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Study of phenolic content and antioxidant activity of Greek red and white wines by means of classical methods and FTIR.

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ABSTRACT

Phenolic and antioxidant contents of wines are very important in terms of both flavour attributes and health benefits. Changes occur during ageing of wine in containers (e.g. wooden barrels) in relation to their antioxidant activity and phenolic content. Vilana, Dafni, Kotsifali and Mandilari single variety Cretan wines, were vinificated to determine their antioxidant activity and phenolic content Wines were aged in different containers after two vinifications. Changes in the above characteristics were determined every three months for a twelve month period. Stainless steel with and without oenosticks containers, American oak, French oak, Acacia and Chestnut barrels were used for wine ageing.

As far as phenolic and antioxidant contents are concerned, ageing of wine in chestnut barrels, Kotsifali and Mandilari (red wines) and in Acacia barrels for Vilana and Dafni (white wines), gave the best results, achieving the highest phenolic content and antioxidant activity after 12 months of ageing.

The phenolic fingerprints of Vilana, Dafni, Kotsifali and Mandilari wines were determined for the first time. The phenolic fingerprint of wines has been recently used for the authentication and discrimination of red wines. In this study, attempt has also been made to use the phenolic fingerprint of white wines, for authentication. Differences were observed in Kotsifali and Mandilari (red wines) and in Vilana and Dafni (white wines) directly after vinification, allowing their discrimination. Also the changes in their phenolic fingerprints were monitored during ageing in different containers for a 12 month period.

The effect of hydroxytyrosol and oleuropein on wine spoilage induced by acetic acid bacteria was also determined. Hydroxytyrosol was better than oleuropein in controlling the increase of volatile acidity, causing wine spoilage. Wines treated with 0.5mg/l hydroxytyrosol showed control of volatile acid production, and may be a promising alternative to sulphites in wine production in the future.

Finally, extracts obtained from olive oil mills and winery by-products were used in Vilana vinification instead of sulphites and antioxidant activity and phenolic content of the wines determined.

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Figure 3.55 Titratable acidity of wine B4 5 weeks since the beginning of the essay. All samples were incubated at 30° C. Statistical differences, are indicated by different letters in columns; Wine treatments: C4: Positive control AAB: acetic acid bacteria, H0.5: hydroxytyrosol 0.5mg/l + acetic acid bacteria, H1: hydroxytyrosol 1mg/l + acetic acid bacteria, OL50: oleuropein 50mg/l + acetic acid bacteria, OL400: oleuropein 400mg/l + acetic acid bacteria; OL800: oleuropein 800mg/l + acetic acid bacteria ……………………………………………………….. 103

Figure 3.56 Volatile of wine B4 5 weeks since the beginning of the essay. All samples were incubated at 30° C. Statistical differences, are indicated by different letters in columns; Wine treatments: C4: Positive control AAB: acetic acid bacteria, H0.5: hydroxytyrosol 0.5mg/l + acetic acid bacteria, H1: hydroxytyrosol 1mg/l + acetic acid bacteria, OL50: oleuropein 50mg/l + acetic acid bacteria, OL400: oleuropein 400mg/l + acetic acid bacteria, OL800: oleuropein 800mg/l + acetic acid bacteria ……………………………………………………….……….............…….. 104

Figure 3.57 Titratable acidity of wine B5 1 month since the beginning of the experiment. All samples were incubated at 30° C. Statistical differences, are indicated by different letters in columns; Wine treatments: AAB: acetic acid bacteria, H1: hydroxytyrosol 1mg/l + acetic acid bacteria, OL1: oleuropein 1mg/l + acetic acid bacteria, OL2: oleuropein 2mg/l + acetic acid bacteria ……………………..……......……....... 105

Figure 3.58 Titratable acidity of wine B5 1 month since the beginning of the experiment. All samples were incubated at 30° C. Statistical differences, are indicated by different letters in columns (Duncan's multiple range tests, p = 0.05); Wine treatments: AAB: acetic acid bacteria, H1: hydroxytyrosol 1mg/l + acetic acid bacteria, OL1:

oleuropein 1mg/l+ acetic acid bacteria, OL2: oleuropein 2mg/l + acetic acid bacteria ……………………………………………………..…………..……. 105

Figure 3.59 Total phenolic content of Vilana wines directly after vinification. Different letters indicate statistical significant differences amongst the treatments. C1: control, O1: OMR 55%, 0.875 gr/100l, O2: OMR 55%, 2.67 gr/100l, O3: OMR 95%, 2.7gr/100l, V1: GP 5 gr/100l, V2: GP, 7.5 gr/100l. (GP: grape pomace extract, OMW: olive oil mill waste water residue) ……………………...…………... 106

Figure 3.60 Total antioxidant activity of the obtained Vilana wines directly after vinification. Different letters indicate statistical significant differences amongst the treatments. C1: control, O1: OMR 55%, 0.875 gr/100l, O2: OMR 55%, 2.67 gr/100l, O3: OMR 95%, 2.7gr/100l, V1: GP 5 gr/100l , V2: GP, 7.5 gr/100l (GP: grape pomace extract, OMW: olive oil mill waste water residue) …………………………..…… 107

ABBREVIATIONS

AAB: Acetic acid bacteria

AAPH: 2,2-azobis(2-amidinopropane)hydrochloride

ABTS+: 2,2΄A-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation

ABAP: 2,2΄A-azobis (2-aminepropane)

AO: American oak barrel

Ac: Acacia barrel

ATR: Attenuated Total Reflection

Ch: Chestnut Barrel

CHD: Coronary heart disease.

DP: Depth of penetration.

DPPH: 2,2-diphenyl-1-picrylhydrazyl

DMPD: N,N-dimethyl-p-phenylenediamine

FO: French Oak barrel

FTIR: Fourier transform infrared spectroscopy

Ge: Geranium

KRS-5: Thallium Bromo-Iodide

LDP: Low density lipoproteins

l : Litres

mg : milligrammes

mM : millimolar

PDS: Potassium peroxodisulfate

ROS: Reactive oxygen species

SPE: Solid phase extraction

SS: Stainless steel container

SO: Stainless steel container with French oak oenosticks

TEAC: Trolox Equivalent Antioxidant Capacity

TBA: Thiobarbituric Acid

TPC: Total phenolic content

VQPRD : '*Vin de Qualité Produit de Région Déterminée'*, Quality Wine Produced in Determined Regions

ZnSe: Zinc selenide

GP : Grape pomace extract

OMW : Olive oil mill waste-water residue

μM: micromolar

CHAPTER ONE

GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 General introduction

The main ingredients of wine are water and alcohol. However, their flavour, astringency, bitterness, aroma and character is mainly attributed to a large group of compounds with phenolic structure (Rivero-Pérez et al., 2008). Phenolic substances in wine attribute a great deal to their character. Phenolic content and type varies considerably between different wines, and is one of the biggest factors in discriminating between wines. Grape and as a consequence wine phenolics vary a great deal amongst varieties.

Some natural products, foods and beverages are known for their antioxidant properties and their consumption, are thus constantly increasing. Natural phenolic substances are known for the antioxidant properties and the benefits they can give to human health (Sun et al., 2002). Phenolic compounds in wine, possess great antioxidant properties and it is widely known that consumption of wine in moderation, is beneficial to human health due to the large amounts of antioxidants contained them - which are especially found in red wines and are mainly of phenolic structure (Roussis et al., 2008). For example, people in France generally show a lower mortality rate due to coronary heart disease in comparison to other European countries, although consumption of saturated fat is similar in both France and other countries (mortality from heart disease has been related to high consumption of high-saturated acids). This effect, known as 'the French paradox', has been attributed to the consumption of red wine.

Oxidation of wines by acetic acid bacteria is a factor contributing to wine spoilage due to the amount of the produced acetic acid, spoiling its taste and characteristics. Special care has to be taken during vinification and storage of wine to prevent their growth in the medium and the resulting spoilage.

Furthermore, during vinification, and in order to prevent wine spoilage during ageing and storage, sulphur dioxide is used and added to wines. It is proven and widely recognized that it prevents oxidation and microbial spoilage in wines. There have been great efforts to minimize or substitute the amount of the added sulphur dioxide in wines with natural substances, with little progress being made throughout the years. As costumers' interest in more healthy food and in diet in general, is increasing, so does for the need of the replacement of the artificial substances in them with natural ones.

Another factor influencing the characteristics of wine is the type of barrel used in wine ageing.

The barrel wood influences the wines' flavour and aroma as a great variety of compounds - including phenolic substances - can be transferred from wood into wine. In this way phenolic content and composition in wine may be altered. The right type of barrel must be combined with the correct type of wine in order to improve its quality and not compromise it by changing the wines' aromas in a negative way.

The aim of this research was to study four Greek wines red and white, looking particularly at their phenolic content, antioxidant activity, barrel ageing, spoilage by acetic acid bacteria and alternatives to the use of sulphur dioxide as a preservative.

Objectives of the study were:

A. To characterize four Greek grape varieties in terms of their phenolic fingerprint, content and antioxidant capacity.
B. To determine the influence of the barrel type used in wine ageing has on the phenolic fingerprint, on the phenolic content and antioxidant activity of the above four Greek wines. For this purpose, wines were stored in several different types of barrels and the changes in the above characteristics were monitored with time.
C. To monitor the effect that two natural antioxidants substances, oleuropein and hydroxytyrosol, have on wine spoilage induced by acetic acid bacteria.
D. To monitor the influence on phenolic content and antioxidant activity of substitution of sulphur dioxide in wines by two natural antioxidant extracts obtained from olive oil mills and wineries by-products.
E. To evaluate the use of less common, than already used globally, varieties in winemaking, which could have improved wine characteristics by using different mediums for ageing and natural substances during their production.

1.2 Natural Antioxidants

Antioxidants are a large group of highly reactive substances that have the capability of slowing or blocking oxidant processes so as to minimize the oxidation effects of free radicals.

