorithm (5-point moving, second-degree polynomial) and the baseline was corrected (20 iterations, second-degree polynomial). The average spectrum of each sample was calculated by means of the corresponding function of the software accompanying the spectrophotometer (OMNIC 7.3, Thermo Fisher Scientific Inc.).

- **Chemometrics**

The determination model was developed with Partial Least Squares regression (PLS), using the spectrophotometer’s build-in software (TQ Analyst software). PLS regression possesses the distinct advantage of being more adaptable to modern measuring instrumentation that provide a large number of strongly correlated X-variables, such as FTIR spectroscopy (Wold, Sjöström, & Eriksson, 2001). Another advantage of PLS is that Principal Components are modeled not only on the predictors, but also on the responses, so that it is possible to minimize the variance of both X and Y coordinates of the model (Venkatesan, Dharuman, & Gunasekaran, 2011). The predictive model is built by creating a linear regression after correlating spectral information to observed concentrations of the compound of interest and then projecting the predicted variables and the observable variables to a new space. In this experiment the compound of interest was ellagic acid, so the spectral region selected to perform the corrrelation was the fingerprint region, 1820-950 cm^{-1} (first derivative in range) which includes most information but also because in the same region most of ellagic acid’s characteristic peaks can be found.

- **Total ellagitannins**

As expected, the different containers used resulted in varying ellagitannin contents. In Figure 16, mean ellagitannin concentrations expressed as ellagic acid equivalents are shown according to the type of container and the vintage.

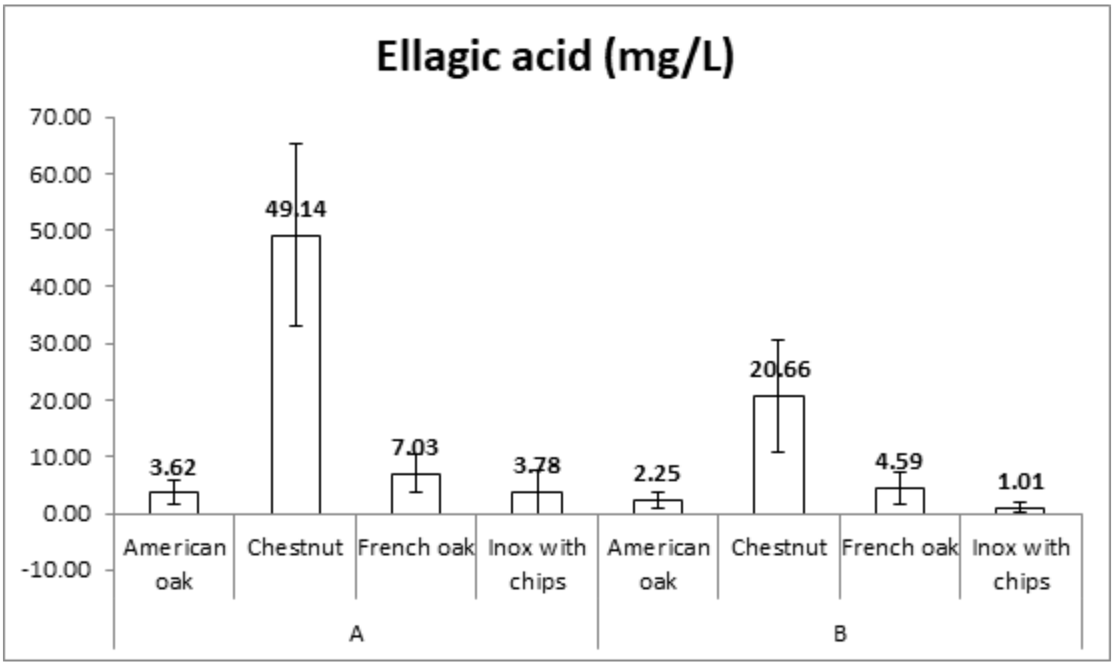


Figure 17. Mean ellagitannin concentrations, expressed as ellagic acid equivalents (mg/L) of all samples (three, six and nine months of contact) according to the type of container and the vintage (A: Vintage 2012, B: Vintage 2013).

As it can be seen in Figure 17, French oak appears to enhance the ellagitannin content of the wines in comparison with the American oak since it is richer in ellagitannins in agreement with (Kyraleou, Kallithraka, Chira, et al., 2015). However, American oak is not as depleted of its ellagitannin content during the second year of the experiment as French oak. This is probably due to the differences in porosity of the particular woods, which may alter the extraction rates of ellagitannins (Jordão, Correia, DelCampo, & González SanJosé, 2012). Similar results were observed for the samples aged with oak chips, where extraction appeared to be smoother, given the cylindrical surface and shape uniformity of the V18 oenosticks, instead of the coarse and small surface of classic oak chips.

As it is indicated by the error bars in the graph, ellagitannin content of chestnut samples is characterized by the highest variability between samplings (i.e. three, six or nine months of contact). Moreover, the extraction rate drops dramatically during the second year. Chestnut heartwood shows the most similar polyphenolic profile to oak, although it is richer in low molecular weight phenols and tannins (Bŕigida Ferńandez De Simón et al., 2014). It is possible that the lower molecular weight of the initial ellagitannin content and the higher porosity of this particular wood resulted in faster extraction rates. Acacia barrels did not appear to contain ellagitannins. Ellagitannins have previously been identified in wines aged in tanks with chips made from Acacia wood (Kyraleou, Kallithraka, Chira, et al., 2015), however not in wines aging in Acacia barrels (Sanz, Fernández de Simón, et al., 2012).

According to Figure 17, the ellagitannin concentration of most samples is lower than 15 mg/L regardless of the time of contact between wine and wood. This is in agreement with previous studies where the concentration of oak ellagitannins in a Bordeaux red wine aged for 18 months in oak barrels, was 10 mg/L (Saucier, Jourdes, Glories, & Quideau, 2006). All ellagitannin concentrations higher than 20 mg/L where determined in samples aged in chestnut barrels and since they over exceed the average oak ellagitannin contents appear as potential outliers. Chestnut is indeed rich in ellagitannins, and research has shown that depending on the geographical origin it may contain four more C-Glycosidic ellagitannins, namely Castacrenins D-G (Tanaka et al., 1997). This richness in hydrolysable tannins in general, has recently been helpful in discriminating wines aging in chestnut barrels from samples in different types of wood (Basalekou, Pappas, Tarantilis, et al., 2017).

The high variability of samples regarding their ellagitannin contents indicates a good representation of commercial wines aged in contact with different types of wood and ensures that the prediction model which will be built using the FT-IR spectroscopy data will cover a wide concentration range.

- **Spectroscopic analysis**

Spectroscopic analysis of hydrolysable tannins in general has revealed that their fingerprint region (i.e. the region of the spectrum that contains most information) presents a distinct absorption pattern highlighting the region 1820–950 cm−1 which can be considered as the most informative (Fig.18).

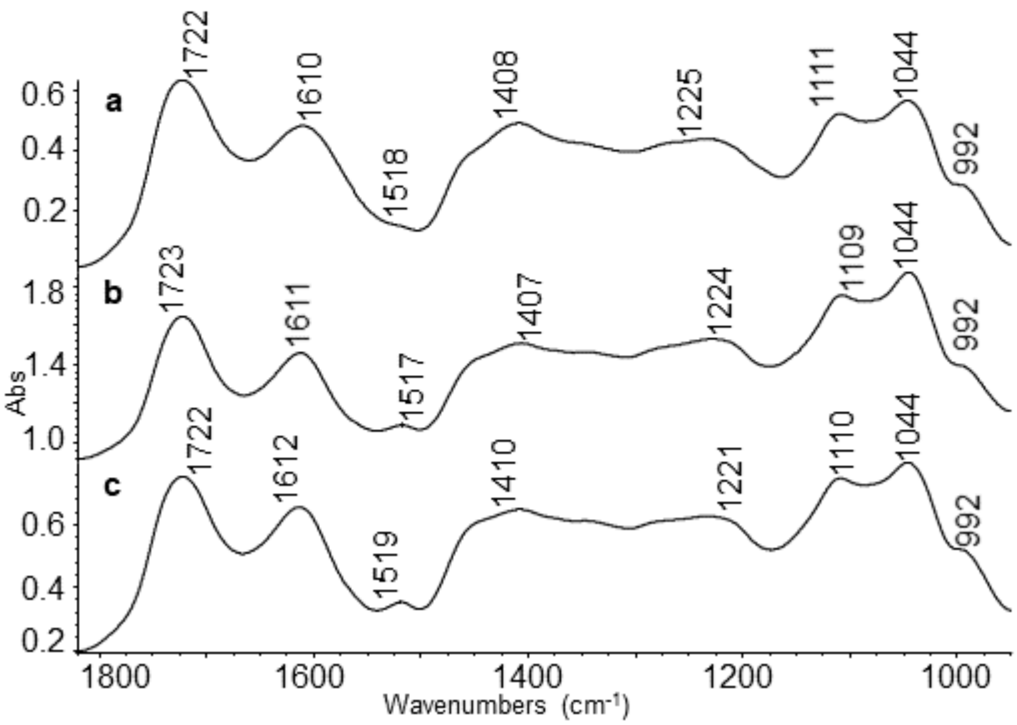


Figure 18. FT-IR spectra (1820-950 cm−1 spectral region) of three wine samples.