Free radicals are molecules with at least one unpaired electron, which is viable (can exist) in that form (Halliwell, 1989; Buenger et al., 2006). The unpaired electron of the radicals, seeks for electrons to pair, stealing them from the donor molecule, resulting in damaging it or in the formation of new free radicals. Free radicals have been related and are suggested to be involved in causing cancer, cardiac diseases, atherosclerosis – a disease of arteries characterized by thickening of artery walls, Alzheimer's disease, ageing and other biological conditions (Li et al.; Halliwell, 1989; Frankel et al., 1999; Sun et al., 2002). Hence, oxidative stress plays a significant role in human health.

Antioxidants are capable of stabilizing, or deactivating free radicals before the latter attack cells and biological targets. Moreover, they have the ability to interrupt the generation of free radicals by possessing properties such as scavenging activity and chelating metal ion activity and by being capable of reducing O_2 concentrations. They are therefore crucial for conserving health and supporting well being (Atoui et al., 2005; Brewer, 2011).

Many research groups are examining the chemical nature and activity of natural antioxidants in fruits, vegetables, grains, herbs, green tea leaves, grapes and wine.(Li et al.; Larson, 1988; Shahidi et al., 1992; Kanner et al., 1994; Shahidi, 2000; Atoui et al., 2005). Most of these antioxidants are polyphenols.

Some of the most widely known and studied antioxidants are vitamins C and E and beta carotene, found in a great amounts in natural beverages and foods. Phenolic compounds such as flavonoids are becoming increasingly known for their antioxidant activity (Percival. 1998). Polyphenols possess ideal structure for free radical-scavenging activities, and have been shown to be more effective antioxidants *in vitro* than vitamins E and C on a molar basis (Rice-Evans et al., 1996; Rice-Evans et al., 1997). The free radical-scavenging activity of natural polyphenols depends on the number and location of free −OH groups on the flavonoid skeleton. Flavonoids with more than one hydroxyl group on their structure possess increased antioxidant activity compared to those with only one hydroxyl substitute (Lupea et al., 2008; Brewer, 2011).

Oxidative damage to DNA, proteins, and other molecules has been involved in the pathogenesis of a wide variety of diseases. If the amount of antioxidants consumed is not sufficient enough, antioxidant potential may be compromised and thus the overall oxidative stress increased (Percival 1998).

Antioxidant activity has been strongly related to the phenolic content of a sample, *in vitro*. Red wines are proven to exhibit 5 to 10 times higher antioxidant activity than white wines. Even though they have less antioxidant activity, white wines are known to contain significant amounts of hydroxycinamic acids, tyrosol and hydroxytyrosol, all substances of phenolic origin, with high antioxidant activity (Fernández-Mar et al., 2012).

1.3 Phenolic compounds

1.3.1 Wine phenolics

Chemically, phenols are cyclic benzene compounds (Figure1.1) that have one or more hydroxyl groups which are associated directly with the ring structure. Although they contain alcohol groups they do not behave as alcohols or have their chemical properties.

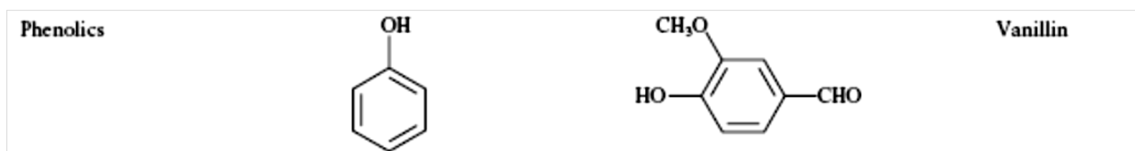

Figure 1.1 Phenol structure (Ribéreau-Gayon et al., 2006)

Wine phenols, are a large and complex group of compounds (Figure 1.2) that play a major role in winemaking and wine. They are very important to wines' characteristics and quality. Phenolic substances are responsible for the differences between red and white wines (especially the colour and flavour of red wine). They play an important role to the taste, mouth-feel, aroma, appearance and antimicrobial properties of wines. The phenols that are contained in wine may come from various parts of grapes such as the fruit and vine stems, can be produced by yeast metabolism, or be extracted from wood cooperage. Their structure varies during wine ageing in

barrels and bottles, and is always dependent on the employed conditions. The phenolic content increases during the early stages of fermentation especially if the juice is in contact with the seeds and skins (Jackson, 2000). Phenolics have health properties and are thought to be responsible for the 'French paradox' (Kanner et al., 1994; Frankel et al., 1995; Meyer et al., 1998; Sun et al., 2002). The 'French paradox' is based on the observation that, despite the high fat intake of people in the country, coronary heart disease (CHD) mortality was quite low, an opposite situation of that expected due to the high fat intake. That has been partially attributed to a regular consumption of wine. It was suggested that wine consumption was acting against and reducing the effect of a diet high in fat, which could lead to coronary heart disease. The phenolic components in red wine were proven to exert antioxidant activity in inhibiting the oxidation of human low density lipoproteins *in vitro* (Kanner et al., 1994). The antioxidant properties of phenolic compounds in red wine in retarding atherogenesis were proposed as an explanation for the French Paradox. The antioxidant and bactericidal properties of wine protected the consumers from cardiovascular diseases.

In red and white wine, phenols that are derived from *p*-coumaric acid and ferulic acid are found and they can cause unpleasant aromas in wine that increase in intensity when combined affecting negatively wine quality. Very small quantities (a few μg/l) of coumarins are found in wood-aged wine. Despite these low levels, they still affect wine's aroma and flavour characteristics. Another family of more complex polyphenols which are present in grapes, wine and oak wood are stilbenes. Resveratrol, a stilbene that has become popular in the recent years for its health related properties, is located in the skins of the grapes and is mainly extracted during the fermentation of red wines.

1.3.2. Flavonoid and non-flavonoid compounds of wine

Phenolic compounds of wine may be divided into two large groups: flavonoids and non-flavonoids.

Phenolic compounds are found in smaller quantities in white wines. In the majority, they are soluble non-flavonoids (hydroxycinnamates), such as caftaric acid and the related derivatives *p*-coumaric acid and ferulic acid. In both red and white wine, phenols that are derived from *p*-coumaric acid and ferulic acid give to wine unpleasant 'animal' aroma that increases in intensity when combined. Examples of phenolic compounds found in wines are shown in Table 1.1.

Table 1.1 Phenolic compounds in grape and wine (Jackson, 2008)

General type	General structure	Examples	Major source[a]
Nonflavonoids			
Benzoic acid	COOH	Benzoic acid	G, O
		Vanillic acid	O
		Gallic acid	G, O
		Protocatechuic acid	G, O
		Hydrolyzable tannins	G
Benzaldehyde	CHO	Benzaldehyde	G, O, Y
		Vanillin	O
		Syringaldehyde	O
Cinnamic acid	CH=CHCOOH	p-Coumaric acid	G, O
		Ferulic acid	G, O
		Chlorogenic acid	G
		Caffeic acid	G
Cinnamaldehyde	CH=CHCHO	Coniferaldehyde	O
		Sinapaldehyde	O
Tyrosol	CH_2CH_2OH, OH	Tyrosol	Y
Flavonoids			
Flavonols	R_1, OH, R_2, HO, O, OH, OH, O	Quercetin	G
		Kaempferol	G
		Myricetin	G
Anthocyanins	R_1, OH, R_2, HO, O, +, OR, OH, O	Cyanin	G
		Delphinin	G
		Petunin	G
		Peonin	G
		Malvin	G
Flavan-3-ols	OH, OH, HO, O, OH	Catechin	G
		Epicatechin	G
		Gallocatechin	G
		Procyanidins	G
		Condensed tannins	G

[a] G: grape, O: oak, Y: yeast

[a]Data from Amerine and Ough (1980) and Ribéreau-Gayon (1964).
[b]G, grape; O, oak; Y, yeast.

Flavonols and other flavonoid phenols are extracted slowly. Therefore they are only found in significant quantities in grape juice macerated with the grape pomace (skin, seeds, stems and pulp that remains after pressuring grapes, during vinification). Those found are primarily catechins and catechin-gallate polymers. In white wines, the yellow colour is mostly attributed to the limited extraction during maceration and oxidation of flavonols (such as kaempferol and quercetin). Furthermore, as the extraction of phenols is affected by many factors, such as grape maturity and vinification conditions and procedures, their content in wine shows great variation, notably greater than that of any other wine compound. In addition, the quantitative and

qualitative changes in the concentration and structure of phenolics during ageing are very intense and greater than in any other wine constituent (Jackson, 2000).

1.3.2.1 Flavonoid compounds of wine

Flavonoids are a group of phenylpropanoids and are characterized as molecules consisting of two phenols joined by a pyran. They have a C6-C3-C6 skeleton, common in all flavonoids, consisting of the phenolic rings (A and B rings) linked together by a pyran ring (C ring). Flavonoids are hydroxylated on their C_5 and C_7 carbons on A ring and $C_{4'}$ carbon on the B ring (Moreno-Arribas et al., 2008) (Figure 1.2). Classes of flavonoids differ in the level of saturation of the C ring (Brewer, 2011).

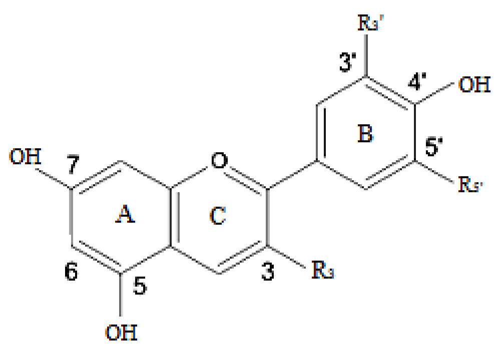

Figure 1.2 Basic flavonoid structure (Ribéreau-Gayon et al., 2006)

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Flavonoids have a varying yellow colour. The most common flavonoids in wines are flavonols, anthocyanins and catechines (flavanols). The most widespread are flavonols, which are pigments that are found in the skins of both red and white grapes. They are synthesized in the endoplasmic reticulum and afterwards, stored in the central vacuole of the cell (Jackson, 2000).