In that region, eight bands can be found. First at 1723-1722 cm^{-1} assigned to the stretching vibration of the carbonyl function (Wang et al., 2017). The stretching vibration peaks of the benzene ring appear at 1612-1610, 1519-1517 cm^{-1} and 1410-1407 cm^{-1} (Wang et al., 2017). The fifth at 1225-1221 cm^{-1} has been correlated to C-O stretching (Socrates, 2001). Absorbance at 1111-1109 has been assigned to C-C (Kyraleou, Pappas, et al., 2015) stretching in combination with C-O-C asymmetric stretching (Socrates, 2001). Symmetric stretching of C-O-C appears at 1044 cm^{-1} and the peak at 992 cm^{-1} corresponds to out-of-plane vibrations of aromatic CH and (1→3) glycosiding links (Kyraleou, Pappas, et al., 2015).

Given Spectroscopic analysis of pure ellagic acid in particular, showed a band observed at 1714 cm^{-1} corresponding to C=O stretching, an absorption band observed in the region 1320- 1000 cm^{-1} corresponded to the ester linkage, and another two bands observed at 1614 and 1508 cm^{-1} are due to aromatic ring vibrations (Bulani et al., 2016). The same peaks and bands are found in hexahydroxydiphenic acid spectrum, which is the structural component of ellagitannins, however, ellagic acid was selected in order to avoid confusion with HPLC's total ellagitannin interpretation as ellagic acid equivalents. Previous research on wine samples has shown that most information such as various IR-bands appears in the fingerprint region (Bevin, Fergusson, Perry, Janik, & Cozzolino, 2006). In order to include the most characteristic peaks of ellagic acid found in wine samples, the spectral region selected for the analysis was the region between 1820 and 950 cm^{-1}. In order to enhance the sensitivity, the 1st derivative in range was used as it is useful for revealing peaks that appear as shoulders in the original spectra. In this case, the software measures an estimate for the first derivative at each data point. Although spectroscopy assumes a direct and linear relationship between absorbance and concentration, it is not always possible to collect spectra where this is completely true. Placing the data in another format such as a derivative form often allows the software to better model these relationships.

- **Chemometrics**

Partial Least Squares regression was used for the construction of the calibration model as it is one of the most used models in cases of analyses that contain absorptions to generate equations that best fit reference values of a given data set. The accuracy of the PLS model is determined by their correlation coefficient (R^2), the Root-mean-Square Error of Calibration (RMSEC) and Prediction (RMSEP). In order to build a predictive model for the ellagitannin concentration, the content of ellagic acid determined by HPLC (produced after hydrolysis of the samples) was selected as the calibration variable and then the correlation with the selected spectral region of the wine samples was examined. Correlation with specific monomers would not be applicable in our case as they have not been fully studied, so a structural element of all ellagitannins was selected (ellagic acid/hexahydroxydiphenic acid). Quantification of specific compounds using FT-IR can be achieved easily after spiking samples with known concentration of the compound of interest, provided there can be a noticeable change in the sample's spectrum, as appearance of a new peak, or enhancement of an existing one. However, in complex matrices, selecting a spectral band or a set of spectral bands instead of specific peaks has proven to be equally helpful for quantification purposes (Mallah et al., 2015). The samples aged in Acacia barrels were not considered in this study as their ellagitannin content was below the limit of detection. Although samples used in Chestnut barrels would provide more data points in the linear regression and could potentially provide better calibration results for samples with higher ellagitannin concentrations, they were ultimately excluded as well. The predictive models built with the inclusion of Chestnut samples, showed poor predictive ability and low correlation coefficients (R^2), indicating that the relationship between calculated and actual values was far from linear (data not shown). Chestnut barrels may be exceptionally rich in ellagitannins, however they also have been found to contain ellagic acid derivatives such as ellagic acid deoxyhexose, dehydrated ellagic acid dimer and valoeic acid dilactone (Sanz et al., 2010), the ellagic acid moiety of which can also be recorded in the samples' spectra thus interfering with the results.

The final model was built not taking into account the grape variety as a variable (i.e. the models were not constructed by variety) in order to obtain data that can be applied regardless of the type of grape used for vinification. In this experiment, the model's correlation coefficient (R^2) was found 0.93, while the Root-Mean-Square Error of Calibration RMSEC was 1.17 and the Root-Mean-Square Error of Prediction, RMSEP was 1.57 (Fig. 19). The proximity of RMSEC and RMSEP values further highlights the performance of the model. Calibration and prediction values for all models as well as Predicted Residual Error Sum of Squares (PRESS) are given as supplementary material (Table S5, Fig. S5 respectively). To build the model 69 samples were used (four where excluded as outliers), four of which were used for validation purposes. The Root Mean Square Error of Cross Validation RMSECV was 2.85 for the 14 factors used to build the model.

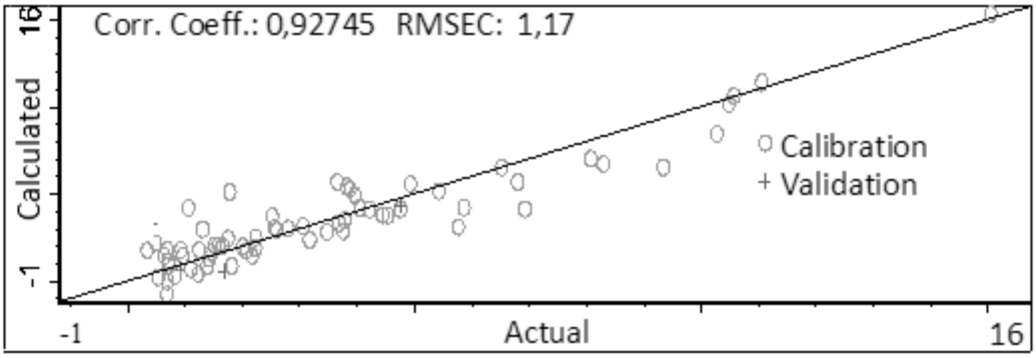


Figure 19. Correlation between the actual total ellagitannin and the calculated values by the constructed model.

To further examine the reliability of the model, an extra set of samples, not included in calibration or validation standards sets, was employed. The set contained six samples from Vilana and Mandilari wines aged for 12 months in tanks with oak chips and in American and French barrels. All sample's spectra were recorded by FT-IR and subsequently they were uploaded to TQ analyst in order to calculate their ellagic acid concentrations using the model built. For comparison reasons, all wine's ellagic acid concentrations were also determined by HPLC.

Table 9. External validation set results (Actual: ellagic acid concentration measured by the HPLC method, Calculated: ellagic acid concentration calculated by the model)

Sample ID	Variety	Container	Aging time	Actual (mg/L)	Calculated (mg/L)	Difference (mg/L)
B2B12	Vilana	Oak chips	12 months	2.17	2.17	0
B3B12	Vilana	American oak	12 months	2.8	2.41	- 0.39
B5B12	Vilana	French oak	12 months	6.66	1.85	- 4.81
M2B12	Mandilari	Oak chips	12 months	2.76	4.33	+ 1.57
M3B12	Mandilari	American oak	12 months	3.26	4.55	+ 1.29
M5B12	Mandilari	French oak	12 months	4.48	3.43	- 1.05

As can be seen in Table 9, the actual (by HPLC) and calculated (by the model) values show small deviations, with the highest recorded in the Vilana sample that aged in French oak. The differences for the rest of the samples are in the range of 0-1.57 mg/L. These results highlight the potential of FT-IR for the estimation of total ellagitannin concentration. It should be noted however, that when wines bought from the market were analysed, estimation rates were lower, as IR models require either large training sets, or training sets that are representative of the samples that are to be analysed, i.e. same geographical region or grape variety (data not shown). To our knowledge, this is the first study to show a predictive model regarding the ellagitannin content of wines by FT-IR.

- **Conclusions**

Ellagitannin concentrations in barrel aged wines are of high interest for the wine industry as they not only influence its sensory properties but also take part in the formation of new compounds. Their levels vary depending on the type of wood used, its geographic origin and the time of contact between wine and wood. Analytical methods for their identification and quantification are cost effective and laborious, and ellagitannin monomers have not been fully studied and characterized, and thus calibration standards of high purity did not exist. FT-IR appeared to be a promising technique for the determination of total ellagitannin concentration in samples aged in different types of oak, although it is not so efficient for samples aging in Chestnut barrels probably due to the presence of ellagic acid derivatives. However, it can estimate accurately the ellagitannin content of wine samples aged in different French and American barrels or being in contact with oak sticks which are the most common woods used in wineries and for contact times ranging from three to twelve months. In addition, no sample pretreatment or solvents are required minimizing this way the environmental issues regarding industrial chemical waste, while FT-IR analysis itself is faster, less costly, and non-demanding of specialized personnel compared to using an HPLC. The ease of using a spectrophotometer and the range of accuracy of the results could also assist winemakers for the in-situ monitoring of ellagitannin extraction rates during aging at the winery.

- **Supplementary Information**

Table S5. Actual and predicted values of ellagic acid for wine samples

Sample	Usage	Actual	Calculated
1	Calibration	4,76	4,12
2	Validation	0,91	0,62
3	Calibration	0,7	-0,01
4	Calibration	6,93	4,14
5	Validation	0,42	3,27
6	Calibration	0,69	1,78
7	Calibration	1,59	2,08
9	Calibration	0,92	1,81
10	Calibration	1,38	0,82
11	Calibration	3,96	4,93
12	Calibration	1,1	0,69
13	Calibration	3,17	2,39
14	Calibration	2,23	2,52
17	Validation	1,69	0,49
18	Calibration	1,06	4,23
19	Calibration	1,81	0,88
20	Calibration	0,52	0,21
23	Calibration	0,96	1,45
24	Calibration	1,51	2,05
25	Calibration	0,34	1,77
26	Calibration	1,23	0,43
27	Calibration	4,54	3,77
30	Calibration	0,82	0,27
31	Calibration	1,78	5,13
32	Calibration	0,69	-0,77
33	Calibration	1,31	2,98
34	Calibration	0,71	1,13
36	Calibration	1,41	1,31
38	Calibration	10,49	10,21
39	Calibration	0,71	0,92
40	Calibration	4,45	3,79
41	Calibration	2	1,97
45	Calibration	3,68	3,32
50	Calibration	3,06	3,15
51	Calibration	6,81	5,71

52	Calibration	2,06	1,74
53	Calibration	0,48	2,19
54	Calibration	1,65	2,04
55	Calibration	5,76	3,12
58	Calibration	0,64	1,42
59	Calibration	3,65	5,72
60	Calibration	10,57	10,62
61	Calibration	3,75	2,85
62	Calibration	2,16	1,42
63	Calibration	2,52	3,71
64	Calibration	2,23	1,92
65	Calibration	3,82	5,42
67	Calibration	3,88	5,3
68	Calibration	2,55	3,07
69	Calibration	3,47	2,81
72	Calibration	5,42	5,17
76	Calibration	8,3	6,72
77	Calibration	9,33	6,53
78	Calibration	3,78	3,52
79	Calibration	2,61	2,95
80	Calibration	5,86	4,24
88	Calibration	15,05	15,33
89	Calibration	4,93	5,57
91	Calibration	1,25	1,8
95	Calibration	8,07	7,04
96	Calibration	4,04	4,19
97	Calibration	11,05	11,39
98	Calibration	1,75	2,46
100	Calibration	2,8	3,02
104	Calibration	4,22	4,12
109	Calibration	6,52	6,54
112	Calibration	1,45	1,62
113	Calibration	10,28	8,48
114	Validation	4,76	4,32

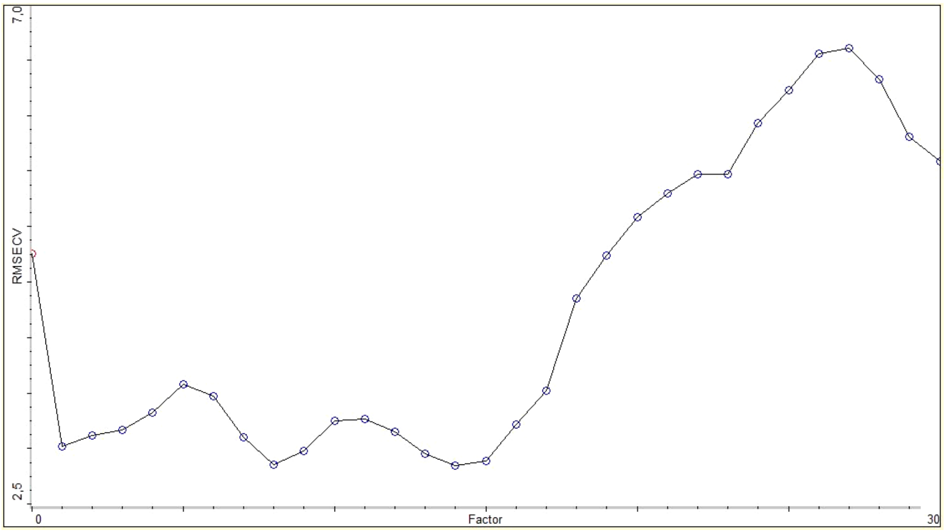


Figure S5. Predicted Residual Error Sum of Squares (PRESS) values and Root Mean Square Error of Validation (RMSECV) for ellagic acid prediction.

iv. Proanthocyanidin content as an astringency estimation tool and maturation index in red and white winemaking technology

Introduction

Wine astringency has been an ongoing research topic over the last decade as it is related to wine quality and consequently to consumer preference. It has been described as a “drying, roughing or puckering sensation” elicited by phenols present in wines, more specifically by tannins (Gawel, Iland, & Francis, 2001).

Tannins are a large group of compounds that share certain characteristics such as the ability to precipitate proteins, an attribute to which they owe their name. They can be further separated to two main groups, hydrolysable tannins and condensed tannins. Hydrolysable tannins are oligomeric derivatives of gallic and ellagic acid, and condensed tannins are dimmers or polymers with C-C bonds between flavan-3-ol subunits (Benaiges & Guillén, 2007). In grapes, only condensed tannins can be found, however in wine both groups are present since hydrolysable tannins are extracted into wine from oak during the course of barrel aging. Between condensed tannins, also referred to as proanthocyanidins, the most important ones are flavan-3-ols (catechins) and their condensed forms (+)-catechin (C), (-)-epicatechin (EC), (+)-gallocatechin (GC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG) and (-)-epigallocatechin gallate (EGCG) (Benaiges & Guillén, 2007), all of which can be used as subunits during polymer formation. Although these forms as well as the biosynthesis of flavan-3-ols itself are well studied, the mechanism of polymer formation is still unknown (Downey, 2010).

Condensed tannin polymers have a terminal subunit and a series of extension subunits linked by interflavanoid bonds (Hanlin, Hrmova, Harbertson, & Downey, 2010). In this skeleton, according to most studies, the main terminal subunit usually is (+)-catechin and the extension subunit (-)-epicatechin (Downey, 2010). As for the length of the polymer found in grapes, it ranges between five to forty subunits, depending on the source of the tannins, i.e. seed or skin tannins (Hanlin et al., 2010).

Tannin concentration, as well as polymer composition and polymer size are factors of great importance for the winemaker as they have a serious impact on wine mouthfeel. For example, as the polymer chain becomes longer, astringency increases (Vidal et al., 2003). The same happens when the molecular weight of tannins increases, along with their ability to bind with proteins however molecular weight alone is insufficient to characterize proanthocyanidins regarding their influence on astringency (Chira et al., 2012).

In order to fully study this group, the degree of polymerization (DP), which corresponds to the average number of flavan-3-ol subunits that compose the tannin chain, was introduced (Guyot, Le Guernevé, Marnet, & Drilleau, 1999). The most successful method for this analysis has been the depolymerization method which consists of treatment of tannins with acid in the presence of a nucleophile such as phloroglucinol and consequent release of terminal and extension subunits (Herderich & Smith, 2005). This way the subunit profile can be revealed with the help of High Performance Liquid Chromatography (HPLC) analysis and structural characteristics of the proanthocyanidins as the mean degree of polymerization (mDP) and the type of individual subunits can be determined.