Flavonoids can be found free or polymerized to sugars, flavonoids and non flavonoids or a combination of both. If they are combined with sugars and non flavonoids they are called glycosides and acyl derivatives. Examples of such esterified flavonoids found in red wine grapes are kaempferol, quercetin and myricetin. In white wine grapes only kaempferol and quercetin can be found (Ribéreau-Gayon, Glories et al. 2006). Despite the fact that they can be found in both red and white wines, flavonoids characterize red ones more than they do white. In red wines, they constitute more than 85% of the phenol content (1000 mg/l). In white wines, flavonoids constitute less than 20% of the total phenolic content (50 mg/l) (Jackson, 2000). The

polymerized flavonoid that is most frequently found in both grapes and wine is dihydroquercetin (known as taxifolin).

Procyanidins occur primarily as monomers in grapes (Dumon *et al.,* 1991). In wine, they tend to polymerize occurring as condensed tannins.

Apart from grape variety and the amount of flavonoids that are present in the grape - which in turn depends on the climatic conditions and berry maturity, the amount and degree to which flavonoids are extracted during wine production, depends on many factors. Traditional fermentation due to longer maceration in contact with the seeds and skins, extracts more phenolic compounds than other types of vinification (wine production). Their extraction also depends on the pH, the content of sulphur dioxide and ethanol of the juice, as well as the temperature and duration of fermentation (Jackson, 2000).

i) Anthocyanins.

Anthocyanins exist in grapes as glucosides. Anthocyanidins conjugate with glucose to form anthocyanins. This conjunction is responsible for the increase of the chemical stability and water solubility of the anthocyanidins. The colour of anthocyanins depends on conditions in the medium such as pH and sulphur dioxide content, as well as their molecular structure. They are found mostly in the skin cells of the grape berry, with a concentration gradient from the inside towards the outside of the grape.

Five anthocyanin molecules have been identified in grapes and wines, with two or three substitutes (-OH and -OCH_3). Therefore, grape anthocyanins are divided into five groups: Cyanins, malvins, delphinins, peonins, and petunins (Table 1.2). The proportion and amount of each class varies widely among cultivars. Growing conditions can also play a big role in the amount of each one of them. The proportion of the five molecules influences hue and colour stability of wine. Their chemical resistance to oxidation is affected by the presence of ortho-diphenols on the B ring and the conjugation with sugar and other compounds (Jackson, 2008).

Figure1.3 Basic structure of anthocyanidins (Jackson 2008)

Table 1.2 Types of anthocyanidins in relation with the substitutes of anthocyanidin basic structure.

Anthocyanidins	Type of substitute	
	$R_{3'}$	$R_{5'}$
Cyanins	-OH	-H
Malvins	$-OCH_3$	$-OCH_3$
Delphinins	-OH	-OH
Peonins	$-OCH_3$	-H
Petunins	-OH	$-OCH_3$

Among the five anthocyanins, malvidin is the one found in greater quantities in all grape varieties, varying from 90% in Grenache to just fewer than 50% in Sangiovese. Grape variety and ageing in barrels and bottles affects anthocyanin concentration. Ribéreau-Gayon, Glories et al. (2006) reported concentration of 100 mg/l (Pinot Noir) to 1500 mg/l (Syrah, Cabernet Sauvignon.) after fermentation, which can decrease in a few years of barrel and bottle ageing to 0-50 mg/l. In fact, most anthocyanins conjugate and condense with tannins in wine, forming more complex compounds with markedly more stable colour. These combined anthocyanins although responsible for colour in wine, cannot be identified by standard analyses yet. A small fraction of anthocyanins can be lost, influenced by environmental conditions (temperature, light, oxygen, etc.) that can break them down or by their precipitation in a colloidal matter leading to loss of colour, negatively affecting wine quality (Ribéreau-Gayon et al., 2006).

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ii) Tannins

Tannins are substances that can combine with proteins, polysaccharides and other plant compounds, leading to the formation of stable molecules. They are produced by the polymerization of fundamental phenolic substancess. Therefore, they are characterized as high molecular weight compounds (M_r > 500) with many phenolic groups (Hagerman et al., 1998). The only legally authorised tannins that can be used as wine additives are the hydrolysable tannins despite the fact that they are not naturally found in grapes. The hydrolysable tannins, that are commonly found in oak wood barrels are gallotannins and ellagitannins (Ribéreau-Gayon et al., 2006). As tannins' structure displays a great variability, their analysis is quite complex. Because of their structural diversity their properties can be quite differentiated, and amongst various types of grape and wine they can attribute to flavours and wine aromas in several different ways. Apart from their content, their structure and colloidal status plays an important role on the final taste and flavour of wine.

1.3.2.2 Non flavonoid compounds of wine

The non flavonoid compounds of wine consist of phenolic acids, volatile phenols and stilbenes.

Phenolic acids have no colour in alcoholic solutions or particular taste and flavour. In contrast to flavonoid compounds that have two C6 benzene rings in their structure, phenolic acids are simpler compounds and are characterized by the presence of only one C6 benzene ring in their structure. They are divided in two categories, the hydroxycinnamic acids and the hydroxybenzoic acids (Figure 1.4). Phenolic acids and their derivatives are the most common non flavonoid phenolics found in wines - except for wine aged in oak barrels (Ribéreau-Gayon et al., 2006). Hydroxycinnamic acids are phenylpropanoids, with a C6-C3 skeleton. They are found in wine mostly in their esterified form with sugars, alcohols and organic acids whereas only low amounts are found in the free form. They consist approximately 73% of the phenolic content of white wines, with concentration increasing during wine ageing (Moreno-Arribas and Polo 2008). Hydroxybenzoic acids are characterized by a C6-C1 chain. The phenolic compound that belongs in this group and is found in the largest quantities in wine is gallic acid (Figure 1.5). In general, hydroxybezcoic acids are found in significant smaller quantities in wines in comparison to hydroxycinnamic acids (Moreno-Arribas et al., 2008).

Stilbenes are phenolic compounds that are composed in grapes after microbial attack or UV radiation as a defence mechanism. Their concentration depends on factors such as grape variety,

climatic conditions and pathogenic attack. They do not appear to increase or decrease significantly in content during barrel ageing. They are famous because of their useful properties, the antioxidant properties and the ability to act against cancer and against possible mutations. Resveratrol, the most famous amongst the stilbenes, is formed when grapes are infected by *Botrytis cinerea* mainly in the skin of the grape berries (Moreno-Arribas et al., 2008).

(a)

$R_1 = R_2 = R_3 = OH$; $R_4 = H$: Gallic acid
$R_1 = R_4 = OH$; $R_2 = R_3 = H$: Gentisic acid
$R_1 = R_2 = OH$; $R_3 = R_4 = H$: Protocatechuic acid
$R_1 = R_4 = H$; $R_2 = OH$; $R_3 = OCH_3$: Vanillic acid
$R_1 = R_3 = OCH_3$; $R_2 = OH$; $R_4 = H$: Syringic acid

(b)

$R_1 = R_2 = R_3 = R_4 = H$: Cinnamic acid
$R_1 = R_2 = R_3 = H$; $R_4 = OH$: o-coumaric acid
$R_1 = OH$; $R_2 = R_3 = R_4 = H$: m-coumaric acid
$R_1 = R_3 = R_4 = H$; $R_2 = OH$: p-coumaric acid
$R_1 = R_4 = H$; $R_2 = OH$; $R_3 = OCH_3$: Ferulic acid

Figure 1.4 Chemical structures of (a) hydroxybenzoic and (b) hydroxycinnamic acids found in wines (Gonçalves et al., 2013)

Figure 1.5 Gallic acid (3,4,5-trihydroxybenzoic acid) (Moreno-Arribas and Polo 2008)

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1.3.3 Properties of wine polyphenols

i) Antioxidant properties

Phenols are generally known for their antioxidant properties. The phenolic compounds of wine act as antioxidants, contributing to lowering human low-density lipoprotein, to cardioprotection (protection against heart disease) and to the reduction of cancer (anticarginogenic action). The

antithrombotic ability they have – reducing thrombus formation in blood - is also due to their antioxidant properties. Consumption of wine for two weeks has been proven to reduce the susceptibility of plasma and low-density lipoprotein (LDL) – cholesterol (Rice-Evans et al., 1996; Baydar et al., 2004). Also catechins (a fraction of phenolic compounds), according to Stanley et al. (1999), are absorbed by human blood and have been found to exhibit antioxidant properties *in vitro*.

It has been reported that the by-products of phenolic oxidation in wine produce compounds that bind additional oxygen. As a consequence, oxygen is rapidly diminished and thus is unavailable to oxidize other wine components (Jackson, 2000).

Antioxidant activity of phenolics depends on the conjunction with other molecules, the degree of polymerization, the number and arrangement of the phenolic substituents etc. Flavonoids with the most hydroxyl groups in their structure have higher antioxidant activity. Tannins, due to high polymerisation and many hydroxyl groups in their structure are also proven to have high antioxidant activity (Hagerman et al., 1998).

ii) Antimicrobial properties

Wine is known to have, by partially yet unexplained mechanisms, protective action against several gastrointestinal diseases. This action is mainly attributed to wine phenolic content. The precise mechanism by which wine and its phenols, exerts antimicrobial action is controversial. Not even the phenols that are involved are known. Generally, phenols have various abilities on binding to substances and moreover possess diverse effects on living systems. For example, when tannins bind with proteins, they alter their solubility and structure resulting in limitation of enzyme action (Jackson, 2000). Bacteria and fungi digest substances outside their cells. If enzymes they use in digestion are inactivated, the results are lethal to them. Binding of tannins to the phospholipids and proteins of their membranes interrupts proper function of membranes. Additionally, when phenols chelate with metals they restrict the accessibility of very useful elements like iron and zinc to the microorganisms.