This method has shown that tannins originating from grape seeds have short chain length, with a high percentage of subunits consisting of gallic acid esters (ECG subunits), hence with higher "degree of galloylation" (%G) (Herderich & Smith, 2005). Moreover, mDP correlation with astringency sensation, has shown that polymeric procyanidins with a high mDP present higher astringency compared to oligomeric ones that have lower mDP (B. Sun et al., 2013). Additionally, absolute mDP values have been helpful in characterizing wines not only according to their astringency, but also according to their aging status. Chira and coworkers (2012), after analyzing 23 vintages of Cabernet Sauvignon, came to the conclusion that wines with an mDP between 2 and 4 are characterized mellow and slightly astringent, while wines with mDP higher than 4 are perceived as more tannic. Higher mDP also correlated with younger wines (Chira et al., 2012). However, mDP can give no information on the structure of the chain (sequence of the monomers) and it cannot discriminate between tannin mixes, for example a mix consisting of equal parts of tetrameric and octameric tannins can result in the same mDP as a sample of hexameric tannins (Herderich & Smith, 2005). Finally, there has been some debate on whether mDP results should be accompanied with more information on interflavonoid linkages and conversion yield (conversion of proanthocyanidins to known subunits) (Jorgensen, Marin, & Kennedy, 2004).

Tannin polymerization in wine primarily reflects its tannin composition, which itself is influenced by many factors such as grape variety, viticultural practices, climatic conditions, winemaking practices etc (Chira et al., 2012). Regarding variety, many differences have been documented in the amount and structure of skin and seed tannins of different grape varieties (Asproudi et al., 2015), and as for the impact of winemaking conditions it should be mentioned that red wines contain about 20 times the procyanidin content of white wines (Wamhoff & Gribble, 2012).

Most changes on the degree of polymerization take place during wine aging when many reactions between phenolic compounds occur. These are favored by the presence of oxygen that is diffused into wine through the pores of barrels. It can affect polymerization by leading to acetaldehyde formation, which in combination with procyanidins, can form ethylidene-bridged copolymers (Chira et al., 2012). Tannins extracted from the barrel have also been found to alter condensed tannin size and inductively, mDP (Watrelot et al., 2018).

Numerous reports have been made regarding the mDP of grape tannins, and few have dealt with mDP of tannins in bottle aged wines or wines made with different winemaking protocols (Gonzalez-Manzano, Santos-Buelga, Perez-Alonso, Rivas-Gonzalo, & Escribano-Bailon, 2006; B. Sun, Spranger, Roque-do-Vale, Leandro, & Belchior, 2001). However, there are no reports on the influence of different types of barrel on the evolution of proanthocyanidin composition in wines during maturation. Winemakers have been experimenting with barrel woods other than oak for wine maturation in order to minimize the enhancement of astringency, which is evident especially as far as white wines are concerned. White wines are characterized by low phenolic concentrations and changes in their tannin content can dramatically affect their mouthfeel and final quality. In this study, changes on proanthocyanidin mDP and composition of the wines which stayed in contact with different types of barrel woods for different time periods were monitored. The aim of this project was to understand how to select the appropriate barrel but also to optimize the maturation length based on astringency evolution, which in turn would lead to the production of high-quality wines

Materials and Methods

- **Wines and containers**

Wines from two red (*Vitis Vinifera* cv Kotsifali and Mandilaria) and two white (*Vitis Vinifera* cv Vilana and Dafni) grape varieties differing greatly in their tannin composition were chosen to mature in barrels made of French and American oak, Acacia and Chestnut, woods that in turn also have been found to present differences in their ellagitannin content (Basalekou, Pappas, Tarantilis, et al., 2017). For comparison reasons, a portion of each wine was kept in inox tanks as a control sample. In order to examine the differences of wood size, inox tanks with French oak sticks immerged in them were also employed. Chestnut was only used in the case of red wines. Vinifications were carried out during the 2013 vintage and samples were taken from containers every three months starting right after fermentation completion. For the production of red and white wines, the traditional red and white winemaking protocol -respectively- was followed. All analyses were carried out in triplicate. French (Quercus robur) and American (Quercus alba) barrels (225 L) with a medium toasting were purchased from

'Tonnellerie du Monde World Cooperage', Acacia barrels with a light plus (L+) toasting were purchased from Tonnellerie du sud ouest, and Chestnut barrels with a medium toasting were purchased from Tesias Metsovo, Greece. The oak sticks (French oak) were from Seguin Moreau (Oenostick®, V18).

- **Sensory Analysis**

Astringency was assessed by a trained panel consisting of 11 wine experts. Two repetitions of each sample were required, and red and white wines were evaluated in different tasting sessions, with each session furtherly divided into two sets according to the grape variety used in vinification. The judges were winemakers and enologists from the Wines of Crete network. The selection criteria were their experience in wine sensory assessment, however they were all trained in order to familiarize themselves with the term of astringency but also with the intensity scale (Chira et al., 2012). For this reason, aqueous solutions of aluminum sulfate (3 g/L) were prepared. After establishing identification of sensation, scaling training was employed, using model wine solutions containing different concentrations of the training substances (Chira & Teissedre, 2013b). The judges were presented with 10 mL samples at room temperature and were asked to rate the intensity of astringency using a 0-10 point scale. Breaks were taken between samples, during which time the panelists could wash their mouths with water in order to minimize the risk of carry over effects.

- **Proanthocyanidin Composition**

Proanthocyanidin composition was determined following acid catalysis with phloroglucinol, as described by Chira *et al.*, 2012. Phloroglucinolysis is based on the acid-catalyzed cleavage -in the presence of phloroglucinol- of flavan-3-ol interflavanoid bonds, after which the terminal unit is released as a flavan-3-ol monomer and C4 phloroglucinol adducts of the extension units are formed (Drinkine, Lopes, Kennedy, Teissedre, & Saucier, 2007). According to the method, 10mL of wine (50mL in the case of white wines due to their low phenolic content) were evaporated in vacuum under a low pressure, in a temperature of 40°C in order to remove excessive alcohol. The wine that remained (approximately 4mL) in the evaporator flask was resuspended in water, making up a final volume of 20mL. For proanthocyanidin isolation this solution was passed from C-18 SPE cartridges (Lichrolut C18, 5gr octadecyl bonded endcapped silica, 25mL volume), which were initially activated with 25mL methanol and 25mL distilled water. After adding the diluted wine extract, the cartridge was washed with 50mL of distilled water and left to dry for 15 minutes. The elution of the compounds of interest was performed by adding 50mL methanol. In order to obtain dry powder, the elutes were further evaporated under reduced pressure at 30°C and then lyophilized. Finally, tannin extracts were re-dissolved in methanol to a final volume of 20g/L. To perform phloroglucinolysis, 100μL of the redisolved solution were left to react with 100μL of phloroglucinol solution (50g/L phloroglucinol, 10g/L ascorbic acid, 0.1N HCL, all in methanol) at 50°C for 30 minutes. The reaction was then stopped by adding 1mL sodium acetate 40mM. All solvents and reagents used were of high-performance liquid chromatography grade, purchased by Sigma Aldrich (St Louis, MO, USA).

Reaction products were analyzed by a Shimadzu 2010A LC/MS (Shimadzu corporation, Tokyo, Japan) operating in positive ion mode, coupled to a single quadrupole mass spectrometer equipped with an electrospray ion source (Kyraleou, Kallithraka, Koundouras, et al., 2015). The source's temperature was 70°C, the cone voltage at -30eV, and capillary voltage was at 3.5kV. Absorbance was recorded at 280nm and mass spectra in the range of 50-1500 amu. Separation was performed on a reversed-phase XTerra RR C18 (100 x 4.6mm, 3.5μm) column (Waters, Massachusetts, USA), with a 20μL injection volume at a flow rate of 0.5mL/min, and the elution program was as described by Petropoulos *et al.*, 2017. All analyses were performed in triplicate. All flavan-3-ol monomer, terminal subunits, and phloroglucinol adducts, extension subunits, were expressed in moles.

Calculations:

mDP = SUM of all subunits (terminal and extension) / SUM of all terminal subunits

Percentage of prodelphinidins (%P) =100 x (SUM of terminal and extension EGC subunits / SUM of all terminal subunits)

Percentage of galloylation (%G) = 100 x (SUM of terminal and extension ECG subunits / SUM of all terminal subunits)

(%C) = 100 x (SUM of terminal and extension C subunits / SUM of all terminal subunits)

(%EC) = 100 x (SUM of terminal and extension EC subunits / SUM of all terminal subunits)

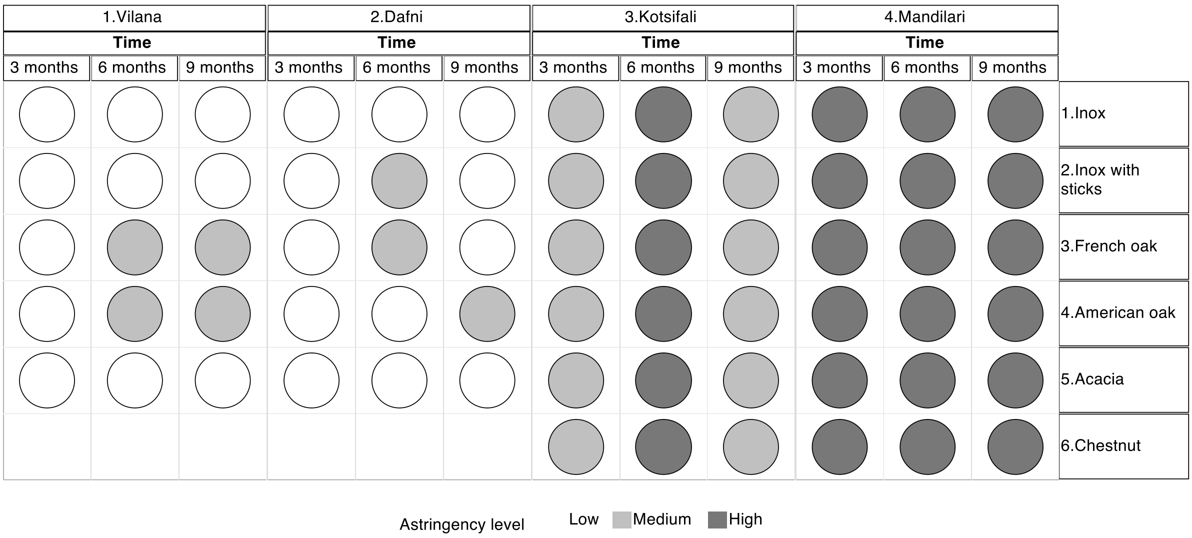
- **Statistical Analysis**

Statistical analysis was performed using the JMP Statistical Discovery software, version 11. For mDP, %G, %P, %C, %EC and sensory analysis results, mean values and standard error were calculated, and analysis of variance (ANOVA) was performed, using Tukey's comparison tests when samples were significantly different ($p < 0.05$). ANOVA was also used to examine the possible interaction effects between the variables of this experiment. In order to determine whether sensory analysis results could be predicted using the results obtained by chemical analysis, Partial Least Squares (PLS) regression was used. PLS regression fits linear models based on linear combinations (factors) of the explanatory variable, obtained in such a way that maximizes the covariance between the explanatory variables.

Results and Discussion

- **Sensory Analysis**

A graphical representation of the results of the sensory analysis carried out during this study is given in Figure 20.



1.Vilana			2.Dafni			3.Kotsifali			4.Mandilari			
Time			Time			Time			Time			
3 months	6 months	9 months	3 months	6 months	9 months	3 months	6 months	9 months	3 months	6 months	9 months	
○	○	○	○	○	○	◍	●	◍	●	●	●	1.Inox
○	○	○	○	◍	○	◍	●	◍	●	●	●	2.Inox with sticks
○	◍	◍	○	◍	○	◍	●	◍	●	●	●	3.French oak
○	◍	◍	○	○	◍	◍	●	◍	●	●	●	4.American oak
○	○	○	○	○	○	◍	●	◍	●	●	●	5.Acacia
						◍	●	◍	●	●	●	6.Chestnut

Astringency level Low ◍Medium ●High

Figure 20. Astringency levels (low, medium, high) according to variety, type of container and time

As expected, wines from white grape varieties were perceived as low to medium astringent, while red wines were more astringent (medium to high). In agreement with the findings of Kallithraka et.al., (2011), Mandilari wines were the most astringent wines tested. On the other hand, Kotsifali wines, which are characterized by their low anthoycanic and tannin content (Basalekou, Pappas, Tarantilis, et al., 2017), have been described as medium astringent with the exception of the wines maturing for 6 months which were perceived as highly astringent. As it can be seen in Figure 20, the type of the container of the red wines did not result in perceived astringency differences by the panel for all sampling periods. A possible explanation is that astringency of red wines was already high enough that any further increase or decrease could not be easily detected. However, in white wines, the influence of the type of barrel wood on perceived astringency is evident. Interestingly, the only type of container (with the exception of the inert inox tank) that does not seem to influence astringency of white wines, even after 9 months of contact, is Acacia barrel.

- **Mean Degree of Polymerization**

In order to examine if there is a relation between astringency and mDP, the mDP values of all wines were recorded every three months for a total period of nine months during maturation. In Figure 21, mean values regardless of the type of container used during maturation can be seen.

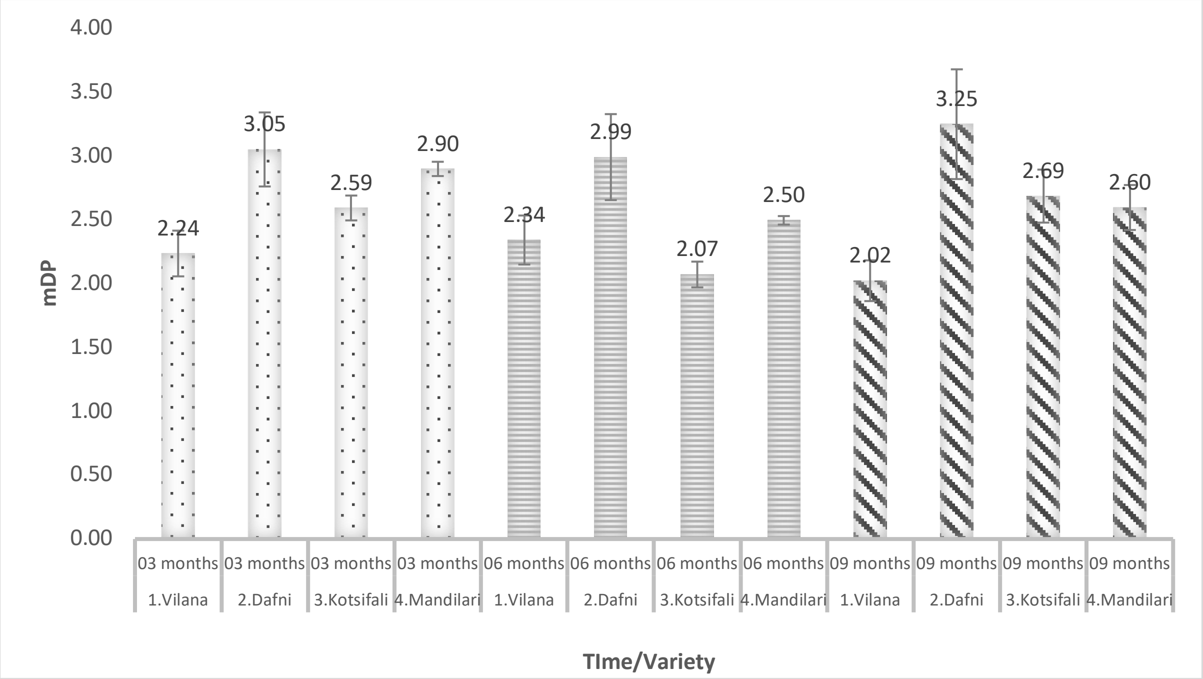


Figure 21. Mean Degree of Polymerization (mDP) during barrel maturation according to the variety and time of contact (3,6, or 9 months after fermentation). The mDP values are presented as the average value of all wine samples of each variety independently of the type of container used during maturation.

It appears that mDP values of white and red wines are quite similar, however there are not any data available concerning white wine mDP values in order to compare with the data obtained in this study. In average, the lowest mDP values recorded were 2.02 and 2.07 (for Vilana and Kotsifali respectively), while the highest values obtained were 3.25 and 3.05 (for Dafni during 9 and 3 months of maturation respectively).

According to the literature, white wine phenolics are characterized by their ring structure and can be found in different forms based on their patterns of hydroxylation, esterification, glycosylation or conjugation with amino acids (Gawel, Smith, Cicerale, & Keast, 2017). They are mostly monomeric; however, dimmers and trimers can also be found in lower concentrations. As short chained groups, they are characterized by a low degree of polymerization, with the dominant flavanols being (+)-catechin and its stereoisomer (-)-epicatechin (Gawel et al., 2017).