Stilbenes, have both antimicrobial and antioxidant properties and are mainly formed when grapes are attacked by pathogens (Stanley et al., 1999). Resveratrol, which has strong antioxidant effects, may be involved in the health benefits derived from moderate wine consumption (Jackson, 2000).

iii) Taste and flavour properties

Phenolic substances play a great role in the organoleptic properties of wines. Their content and proportion affect wine aromas and flavour to a great extent, contributing to the final taste characteristics of wines.

Flavonoid tannins influence taste and mouth-feel. Catechins and their polymers, the procyanidins and condensed tannins, constitute the predominant source of bitter and astringent taste in wine (Jackson 2000). The concentration of certain phenolic groups and molecules can greatly affect the final product. Exceeding certain levels – or even very small quantities - may give unpleasant taste and aroma characteristics to the wine.

1.4. Sulphur dioxide

Sulphur dioxide is very commonly used in foods and drinks processing and storage, winemaking and the pharmaceutical industry, mainly because of its antioxidant and antimicrobial properties and the ability to prevent the enzymatic and non-enzymatic browning of foods and wines that leads to loss of nutritional and sensory properties.

1.4.1 Sulphur dioxide in must and wine

Sulphur dioxide has been used in winemaking since the end of the 18th century. It is the most common chemical that is used worldwide to prevent oxidative spoilage and browning of wine induced by oxidation of wine components (Li et al., 2008).

Bottled wines are not sterile. Bottled wines may contain a considerable amount of viable, but dormant, microorganisms. Under most situations, they do not cause any stability or sensory problems to wines. However, when wines are about to be stored for a long period of time, especially in the case of sweet wines, an antimicrobial substance such as sulphur dioxide, must be added to wine to prevent any unwanted activity of wine microorganisms.

Sulphur dioxide may be added at various times during wine production, but almost always after fermentation. Concentrations of 0.8-1.5 mg/l sulphur dioxide inhibit the growth of most yeasts

and bacteria. The total sulphur dioxide content required to maintain a desirable level depends on the pH of the wine and the concentration of sulphur-binding compounds.

Sulphiting gained in popularity in winemaking by improving wine quality when used on rotten grapes. Its specific antimicrobial properties and its ability to prevent bacterial wine spoilage were later discovered.

Wines can be produced without the addition of sulphites, nevertheless, the absence of sulphites in wines is rare as, even when sulphites are not added to wines, yeast produce small quantities during fermentation.In the majority of cases less than 10 mg/l, even though in a few cases about 30mg/l have been reported.

During storage, sulphur dioxide hinders the development of all types of microorganisms (yeasts, lactic bacteria, and, to a lesser extent, acetic bacteria), preventing yeast haze formation, secondary fermentation of sweet white wines, *Brettanomyces* contamination and the production of ethyl-phenols (Ribereau-Gayon, 2000). Excessive use of sulphites in wine can however result in compromising the wine quality and result in unpleasant flavour and aroma (Li et al., 2008). Due to its diverse properties, it is very difficult to replace it in winemaking with other compounds.

1.4.2 Properties of sulphur dioxide

i) Antioxidant properties:

Sulphur dioxide protects wines from chemical oxidation, oxidation of its phenolic compounds and compounds responsible for its aroma. In the presence of catalysts, it reacts with dissolved oxygen and helps to establish a sufficiently low oxidation–reduction potential, in favour of wine aroma and taste development during ageing and storage. Sulphur dioxide needs several days to consume 8.0–8.6 mg/l of oxygen in a synthetic medium. Sulphites protect oxidizable yeast musts from oxidation. Sulphur dioxide, by combining with oxygen, makes oxygen no longer available for the oxidation of other components. In the absence of sulphites, the depletion of oxygen is very quick and in 4 to 20 minutes on average, it is completed. By sulphiting musts at any time, oxygen is no longer consumed and its concentration remains stable.

Moreover, sulphur dioxide acts against oxidasic enzymes. This act mainly involves wine storage but can also play a significant role during winemaking. During winemaking, sulphur dioxide protects against oxidation by destroying or blocking the activity of oxidases so that the

enzymatic oxidation is inhibited until the fermentation begins. It inhibits oxidation enzymes (tyrosinase, laccase) from becoming active and destroys them over time, protecting musts from oxidation before fermentation.

ii) Antifungal properties

Sulphur dioxide effectively destroys the existing population of microorganisms, in wine. Moderate sulphiting inhibits yeast growth without totally destroying it. If must is sulphited before fermentation, the yeast resistance to the added sulphur dioxide is increased. The combined sulphur dioxide has a direct action on bacteria, acting against them. When it is combined with ethanal (or pyruvic acid) it possess an antibacterial activity 5–10 times weaker than that in the free form. However, it becomes 5–10 times more abundant, making it useful for the control of bacteria (Jackson, 2000).

Sulphur dioxide is also active against acetic acid bacteria but additional studies on this subject are needed. These bacteria resist relatively high concentrations. In the winery, acetic acid bacteria are most effectively prevented by avoiding contact with oxygen in the air and controlling temperature in the winery.

1.4.3 Sulphur dioxide concentration in wine

Sulphur dioxide protects wine aromas by binding ethanal and other similar products, and makes the flat character of wine disappear. Excessive doses must be avoided not only for health reasons; high sulphur dioxide doses can neutralize aroma, or even produce unpleasant aromas and taste such as a smell of 'wet wool' and a burning sensation in the mouth. On the other hand, if the concentration used is not sufficient enough, the stability of the wine cannot be ensured.

Browning of wine caused by phenol oxidation, leads to affecting the sensory properties of wine and loss of its nutritional value (Li et al., 2008). Sulphites prevent browning of wine through their antioxidant properties.

Sulphite exists in many different forms in wine. Concentration or additions of it in wine are expressed in mg/l or ppm of sulphur dioxide regardless of the form used. Limits of sulphites in commercial wines are shown in Table 1.3.

Table 1.3 International Organization of Vine and Wine (OIV) - Maximum acceptable sulphite limits in commercial wines.

Types of wines	Sugar content =or <4 g/l	Sugar content >4 g/l
Red wines	150	300
White and rose wines	200	300
Sweet wines		400

1.4.4 Adverse physiological effects of wine sulphites

Sulphites have been reported to cause many severe adverse reactions, including anaphylaxis, asthma, abdominal pain and diarrhoea, seizures and in a few extreme cases death (Yang et al., 1985). Its use has always been regulated and new techniques have always been sought so as to lower the concentration used.

Since the beginning of the century, the possible toxicity of sulphur dioxide has been the subject of much research. Acute toxicity has been studied in animals. The absorption of a single moderate dose of sulphites is 'slightly' toxic. Concerning its toxicity in humans, studies carried out indicate the appearance of symptoms such as nausea, vomiting and gastric irritation at significantly high absorbed concentrations (4g of sodium sulphite in a single concentration). No secondary effects were observed with a concentration of 400 mg of sulphur dioxide over 25 days. Possible toxicity of sulphites in humans has often been attributed to the destruction of thiamine or vitamin B1. However, the distraction of them is very limited at a pH of around 2, which corresponds to stomach pH. Allergic reaction to sulphites is observed at very low ingested concentrations (around 1 mg) involving mostly asthmatic people. Symptoms to people with sulphite sensitivity are shown in a very small period of time after the consumption of sulphited wine (Vally et al., 2003).

Sulphites have been involved in sulphur dioxide induced bronchial asthma. However, the mechanisms that are responsible have not yet been verified (Vally et al., 2003; Suh et al., 2007).

Red and white wines are proven to vary in the impact they have on consumers as far as sulphur dioxide sensitivity is concerned. When sensitive asthmatics consumed sulphited white wine their tolerance was up to 150 ppm of sulphites. When red wine was consumed the symptoms caused by sulphites appeared at levels of 50-55 mg/l (Vally et al., 2003).

1.4.5 Advantages and disadvantages of sulphur dioxide in wines

Sulphur dioxide is the only permitted wine additive that possesses such wide antimicrobial activity. When sulphur dioxide is not used in bottled wines, stabilization against microbial growth can be achieved only by physical means - such as pasteurization and filter sterilization (Jackson, 2000). **E**xcessive sulphiting slows or completely inhibits malolactic fermentation of red wines, which, when it is desirable, is an important stage in the production of some red wines giving them interesting flavour characteristics.

In conclusion, sulphur dioxide permits the extended barrel maturation, storage and bottle ageing of many types of wines that would be spoiled without its use. Nowadays, especially for health reasons, the possibility and ways of reducing the concentration of sulphites that are added to wines is under investigation. Due to the diverse effects of sulphur dioxide in wine, the use of alternative substance which have the same properties as sulphites, but lacking its disadvantages is a great challenge.

1.5 Acetic acid bacteria

Acetic acid bacteria (AAB) are a group of quite common obligatorily aerobic microorganisms in the *Acetobacteraceae* family. They are well known for their ability to oxidize ethanol to acetic acid, which is a major component in vinegar, and adapt well to sugar-rich and alcohol rich environments (Du Toit et al., 2002; König et al., 2009). They are some of the most common wine spoilage microorganisms because of their effect on acetic acid production and, therefore they represent a great threat to wine quality and commercial value. They are found on grapes, in wine and must (Guillamón et al., 2011).

As they are obligate aerobic microorganisms, their growth depends on the availability of molecular oxygen in the medium. Optimal pH for their growth ranges from 5 to 6, but they can still grow at pH level as low as 3-4 and temperatures between 25-30°C (Bartowsky et al., 2008; König et al., 2009). As mentioned above they oxidize ethanol (sometimes leading to high concentrations of acetic acid). However, at high alcohol concentrations their growth is limited (Du Toit et al., 2002). Acetic acid concentrations damage wine quality when they reach a level > 0.7-1.2 g/l and higher. Some strains can rapidly produce high concentrations of acetic acid. For example, it has been reported that some South African strains of acetic acid bacteria can lead to the production of about 4 g/l of acetic acid in five days in experiments performed in grape juice (Du Toit et al., 2002).