As richer in tannins, red wines would be expected to contain tannins with higher mDP values than white wines. Rinaldi et al., (2015) observed a significant influence on wine astringency based on the tannin extraction method and grape variety. Red wines are richer in total tannins than white wines, due to the maceration process during fermentation where tannins are extracted from the grape skins and seeds to the must. Maceration for white wines is not so common; however, there are cases where it takes place but usually before alcoholic fermentation begins and for a much shorter period resulting in wines with lower total phenolic concentrations (Gawel et al., 2017). It is possible thus, the astringency of white wines to be similar with that of red wines when tasted at equal tannin concentrations. However, this is not the case since red wines are much richer in tannins compared with white wines. Gonzalez-Manzano et al., (2006) also reported that if the maceration period is not long (as in the traditional red winemaking which was followed for the production of the red wines of this experiment), mostly monomers to trimers can be extracted by the grapes, resulting in an mDP value of 2.3, which agrees with the findings of our research.

According to literature, mDP values of red wine have been reported to range from 4.9 to 9.8 for Cabernet Franc and Sangiovese wines respectively, while analysis of different vintages of Cabernet Sauvignon revealed a range from 1.8 (vintage 1978) to 7.6 (vintage 2004) (Chira et al., 2012; Gris et al., 2011). Moreover, Carmenere wines had mDP values that ranged from 7.4 to 13.6 during 2004 and 2006 vintages, while, reported mDP for Tinta Miuda red wine was 22.1 (Mateus, Oliveira, Santos-Buelga, Silva, & De Freitas, 2004; B. Sun et al., 2001). However, recent research regarding an indigenous Greek red variety (Agiorgitiko) reported lower mDP values, ranging from 1.43 to 2.27 (Petropoulos et al., 2017). Since, according to the literature, this parameter is characterized by high heterogeneity it could hardly be considered as an index to be used for characterization or classification purposes. Indeed, mDP has different values depending on the grape variety, the vintage but also the winemaking technique used (for example stem contact or not, carbonic maceration or classic vinification) (Chira et al., 2012; B. Sun et al., 2001). Recently, the mDP values of white wine lees (Chardonnay) were determined and they were found to be much higher than the respective values of the red wine lees, even though the initial total phenolic content was lower (Zhijing, Shavandi, Harrison, & Bekhit, 2018) suggesting that a categorization of red and white wines according to their mDP values might not be possible. In accordance with the findings of Zhijing et al., (2018), in this experiment the highest mDP value was recorder for a white wine (Dafni), while this variety also exhibited the highest variation in mDP values depending on the container used during maturation.

To verify if there is a relation between mDP and astringency, statistical analysis was performed. Contrary to the results reported by some authors (Chira et al., 2012; Chira, Pacella, Jourdes, & Teissedre, 2011; Vidal et al., 2003) but also in agreement with other studies (Kyraleou et al., 2016) in this experiment astringency and mDP levels do not seem to correlate as shown in Figure 22a (R^2=0.0085 and Root Mean Square Error=1.82, p=0.463).

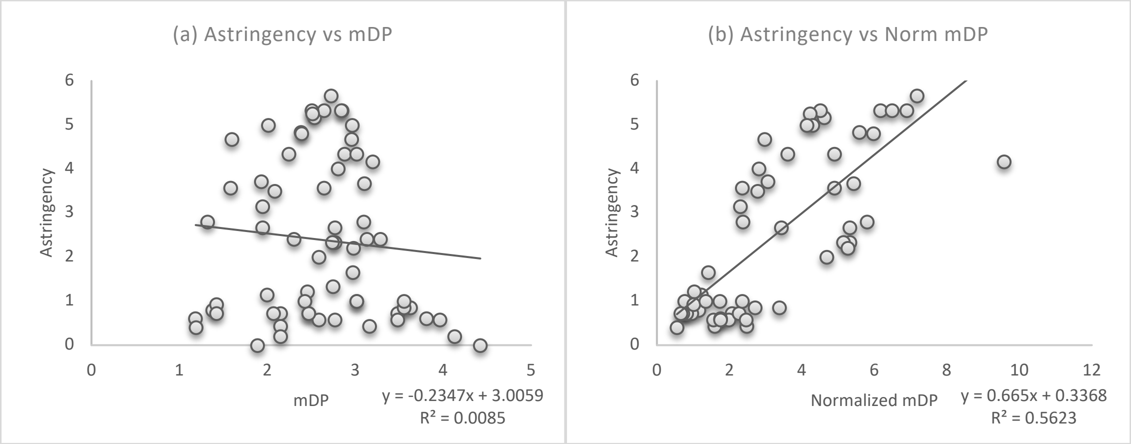


Figure 22 a and b. Correlation between Astringency and mDP values (a) and Astringency and Normalized mDP values (b).

- **Procyanidin composition**

Since mDP is a value calculated as a ratio between the sum of all subunits and the sum of all flavan-3-ol monomers, it does not take into account tannin absolute concentration. Indeed, in this experiment the wine volume used for the analysis of white wine tannin mDP was five times higher than that used for the respective analysis of red wines (50 mL instead of 10 mL used for the analysis of red wines) in order to obtain measurable results.

As mentioned earlier, there has been some debate on whether procyanidin structural characteristics (conversion of proanthocyanidins to known subunits) should accompany mDP results (Jorgensen et al., 2004). Since the proanthocyanidin content in wines has also been found to correlate with astringency (Cáceres-Mella et al., 2013), statistical analysis was performed for a second time, after normalizing mDP values according to each wine's total proanthocyanidin content. For this purpose, the pure weight of the tannins collected after the lyophilization step expressed in milligrams per litre was used as a multiplication factor for the actual mDP values (Normalized mDP = proanthocyanidin (mg/L) * mDP/1000). The results obtained revealed a significant correlation with astringency since they also take into account the phenolic content of the wines (Figure 22b). The resulting equation is the following: Astringency = 0.3368 + 0.665*Normalized mDP, however with a still low R^2 of 0.56 and RMSE=1.21 (p<.0001) suggesting that more information is necessary in order to construct a model based on mDP, which can explain and predict wine astringency.

Moreover, it is important to determine the individual tannin subunit composition of each variety irrespectively of the effect of the container in order to better understand the evolution of tannin structure during maturation and/or aging. For this reason, the percentages of the individual subunits detected in each control wine maturing in inox tanks are shown in Figure 23.

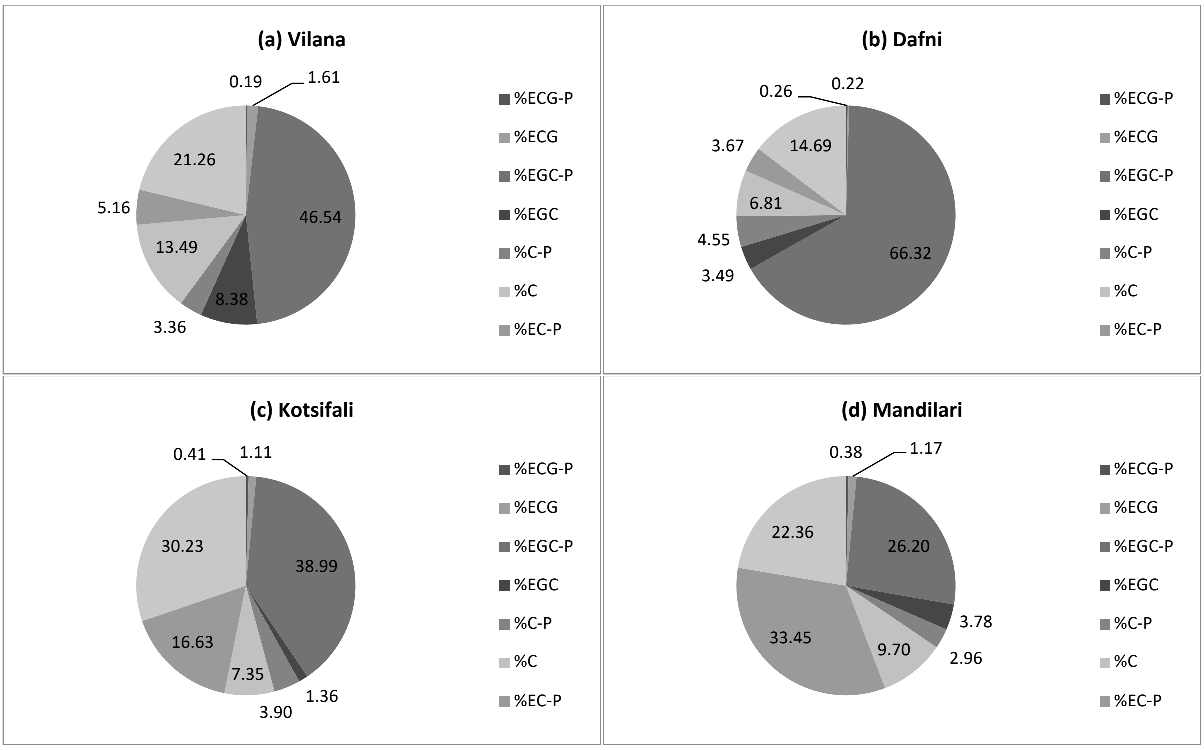


Figure 23. Percentage of monomer subunits (terminal or extension) per variety for control wines (in inox tanks) 3 months after the end of fermentation (ECG-P, EGC-P, C-P, EC-P: extension subunits, ECG, EGC, C, EC: terminal subunits).

As mentioned earlier, tannin polymers are mainly composed of C, EC, GC, EGC, ECG and EGCG subunits. The most prominent difference between the four varieties studied is that for the white varieties Vilana and Dafni, and for the red variety Kotsifali the dominant subunit is EGC, whereas in red variety Mandilari the dominant one is EC. (-)-Epicatechin has been found to be significantly more bitter and astringent than C (S. Kallithraka, Bakker, & Clifford, 1997), while the presence of EGC subunits in proanthocyanidins has been reported to reduce the "coarse" astringent sensation and to be negatively correlated with astringency (Kyraleou et al., 2016; Vidal et al., 2003). (-)-Epicatechin subunits in extension positions, like in the case of Mandilari wines, have also been characterized remarkably important in affecting astringency (Quijada-Morín et al., 2012). These findings are in agreement with the results of the sensory analysis, according to which the most astringent variety, was Mandilari.

The percentages of the subunits detected in white and red wines according to the type of the container and irrespectively of the sampling time are shown in Figure 24 (a and b).

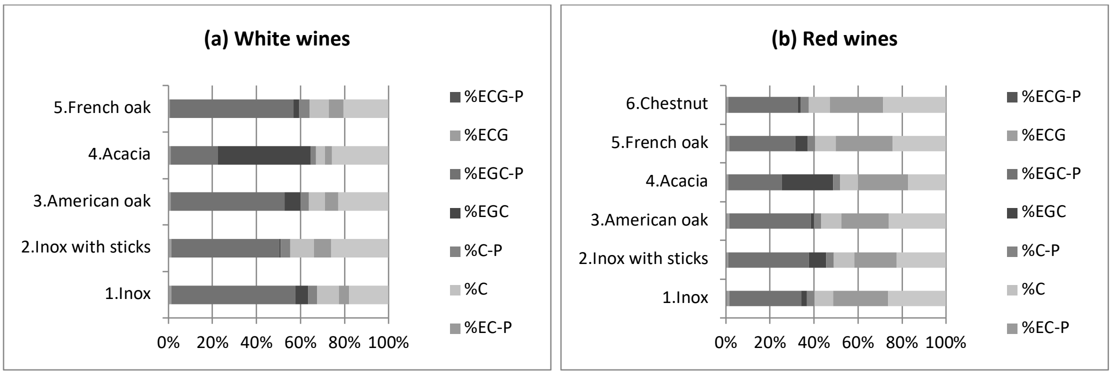


Figure 24 a and b: percentages of monomer subunits according to the type of container as an average for all samplings (3,6 and 9 months in barrel) for white (a) and red (b) varieties.

During maturation, white wine tannins mainly consist of EGC (terminal or extension) subunits, while the highest percentage was observed in wines maturing in Acacia barrels (Sum of extension and terminal EGC subunits: 63.9%) (Figure 24 a and b). In red wines, EC is the dominant monomer except for the case of Acacia barrels and inox tanks with oak sticks, where the dominant one is EGC.

Acacia barrels and Inox containers with sticks seem to be the only containers among all others of the experiment, where EGC subunits are present in higher percentages between wine tannins in both red and white wines. Previous research reported that the occurrence of terminal EGC subunits could reduce precipitation with salivary proteins (Ricardo-da-Silva et al., 1991) , while Quijada-Morín et al., (2012) reported that the amount of EGC in both extension and terminal positions is negatively correlated with the perceived astringency. Recent research on tea tannins reported that EGC is the less astringent than ECG and EGCG (Xu et al., 2018).

According to Figure 24 (a and b), the type and size of wood used during maturation influences the percentage of monomer subunits. In order to examine which of the parameters studied in this experiment exerted the major effect on mDP, Analysis of Variance (Supplementary Table

S2) was performed. It was revealed that although mDP was significantly affected by experimental parameters (variety, type of container and time) the type of container had the highest influence. Moreover, the contact time had the greatest effect on the proanthocyanidin content.

Indeed, the effect of the variety on proanthocyanidin content is decisive since it determines the concentration and type of tannins found in the wine, and thus their polymerization degree. Regarding time, it is well documented that the extraction of phenolic compounds depends on the time of contact, while their content has been found to also to vary as in relation to oxidation, polymerization but also condensation reactions which are favored by the oxygen diffused into the barrels (Rubio-Bretón, Garde-Cerdán, & Martínez, 2018). The type of container on the other hand, also influences proanthocyanidin content, as different types of wood enrich the wine with different types of tannins, also affecting mDP values (B. Zhang et al., 2015). Total flavonoid content in wines maturing for nine months in barrels of acacia, chestnut and oak, showed different evolution patterns. Wines in contact with oak were characterized by the highest concentrations while those in contact with chestnut by the lowest. Moreover, chestnut has been found to be rich in gallotannins and to contain high amounts of phenolic compounds (B. Zhang et al., 2015). Changes in proanthocyanidin concentrations of wines aging in oak barrels have also been monitored by Watrelot et al., (2018), who reported that EC extension and terminal units decreased, while EGC (extension) and mDP increased.

- **General discussion**

In order to examine if the percentage of monomers determined after tannin phloroglucinolysis could provide a more accurate estimation of astringency, Partial Least Squares Regression (PLS) analysis was performed. This type of analysis takes into account all proanthocyanidin subunits and is particularly useful when there are variables that are highly correlated. PLS (NIPALS method) uses the van der Voet T2 test and the leave-one-out validation, after which the optimal number of factors chosen for our experiment was 4. The prediction equation provided by the statistical analysis was the following:

Astringency= [-0.125 * (“%ECG-P”)] + [0.158 * (“%ECG”)] + [- 0.019 * (“%EGC-P”)] + [-0.024 * (“%EGC”)] + [- 0.404 * (“%C-P”)] + [-0.035 * (“%C”)] + [0.109 * (“%EC-P”)] + [-0.065 * (“%EC”)] + 4.921.

Using the equation, the values for the “predicted astringency” were calculated for all samples and after, both predicted and actual values were used for correlation purposes (Figure 25).

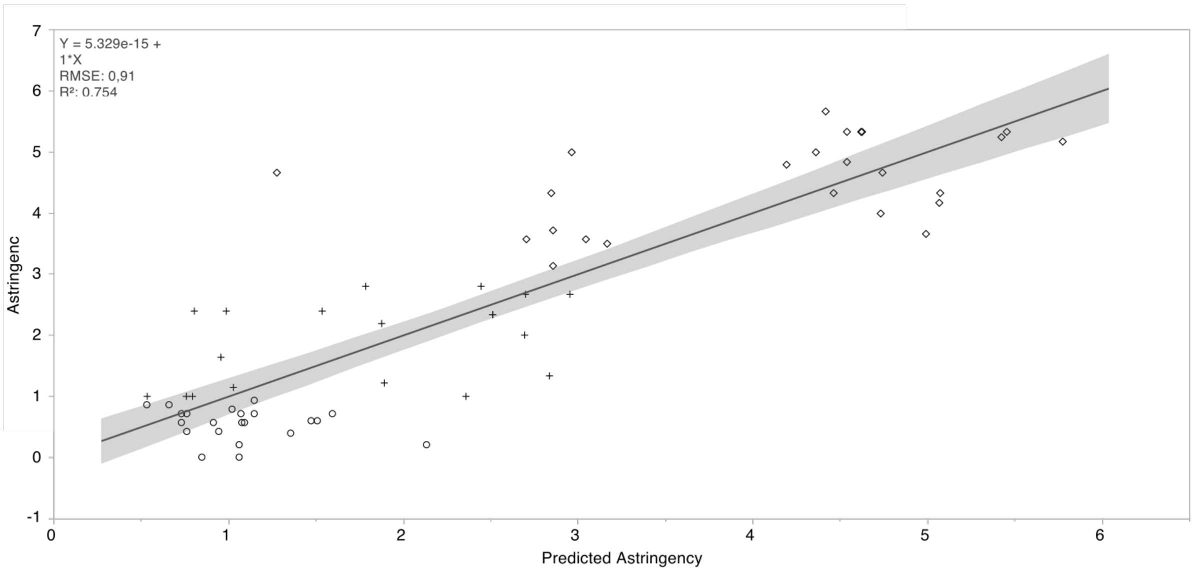


Figure 25. Predicted versus Actual Astringency results, based on the results of PLS regression analysis.