Acetic acid bacterial growth depends on factors such as the phase in the winemaking process, the treatments that have been used and the control of wine storage. Their numbers reduce considerably under the essentially anaerobic conditions present during alcoholic fermentation.

However, once alcoholic fermentation is complete, the processes associated with racking, filtration, and storage of wine can lead to oxygen enrichment, which activates the metabolism of acetic acid bacteria and increases their growth.

They have been isolated from the top, middle and bottom of the tanks and barrels, suggesting that AAB can actually survive under the semi-anaerobic conditions occurring in wine containers (Du Toit et al., 2002). Inadequate storage conditions - either in barrels or in bottle - can also stimulate the growth of these bacteria and increase volatile acidity. The number of bacteria usually decreases rapidly after bottling, because of the relatively anaerobic conditions present

within a bottle. Although they are obligate aerobes, acetic acid bacteria can survive under the almost completely anaerobic conditions during winemakingWhen oxygen is present in the medium, they are able to still grow (Guillamón et al., 2011). Their growth is increased by any process that involves aeration or oxygenation.

1.6 Oleuropein and Hydroxytyrosol

Oleuropein and hydroxytyrosol (Figure 1.6), are phenolic compounds contained in olive fruit, olive leaves and olive oil (Saija et al., 1998). Oleuropein consists of hydroxytyrosol (4-(2-hydroxyethyl)benzene-1,2-diol) (HT), elenolic acid and one molecule of glucose.

hydroxytyrosol oleuropein

Figure1.6 Structural formulas of oleuropein and hydroxytyrosol molecules (Rietjens et al., 2007)

It has been found that oleuropein acts beneficially on human health and is proven to possess antioxidant, anticancer, anti-inflammatory, antimicrobial and antidiabetic properties (Hassen et al.; Gikas et al., 2007).

The biological properties of oleuropein and hydroxytyrosol, may be related to a certain extent, to their antioxidant activity and their ability to scavenge free radicals (Saija et al., 1998).

In the past, olive leaf infusion had been used against diseases such as malaria. Nevertheless, research in olive leaves properties begun only after the second half of the 20th century, demonstrating that their properties and benefits in human health were driven by their phenolic content. Oleuropein, found in the largest amount than any other phenol in olive leaves has been

used in a great number of medical treatments. Oleuropein prevents cardiac diseases by protecting membrane lipid oxidation, improves lipid metabolism influencing in positive way obesity problems, has a protective effect against cancer and possesses antiviral properties. Derivatives of oleuropein such as hydroxytyrosol, are used against several diseases (Japσn-Lujαn et al., 2006). Red and white wines have be found to contain some significant amounts of hydroxytyrosol varying from 1,72 - 1.92 mg/l in white wines and 3,6-4,2 mg/l in red wines (Di Tommaso et al., 1998).

Elenolic acid, one of the structural subunits of oleuropein, has been demonstrated to exhibit strong antiviral properties. In addition, olive leaf extracts have a great impact on many viruses such as those causing herpes, influenza and encephalomyocarditis. They have been found to be susceptible to these extracts'. Moreover, oleuropein forms non-covalent complexes with the peptide that is known to be an etiologic factor of Alzheimer's disease (Gikas et al., 2007).

1.6.1 Oleuropein resources

Oleuropein can be found in olive oil wastes, olive leaves and olive oil. Alperujo, is a semi – solid residue obtained in olive mills during the production of olive oil. Its high moisture content (65–70%) prevents its use in the olive-oil industry for a second extraction. The cost of the procedure is high and in not compensated by the quality of the produced oil. The semi-solid olive oil residues have a rich phenolic content. Their antibacterial and antioxidant activities are attributed mainly to their phenolic content making the olive oil residues cheap cost but a source of antioxidants. The olive oil mill residues are proven to be up to 100 times higher in phenolics than that in olive oils. Olive leaves are rich in oleuropein and have very powerful radical scavenging properties. The concentration of oleuropein in olive oil is strongly affected by the extraction conditions.

1.7 General principles of winemaking

The first step of vinification, after removing all leaves and any other material from the berries, is the crushing of the fruit in order to release its juice. Maceration, the extraction of several components of the grape pulp, seeds and skin, is the first step of vinification, and is promoted by the action of several enzymes.

As far as white wines are concerned, maceration is limited. Contact of grape juice with pomace lasts for a few hours. On the contrary, in red wines maceration lasts for a long period of time and it occurs at the same time as alcoholic fermentation. The alcohol produced increases the extraction of anthocyanins and tannins from the skin and seeds. Extraction of phenolic compounds takes place, which gives the wines their special characteristics (aroma, flavour, appearance, ageing ability). Most of the time, the must is introduced with a yeast strain with known characteristics that will enhance alcohol production, and help the development of the flavour and bouquet of wine (Jakson 2008).

In some type of wines, malolactic fermentation follows alcoholic fermentation. During maturation, attention is being paid to avoid exposure with oxygen that leads to oxidation and microbial spoilage. After weeks or even months, wines are racked to separate wine from solids that have precipitated. At bottling, a small amount of sulphur dioxide is added to the wines to prevent microbial spoilage and oxidation.

1.7.1 The role of barrels in wine making

Ageing of wine is a technique commonly used in wineries to increase the stability of wine and achieve more complex aromas. It commonly takes place in wooden barrels. As a result of ageing, wine gains in quality, with its colour becoming more stable, aroma and flavour being enriched and clarity improved. (Ibern-Gómez et al., 2001; Fernández de Simón et al., 2003; Garde-Cerdán et al., 2006; De Rosso et al., 2009; Chira et al., 2014).

Barrels that are used for this purpose may be made from oak (American, French or Spanish oak), chestnut wood and acacia wood, showing important differences in their chemical

characteristics (De Rosso et al., 2009; Ortega-Heras et al., 2010; Fernández de Simón et al., 2014). These are the only two approved by International Organization of Vine and Wine (OIV) for wine ageing (Fernández de Simón et al., 2014). Wood from Castanea sativa (chestnut wood) was commonly used in construction of barrels in the Mediterranean region in the past due to its low cost and porosity (De Rosso et al., 2009).

During ageing, oxygen can penetrate, due to woods' porosity, through the wood into the barrel and wine, leading to the formation of stable anthocyanins and tannins. A result of this process is that the colour of wine becomes more stable and its astringency is decreased (del Álamo Sanza et al., 2004; De Rosso et al., 2009; Oberholster et al., 2015). It has been reported that in oak barrels, oxygen penetrates at a rate of 10-45 mg/l per year (De Rosso, Panighel et al. 2009). Nevares and Del Alamo (2008) estimated that the rate of oxygen penetration in new French oak barrels is between 1.66 ml/l per month and 2.5 ml/l per month.

Also, during wine ageing, many compounds, volatile and non-volatile, are extracted from the wood into the wine. Compounds such as polyphenols, coumarins, polysaccharides, terpenes and fatty acids are transferred from the wood into the wine. The hydrolysable tannins gallotannins and ellagitannins, are the major phenolic compounds that are extracted from wood during wine ageing. It has been reported that ellagitannins concentrations are lower than expected in wine aged in oak barrels (Fernández de Simón et al., 2003; De Rosso et al., 2009). Ageing of wine is depented on the phenolic composition of wine, which is also affected by contribution phenolics in the wooden barrels (Hernández et al., 2007). Wood of different species shows differences in their chemical properties and as a result the extractable components that can contribute to wine flavour are differentiated. As reported by De Rosso et al. (2009) acacia wood is characterized by significant contents of benzene aldehydes, chestnut wood by rich content in polyphenols and cherry wood by release of methoxyphenols, and oak by –methyl γ octalactones and polyphenols stable to oxidation. According to Fernández de Simón et al. (2003) the extractable compounds in oak wood varies with the oak wood characteristics and geographical origin of the wood, the oak species and on factors such as the seasoning and toasting (a hydrothermal procedure) during barrel manufacture. Of course the variety of grapes has a significant role in the extraction of any compounds from the barrel by the wine (Garde-Cerdán et al., 2006).

Apart from the use of wood barrels ageing of wine may be done in tanks that contain small fragments of toasted oak (sticks, powder etc) (Hernández-Orte et al., 2014). This technique is becoming popular nowadays and apart from giving the wine taste, aroma and wooden character, as it is also cheaper than wooden barrels (Ortega-Heras et al., 2010; Hernández-Orte et al., 2014; Tao et al., 2014). Using tanks with wood fragments, instead of wooden barrels, can

decrease the cost of wines by 10 times. This technique has been used in countries such as Chile, Argentina, South Africa, Australia and United States for several years (Hernández-Orte et al., 2014).

1.8 Determination of antioxidant activity, phenolic content and fingerprint of wines.

1.8.1 Methods used for the determination of antioxidant activity in biological samples.

Many analytical methods are used to measure the antioxidant activity of biological substances. (Fogliano et al., 1999; Re et al., 1999; Sanchez-Moreno, 2002; De Beer et al., 2003; Villaño et al., 2004; Buenger et al., 2006; Rivero-Pérez et al., 2008). Table 1.4 summarises the different methods used to determine total antioxidant activity. The two most widely used chromogen compounds to measure the antioxidant activity of biological material are the ABTS+ and the DPPH radicals (Arnao, 2000).