According to Figure 25, the subunit percentages provided a better model for the prediction of astringency, with R2=0.754 and Root Mean Square Error=0.91 ($p<0.01$). According to our knowledge, this is the first time that a model for the prediction of astringency is provided using both red and white wines in the calibration set, and which could be used as an index during wine maturation.

According to previous works, the intensity of astringency is related to total concentration of grape phenolic compounds, total proanthocyanidin content, their mean degree of polymerization (mDP) and subunit composition (Chira et al., 2012; Kyraleou et al., 2016; Quijada-Morín et al., 2012). However, comprehensive investigations on the relation between grape proanthocyanidin content and composition with wine or grape sensory properties are rather fragmentary and the results are contradictory. Several published articles have examined either the influence of total tannin or phenolic content (Jeffery, Mercurio, Herderich, Hayasaka, & Smith, 2008; S. Kallithraka et al., 2011) or the effect of tannin composition (Quijada-Morín, Williams, Rivas-Gonzalo, Doco, & Escribano-Bailón, 2014; Wollmann & Hofmann, 2013) on grape or wine astringency. However, even when both proanthocyanidin concentration and composition have been examined simultaneously (Chira et al., 2011; Ćurko et al., 2014; Kyraleou et al., 2016; Quijada-Morín et al., 2012) the results are not in agreement. Chira et al., (2011) reported that mDP exhibited a higher influence on wine astringency compared with total phenolic compounds or total tannins. In addition, Kyraleou et al., (2016) demonstrated that astringency is more highly correlated with grape total phenolic and proanthocyanidin content than tannin structural composition, whereas the results presented by Quijada-Morín et al., (2012) showed that astringency in wine is more affected by subunit composition than by the total concentration. The presence of galloyl groups (%G), is also a critical factor for astringency. Nevertheless, controversies have also been reported in the literature regarding this issue. %G values correlated positively with perceived astringency in several studies (Chira et al., 2011; Ćurko et al., 2014) while others either report absence of correlation (Kyraleou et al., 2016; Wollmann & Hofmann, 2013) or negative correlation as in the case of grape seed extracts studied by Chira et al., (2015). Interestingly, in the case of EGC, most of the published data are in agreement that it is negatively correlated with astringency perception (Chira et al., 2015; Kyraleou et al., 2016; Quijada-Morín et al., 2012; Vidal et al., 2003). Therefore, the profile of the monomers composing each polymer should also be evaluated in order to study their possible effects on astringency.

- **Conclusions**

The evolution of mDP during wine maturation and aging is mostly influenced by the type of container used but also by the grape variety used. For this reason, mDP cannot be used as an independent variable for astringency estimation and quality classification of the wines. In this study proanthocyanidin mDP values were not significantly correlated with the perceived astringency. However, when total proanthocyanidin content was also considered, a statistically significant correlation was obtained with astringency. Interestingly, the strongest correlation was observed between astringency and the type of subunits comprising the tannin chains indicating that this might be a valuable tool used for the estimation of astringency during wine maturation in barrels. Additionally, the mDP values of white wines were evaluated for first time, showing that their tannin oligomers and polymers are characterized by different dominant monomers. Finally, the proanthocyanidin subunit composition revealed that the use of Acacia barrels in white wines during maturation did not result in astringency enhancement even when used for longer contact periods (at least 9 months). This finding could provide winemakers with a useful tool when deciding how long but also in what type of barrel wood to mature a wine, red or white.

- **Supplementary Information**

Table S2. ANOVA interaction effects of type of container, time and variety on mDP values and proanthocyanidin content.

mDP	F ratio	Prob > F	proanthocyanidins	F ratio	Prob > F
Container	12.1767	<.0001*	**Container**	0.7249	0.6061
Time	2.2686	0.1080	**Time**	11.9758	<.0001*
Variety	14.2233	<.0001*	**Variety**	2.7926	0.0438*

V. GENERAL CONCLUSIONS AND DISCUSSION

GENERAL CONCLUSIONS AND DISCUSSION

Maturation is a very important process, however there are very few tools to monitor it. It represents a very crucial period for the wine, as it can improve its quality or even deem it unsuitable and faulty. Monitoring is mostly done indirectly through the estimation of key compounds that are affected by it, such as phenolic compounds, or by the evaluation of key sensory aspects such as astringency and aroma. However, none of these compounds or characteristics are suitable for an overall description of the process, as not only each analyte might be suitable for the evaluation only of one specific parameter of maturation, such as the type of container, but also because the types of interactions taking place during maturation are dependent on variables that can not be determined. FT-IR spectroscopy can provide a unique spectrum for each wine, based on the combination of its atoms. Therefore, it is suitable for providing multidimensional information, however without specific insights if not combined with information resulting from chemical analyses. This means FT-IR can be used to build a database of wines and it can group samples based on their most prominent attribute with the help of statistics, however it provides limited information on what compound or characteristic was the basis of the discrimination. Since chemical analyses can provide information on that characteristic, it seems that FT-IR would be optimally utilized, when it is used complimentary to targeted chemical analyses.

Regarding the alteration of the astringency during maturation, many methods have been built to measure it without the need for a tasting panel, however this has been a difficult task as it is not a sensation elicited by one compound. On the contrary, many wine components seem to affect it. Moreover, even though it is directly influenced by the presence of tannins in wine, their concentration cannot be directly correlated to its intensity, as it greatly affected by their structure and size. The mean Degree of Polymerization has been found to correlate with the perceived astringency of wines with various characteristics, which makes it very appealable for use as a tool to control its levels during maturation. The information provided along with the ellagitannin prediction, coupled with FT-IR data could be used for a more complete control of maturation.

In this study, FT-IR spectroscopy was found to be useful in wine discrimination on the basis of variety, the type of container used during maturation, and the time of contact between container and wine, which means it can be used for wine authentication purposes. Poor validation results were obtained in the case of container discrimination, due to high sample variability. However, there was a clear separation of wines maturing in barrels made from

Acacia and Chestnut and wines maturing in oak (French or American, in the form of barrel or sticks) or Inox tanks, thus, FT-IR can be used for the regulatory control of wines, since Acacia is the only wood not currently allowed by the OIV for maturation.

The issue of high sample variability observed during container discrimination, implies that the type of container affects the wine spectra profile less than other parameters, such as variety or time of contact. This obstacle could possibly be overcome, by comparing samples by the same variety, that matured in different containers. However, this was not possible to evaluate during this study, as dividing the samples according to variety, would decrease the number of samples below statistically safe levels.

Regarding wine aging, FT-IR combined with PLS allowed developing regression models to provide approximate quantitative values for chemical age indexes i and ii which are significantly correlated with maturation time and are less dependent to the variety, the vintage or the type of container used for maturation. Linear relationships were found with correlation coefficients (r) 0.93 and 0.91 for the chemical age index i and 0.95 and 0.88 for index ii for Mandilari and Kotsifali samples respectively, with low RMSEC values in each model as well. Chemical indexes i and ii can provide insight on the way a red wine matures, they are not dependent to the type of wine and their estimation does not require the use of sophisticated machinery, it is however quite laborious.

Total ellagitannin concentration on the other hand, is laborious and does require the use of High Pressure Liquid Chromatography. It was also successfully determined in wines maturing in contact with various types of wood, except Chestnut, while Acacia was not found to contain any ellagitannins. However, ellagitannin estimation in different types of oak was accurate. This would provide a very helpful tool for wineries, which mostly utilize oak for maturation purposes, for a fast and on-site evaluation of ellagitannin extraction rates.

The estimation of ellagitannin extraction rates, combined with the estimation of Chemical age i and ii would provide winemakers with a helpful insight on the maturation process.

However, most information on maturation originated from the evaluation of total proanthocyanidin content. Its estimation for a nine-month period, revealed a strong correlation with astringency, especially in terms of subunits comprising the tannin chains. Based on this correlation and with the help of PLS regression an equation predicting astringency was provided, with $R^2 = 0.754$ and RMSE = 0.91. Moreover, white wine mDP values were evaluated for the first time. The proanthocyanidin subunit composition revealed that the optimum type of wood (between the woods of the experiment) for white wine maturation was Acacia wood, as it did not seem to enhance their astringency even after 9 months of maturation.

These findings underline the beneficial use of FT-IR in wine analysis either in terms of predicting astringency sensation, the levels of extracted ellagitannins or wine’s chemical age, but also in terms of authentication. FT-IR is cost effective, simple and rapid, without the requirement of chemical reagents and sample pretreatment steps and its use as a monitoring tool of wine maturation can be recommended.

VI. REFERENCES

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