However, all the different methodologies used in the evaluation of antioxidant activity have resulted in different results between laboratories that are difficult to compare. There is a need for a standardized and reliable method that can be used worldwide to evaluate the antioxidant activity of wines in such way that results between laboratories can be compared (De Beer et al., 2003; Buenger et al., 2006)

Table 1.4: Some of the assays used to measure antioxidant activity of substances (Buenger et al., 2006)

Method / assay	**Name-giving molecule / abbreviation**	**Measurement**
DMPD	N,N-dimethyl-p-phenylenediamine	Analyses the ability to reduce the radical cation
DPPH	2,2-diphenyl-1-picryl-hydrazyl	Analyses the ability to reduce the radical cation
FRAP	Ferric reducing ability of plasma	Uses metal ion to produce oxidation and analyses the ability to reduce
ORAC	Oxygen radical absorbance capacity	Measures the inhibition in the loss of fluorescence due to the oxidation by peroxylradicals
TBA	2-Thiobarbituric acid	An indirect fluorometric screening test of total oxidative stress
TEAC	Trolox equivalent antioxidant capacity assay	Compares the ability of an antioxidant to scavenge the ABTS+ cation with that of Trolox
TRAP	radical-trapping antioxidant capacity	Analyses the delay in oxidation Compares the ability of an antioxidant to scavenge the ABTS+ cation with that of Trolox.

The TEAC assay evaluates the capacity of antioxidants (hydrogen donors) to scavenge the pre-formed radical cation of 2,2΄A-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS+) whereby concentration of the antioxidant as well as the duration of the reaction on the inhibition of the radical cation absorption, are taken into account (Re et al., 1999; Long et al., 2001; Villano et al., 2004; Buenger et al., 2006). Generation of the radical before the addition of the antioxidants prevents interference of compounds that would affect the radical formation. Thus the assay can prevent overestimation of antioxidant activity (Sanchez-Moreno, 2002).

The DPPH assay reflects the ability of antioxidants to scavenge the stable radical 2,2-diphenyl-1-picrylhydrazyl. Reaction of DPPH with the antioxidants can be followed by monitoring the fall in absorbance at 517 nm of the DPPH-antioxidant mixture (Long et al., 2001).

The TBA assay measures the inhibition of the formation of malondialdehyde, which is one of the degradation products of lipid peroxidation, by the antioxidant (Buenger et al., 2006).

Each assay has its own advantages and disadvantages that should be taken into account when assessing the antioxidant potential of a substance or biological sample. ABTS and DPPH, the most widely used chromogens used in order to determine antioxidant activity of substances, have a number of quite important differences. According to Buenger, Ackermann et al. (2006), as far as interlaboratory reproducibility of each assay is concerned, the TEAC assay exhibited 9–40% variation, followed by the DPPH assay (6–57% variation) and ABAP assay, which exhibited 10–67% variation. Based on the results of that study, TEAC and the DPPH assays are the most easy to perform and give the most reproducible results.

ABTS+ is soluble in both aqueous and organic media and antioxidant activity of hydrophilic and lipophilic compounds can be measured (Arnao, 2000). DPPH can be dissolved only in alcoholic (thus organic) media. It presents a peak at 515 nm absorbance, whereas ABTS+ (produced by the reaction of ABTS with potassium persulfate) presents peaks at 414 nm, 645 nm, 734 nm and 815 nm as shown in Figure 1.7 (Re et al., 1999; Arnao, 2000). Especially, as far as red wines are concerned, there can be a great interference in measuring antioxidant activity at low wavelengths, due to the colour of the containing pigments. The more colour in the sample - despite the small volumes used in the procedures - results in a decrease of absorbance and thus, leads to lower antioxidant activity quantification. Therefore, employing a wavelength such is 734nm, instead of 525nm (DPPH absorption peak) or 414nm (one of ABTS+ absorption peaks) is much more preferable, in order to minimize sample interferences (Re et al., 1999; Villano et al., 2004).

Antioxidant activity measured by the TEAC method is based on the bleaching of the blue-green ABTS radical cation that can be monitored by the decrease in absorbance at 734 nm. The ABTS radical cation can be prepared by employing different oxidants such as MnO_2, 2,2-azobis(2-amidinopropane)hydrochloride (AAPH) and potassium peroxodisulfate (PDS) (Carola Henriquez, 2002). The oxidant that has been reported to have the best results is PDS (Carola Henriquez, 2002).

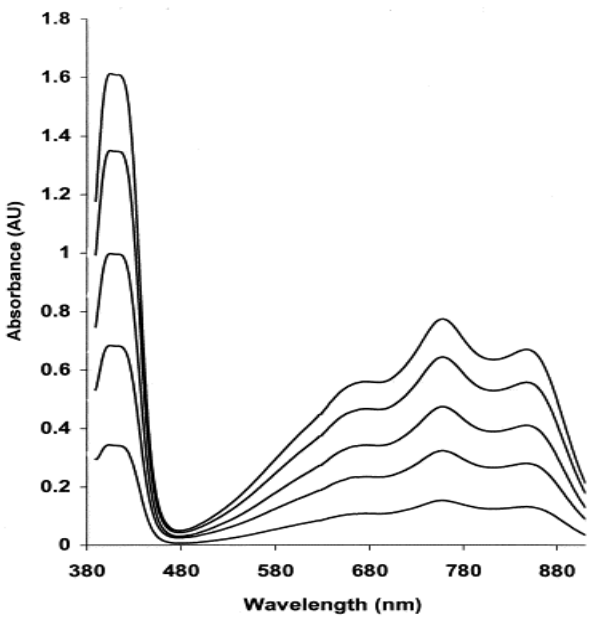

Figure 1.7 Absorption spectrum of ABTS+ (Re et al., 1999)

1.8.2 Methods used for the determination of total phenolic content

The most commonly used methods for the determination of total phenolic content in wines are the Folin-Ciocalteu (FC) method and the OD 280 value. The FC method has been adopted by OIV as the official method for the determination of wine phenolics (Waterhouse, 2001), by measuring indirectly total phenols. It is based on reaction (electron transfer) of the yellow coloured Folin-Ciocalteu reagent (a mixture of phosphotungstic acid and phosphomolybdic acid), with wine phenols, resulting in a blue coloured mixture with absorption maximum in the region of 765nm (Ribéreau-Gayon et al., 2006). Corrections of the obtained results must be made in case of high ascorbic acid content and for sweet wines, as sugars such as glucose and fructose can cause minor interferences. Additionally, interferences can be caused by wine sulfites, but only in white wines with low phenol content (< 250 mg/l) and high sulfite levels (> 50 mg/l) (Waterhouse, 1999; Waterhouse, 2001; Lorrain et al., 2013).

The OD 280 value is based on the benzene cycles absorption at 280nm. This procedure is very fast and easy, however, phenolics such as chalcones and cinnamic acids do not have an absorption maximum at this wavelength. Another disadvantage of this procedure is the interferences from non-phenolic compounds that also contain aromatic rings (Lorrain et al., 2013).

1.8.3 Phenolic fingerprint

1.8.3.1 Infrared radiation and interaction with molecules

The infrared region of the light spectrum ranges from 12800cm^{-1} to 10cm^{-1}. The infrared spectrum is divided into near, far and mid-infrared radiation (Skoog et al., 1998). The mid-infrared light spectrum ranges from 4000 to 400 cm^{-1}. The margins of infrared region are not clearly defined, so differences can be found at the margins between references. The mid-infrared spectrum (4000–400 cm^{-1}) can be approximately divided into four regions: the X–H stretching region (4000–2500 cm^{-1}), the triple-bond region (2500–2000 cm^{-1}), the double-bond region (2000–1500 cm^{-1}) and the fingerprint region (1500–600 cm^{-1}) (Stuart, 2005; Schrader, 2008).

Almost any compound having covalent bonds, whether organic or inorganic, absorbs various frequencies of electromagnetic radiation in the infrared region of the spectrum. Molecules absorb only selected frequencies of infrared radiation. Every type of bond has a different frequency of vibration. Frequencies that might be absorbed by two same types of bonds in two different compounds might be the same but the overall spectrum of the two compounds will be different (Pavia et al., 2008).

When molecules absorb infrared light they vibrate. When organic molecules are exposed to infrared radiation their chemical bonds can vibrate or rotate, due to the energy absorption at specific wavelengths in the IR region. Chemical bonds can stretch, bend, twist or rotate. The infrared spectrum of a substance contains much information about both structure and concentration of chemical groups in a sample. Analyzing infrared spectra can tell what molecules are present in a sample and at what concentrations (Skoog et al., 1998).

As mentioned above, vibrations can involve stretching which is a change in the length of the bond, or bending which is a change in the angle of the bond, as shown in Figure 1.8(a). Some bonds can stretch in-phase (symmetrical stretching) or out-of-phase (asymmetric stretching) as shown in figure 1.8 (b) (Stuart, 2005).

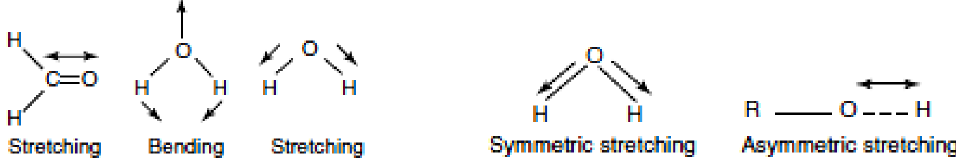

Figure 1.8 (a) Stretching and bending vibrations, (b) symmetric and asymmetric stretching vibrations (Stuart, 2005)

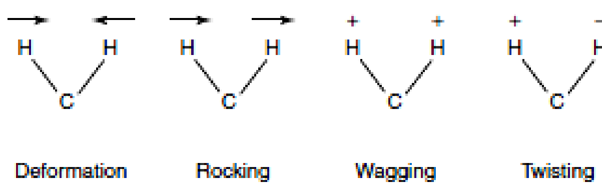

Figure1.9 Bending vibrations (Stuart 2005)

Bending vibrations are summarized in figure 1.9. The hydrogens can move in the same direction or in opposite directions in the plane, discriminating two types of bending: out of the plane and in plane bending (Figure 1.10).

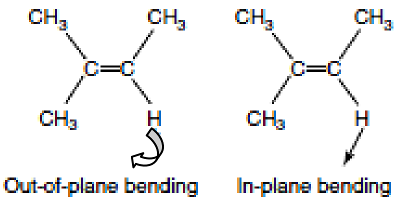

Figure 1.10 Out of plane and in plane bending vibrations (Stuart 2005)

Comparison of the IR spectrum of an unknown material with that of known standards is a useful tool for indentifying the components of the unknown material. In the absence of standard spectra, the fact that many functional groups possess absorption bands that are characteristic of that moiety can be used for the identification of the unknown material (McKelvy, 2006).

The peak positions in an infrared spectrum correlate with molecular structure, so samples can be identified by comparing the unknown spectrum to a reference spectrum observing how well the peak positions, heights, and widths in the two spectra match.

Infrared spectroscopy is a quick, comparatively inexpensive high sensitivity method, using a minimum amount of material in order to give a spectrum capable of giving information about the contained in the material under investigation, components. However, complex samples or mixtures may lead to complex spectra making difficult to determine what peaks are from what molecules. Purification of the mixture makes its spectrum simpler and much easier to interpret. Any of the purification techniques that chemists use can be applied to purify infrared samples. Furthermore, attention must be paid to water in samples. Water is a problem because it absorbs in the IR region; it has broad and intense peaks that can interfere with and cover the spectra of molecules dissolved in it (Smith, 2011).

1.8.3.2 Fourier transform infrared spectroscopy (FTIR) – Attenuated Total Reflectance (ATR) technique

There are several types of infrared spectrometry techniques used, but the most widely used one is FTIR (Smith 2011). FTIR is capable of extracting information from samples which is difficult or even impossible to obtain by techniques such as nuclear magnetic resonance and mass spectrometry. Nowadays, applications of FTIR spectrometry include simple, qualitative as well as quantitative analysis, identification of unknown compounds, and investigation of biological materials (Gremlich, 2008). For routine FTIR, microgram samples are used (Smith, 2011).

There are several sampling techniques for FTIR analyses which are divided into two families, the transmission and the reflectance modes. In transmission modes, the infrared beam passes through a thin film of sample and impinges on a detector (Figure 1.11). This technique works on many different types of samples but can be very time consuming due to the preparation of sample that may be needed (dilution, grinding, pressing).

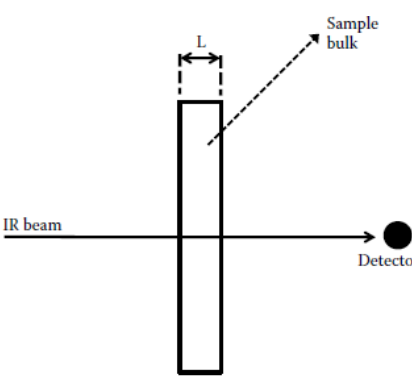

Figure 1.11 Transmission sampling in FTIR analyses (Smith, 2011)

In reflection mode the light is reflected from the surface of the sample. Specular reflectance, diffuse reflectance (DRIFTS) and attenuated total reflectance (ATR) are some of the different types of reflectance methods.

Attenuated total reflectance (ATR) is based on internal reflectance (radiation is not transmitted through the sample). The sample is placed on a crystal (the sensing element or internal reflection element) and light is reflected several times. Several different types of sensing elements can be used in ATR, such as diamond, Geranium (Ge) and Zinc selenide (ZnSe) crystals (Table 1.5). A spectrum is obtained as a result of the contact between the element and the sample. The sample surface is studied to a depth of about 1μm (Smith, 2011). The wave that is created, interacts with the sample resulting in the absorption of radiation by the sample at each point of reflection (Gremlich, 2008). In many cases, samples can be measured in their natural state, by placing the sample on the crystal, followed by removing the sample and cleaning the crystal (Griffiths et al., 2006; Smith, 2011). It is a fast and easy to employ technique as it involves none or minor sample preparation and can be applied to a wide variety of samples, liquids, semi-liquids, solids, polymers and powders and is a non destructive method.

Table 1.5: Types and properties of ATR crystals (Smith, 2011)

Crystal material	Wavenumber range (cm^{-1})	pH range	Comments
Diamond	30.000-2200, 2000-400	1-14	Tough, durable, absorbs in mid-infrared, expensive
Thallium Bromo-Iodide (KRS-5)	20.000-250	5-8	Soft, highly toxic, rarely used
Zinc selenide (ZnSe)	15.000-600	5-9	Attacked by strong acids and bases, once very common
Silicon (Si)	8900-660	1-12	
Geranium (Ge)	5500-600	1-14	Durable, shallow DP

1.8.3.3 Interpretation of phenol frequencies.

The interpretation of the absorption spectrum of an unknown sample is critical to understand the structure and chemistry of the sample. Throughout the years, much information has been published, revealing the fundamental absorption frequencies of several chemical groups. Those frequencies have been used to reveal the relationship between the obtained spectrum and the structure of known molecules in the sample. Physical and chemical data from the sample must be taken into account. In order for someone to interpret successfully a sample, attention must be paid apart from locating particular bands within a spectrum, to noticing the absence of others as well (McKelvy, 2006).

Phenols, either in the liquid or solid state, exhibit strong and characteristic bands in the IR region due to vibrations caused by O–H stretching and C–O stretching (Table 1.6). C–O stretching in phenols (as well as in alcohols) produces a strong band in the 1300–1000 cm^{-1} region (Stuart, 2005). The O–H bending vibrations that phenols produce, in the fingerprint region (1500–600 cm^{-1}) couple with other vibrations and result in complex band production (Stuart, 2005)[b].

Due to the hydrogen bonding of the OH groups, the bands caused by 0-H stretching vibrations are very broad regardless the sample state (liquid or solid, concentrated solutions or mixtures).

Along with O-H stretching bands, the position of the intense C-0 stretching band also allows for phenols to be detected in the samples (Gremlich, 2008).

Table 1.6 Some characteristic frequencies of phenols and aromatic ring (Coates, 2006)

origin	**Group frequency wavenumber (cm-1)**	**Assignment**
O-H	3640-3530[a]	Phenols, OH stretch
C=C-C	1615-1580	Aromatic ring stretch
C=C-C	1510-1410	Aromatic ring stretch
OH	1410-1310	Phenol OH bend
C-O	1200[b]	Phenol, C-O stretch
C-H	1225-950	Aromatic C-H in plane bend
C-H	900-670	Aromatic C-H out of plane bend

[a] Frequency is influenced by the nature and position of other ring substituents.

[b] Approximate centre of range of the group frequency.

1.8.3.4 Wine analysis

Various techniques have been used to analyze wines and characterize several of their components, amongst them phenolic compounds, alcohol content, total acidity, pH and flavonoid content. ATR-IR spectroscopy (Edelmann et al., 2001; Patz et al., 2004; Bevin et al., 2006; Schmidtke et al., 2012; Friedel et al., 2013; Silva et al., 2014), HPLC(Sen et al., 2014), HPLC-MS (Sagratini et al., 2012; Pati et al., 2014; Sen et al., 2014) and NIR (Cozzolino et al., 2004; Urbano Cuadrado et al., 2005) are some of them.

Solid-phase extraction of grape skin extracts or wines followed by reversed phase HPLC-UV−vis methods has been used to analyze the phenolic fingerprint of wines. HPLC in combination with mass spectrometry is also used to determine the anthocyanin and flavonoid composition and content of wines (Etiévant et al., 1988; Berente et al., 2000; Sen et al., 2014).

Fourier transform infrared spectroscopy is widely used for the analysis of food and wine components (Silva et al., 2014). In order to determine wine authenticity, mid - infrared spectra has been used. (Patz et al., 2004; Bevin et al., 2006). The development in sampling accessories, such as ATR cells, has improved IR analysis. Handling of the sample is therefore simpler, the time needed for the analysis is shorter and measurement problems often found with transmission cells are avoided. Comparatively, recent studies have shown that employing ATR-IR spectroscopy is an easy and fast way to determine the phenolic fingerprints of wines, to differentiate them, authenticate them and determine their phenolic content (Edelmann et al., 2001; Tarantilis et al., 2008).

CHAPTER TWO

MATERIALS AND METHODS

2.1 Total phenolic content, fingerprints and antioxidant activity of Vilana, Dafni, Kotsifali and Mandilari wines.

The white grape varieties Dafni and Vilana and the red grape varieties Kotsifali and Mandilari, all of them of Greek origin, were used for the production of single grape variety wines, in order to determine their phenolic fingerprint in the mid-ir region. Their total phenolic content and antioxidant activity were also determined immediately after vinification.

After vinification, stainless steel and stainless steel with oenosticks containers and American oak, acacia, French oak and chestnut barrels were used for red wine ageing. The above types, chestnut barrels excluded, were also used for white wine ageing. Due to wine component transformations during ageing and the extraction of phenolic substances from the wood of the barrels into the wine, changes in phenolic composition occur. Therefore phenolic fingerprints, total phenolic content and antioxidant activity of wines were monitored to determine the changes, in relation to barrel type and time.

For this study, wines were vinificated in September 2012 and September 2013 in Lirarakis GEA, AE. winery.

Table 2.1 Wine characteristics directly after vinification

	Vilana	Dafni	Kotsifali	Mandilari
	1st vinification			
Alcohol content %	12.4	13.1	12.15	13.3
Sugar content (gr/l)	1.56	2.25	2.02	1.96
Volatile acidity (acetic acid gr/l)	0.22	0.24	0.29	0.32
Total acidity (tartaric acid gr/l)	6.07	4.5	5.65	6.45
pH	3.45	3.73	3.6	3.45
	2nd vinification			
Alcohol content %	14.3	12.75	13.6	13.0
Sugar content (gr/l)	2.14	1.05	1,53	1.56
Volatile acidity (acetic acid gr/l)	0.39	0.30	0.41	0,28
Total acidity (tartaric acid gr/l)	6.54	5.3	5.55	5.55
pH	3.38	3.44	3.45	3.35

2.1.1 Determination of total phenolic content of wines (Folin-Ciocalteau method)

In order to determine wine total phenolic content the Folin-Ciocalteu micro-method was employed (Waterhouse, 1999; Waterhouse, 2001; Staško et al., 2008).

Materials that were used were Folin-Ciocalteu (2N) reagent, Gallic acid solution (stock solution 500mg/l) and $NaCO_3$ solution (5gr/l). Nanopure water was used throughout the experiments.

Determination of total phenolics was made photometrically at 765 nm using a Hitachi U-2000 spectrophotometer.

PROCEDURE:

- 20 μl of sample were added to 1580 μl H_2O.
- 100 μl of Folin-Ciocalteu reagent were added to the above mixture and mixed well, allowed to stand for 30 sec – 8min. 300 μl of sodium carbonate solution was add and mixed well.

- After incubation for 30' at 40° C in order for the reaction to be completed, absorption of the final mixture was measured at 765nm.

Red wines were diluted 5 times with EtOH 10% right before measurements. No dilution was performed in the case of white wines. Measurements were performed in triplicate. Gallic acid was used as standard substance. A gallic acid standard curve was prepared prior to each measurement.

2.1.2 Determination of antioxidant activity of wines (Trolox Equivalent Antioxidant Capacity method).

Antioxidant activity of wine is related to their phenolic content as they are the main antioxidant components. In order to determine antioxidant activity of wines the method described by Rice-Evans (1997) and Re et al. (1999) was employed, with a minor modification as reported by Ozgen et al. (2006). The method was based on the decolorization of a preformed ABTS radial cation ($ABTS^+$) by any antioxidant that may be present in the sample.

Materials used in the experiments were Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, a water soluble analogue of vitamin E), ABTS [2,2´-(3-ethylbenzothiazoline-6-sulfonic acid)] 99.8%, Potassium persulfate (PDS), Ethanol HPLC grade (99.9%) and nanopure water.

1. ABTS radical cation was performed by preparing a dilution of 7mM ABTS in H_2O and 2.45 mM PDS (final concentration).

   The solution was incubated for 12-24 h, in the dark at room temperature in order for the $ABTS^+$ to be stabilized.
2. $ABTS^+$ was diluted in sodium acetate buffer (it increases the stability of the radical and is most appropriate for products that contain phenolics according to Ozgen et al. (2006), until the absorption was 0,7 ±0,02 at 734 nm.
3. 50μl of wine sample were added (or standard solution) to 1ml of diluted $ABTS^+$. After incubation of the sample at 37° C, the absorption at 734 nm is measured.
4. Calculation of TEAC was made using the % inhibition of absorption induced by $ABTS^+$, compared to that of Trolox, in Trolox equivalents (mg/l).

All wines were diluted before they were used with 10% (v/v) ethanol. The dilution was 50 times (50x) for red wines and 10 times (10x) for white wines. All measurements were made in triplicate. Before each measurement, a Trolox standard curve was prepared.

Prior to each measurement, in order to determine total phenolic content of wines a standard curve of gallic acid was prepared (Figure 2.1.a). TPC was calculated with the use of the standard curve, in gallic acid equivalents (mg/l). Similarly, a standard curve of Trolox was prepared before each measurement in order to determine TEAC. A typical Trolox standard curve is shown in Figure 2.1.b. Total antioxidant activity of the samples was calculated by relating the % inhibition in absorbance at 734nm to that of Trolox.

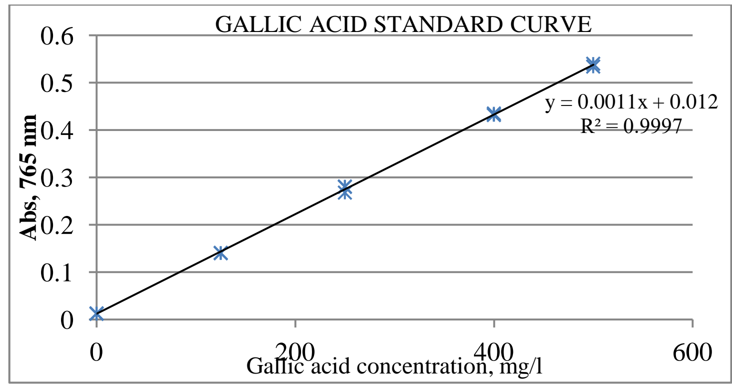

Figure 2.1.a Gallic acid standard curve.

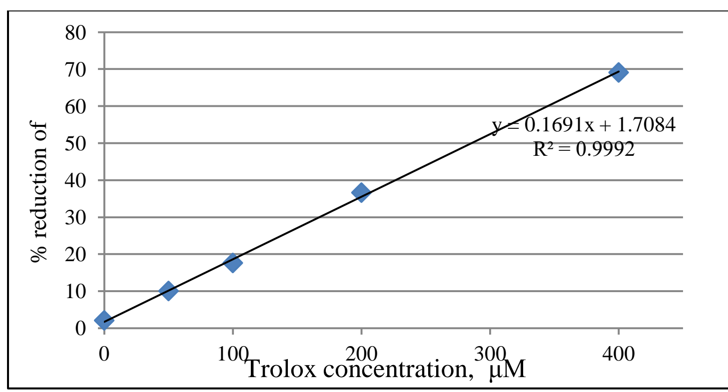

Figure 2.1.b Trolox standard curve.

2.1.3 FTIR fingerprint

Mid-infrared spectroscopy was used to differentiate and authenticate four Greek red and white wines of different origin directly after vinification, that were afterwards stored in different kinds of barrels, in order to determine the difference in phenolic composition during ageing due to type of barrel used in the procedure. Extracts of wine phenolic components were investigated by Fourier transform infrared (ATR-FTIR) spectroscopy (Edelmann et al., 2001).

The wine extracts were obtained by solid-phase extraction with C-18 columns and elution with methanol. The spectral region 1800–900 cm^{-1} was used to 'fingerprint' wine on the basis of grape variety and type of barrel used.

Each wine sample was filtered through a 0.45 μm Whatman filter. 3ml of each filtered sample was diluted with 15ml distilled water. The solutions (18 ml) were passed through a C18 solid phase extraction (SPE) cartridges under vacuum. Cartridges were preconditioned using 10 ml methanol and 10 ml distilled water for every syringe. Afterwards the solutions were p washed with 20 ml of distilled water. The extract was removed by a vacuum pump. At the end, the syringes were washed with 3 ml of acidified methanol (0.01% hydrochloric acid) and phenolic fractions collected (Figure 2.3).

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Figure 2.3 Isolation of phenolic substances of wines.

FT-IR spectra were obtained using a Shimadzu IRPrestige-21, Fourier transform infrared spectrophotometer (Figure 2.3). 150 μL of methanol extract was placed on the FTIR crystal (samples were air-dried prior to use) and spectra obtained.

Figure 2.3 Shimadzu IRPrestige-21 Fourier transform infrared spectrophotometer

2.1.4 General characteristics of Mandilari, Kotsifali, Vilana and Dafni grape varieties used in the experiments.

Mandilari, Kotsifali, Vilana and Dafni are grape varieties cultivated in Greece and more precisely in the island of Crete, very commonly used in the vinification of Greek wines. The general characteristics of each variety are listed below.

a. Mandilari:

One of the richest in colour, this Greek red grape variety has a vigorous vine growth. It reaches maturity late in August in the island areas and late September in northern Greece. It produced grapes with big berries whose weight reaches 2.8g. Mandilari produces wines very rich in colour, with medium acidity and low to medium alcoholic content. It is cultivated mainly in Crete and the Aegean area. Mandilari is produced alone or is blended with other grape varieties such as Kotsifali and Monemvasia to produce wines that are characterized as "VQPRD ('*Vin de Qualité Produit de Région Déterminée'*, Quality Wine Produced in Determined Regions) (Stavrakas 2010).

b. Kotsifali:

A Greek grape variety mainly cultivated in the area of Crete. Kotsifali produces VQPRD wines 'Peza" and 'Archanes' when combined with grape variety Mandilari. Kotsifali is known for high sugar concentration and wine colour instability. The wines originating from Kotsifali, have a high alcohol content, intense and very interesting aromas, but unstable colour. The last

characteristic is one of the reasons for it being vinificated along with Mandilari, with the latter contributing a great deal to the final colour of the produced wine (Stavrakas 2010).

c. Vilana:

Vilana is a white Cretan grape variety. Grapes reach maturity in the middle of September. It has medium size round berries. Their weight reaches 2.4g. Wine produced by this variety, have a good acidity, medium flavour and medium to high alcoholic degree. The wine is susceptible to oxidation, therefore care must be taken and attention must be paid to avoid this phenomenon during vinification. Vinificated on its own produces VQPRD wine ‘Peza’ and in combination with grape variety Thapsathiri, VQPRD wine ‘Sitia’ (Stavrakas 2010).

d. Dafni:

This is a white grape variety cultivated in the region of Crete. It is one of the most ancient and rare Cretan varieties that until recently was abandoned. The variety owes its name to Bay Laurel due to the resemblance to its aromas, which in this grape variety, are mainly distributed in the berry flesh. Dafni is a very vigourous variety, with medium productivity. It has large sized round berries, medium sweetness of the flesh and is very aromatic. The produced wine has low to medium alcohol content, medium acidity and interesting aromas resembling those of *Laurus nobilis* (Stavrakas 2010). Table 2.2 shows the general oenological characteristics of the wines made of the above grape varieties used in the experiments after 3, 6, 9 and twelve months of ageing.

Table 2.2 General characteristics of wines during ageing in containers. TA: titratable acidity, gr/l, VA: volatile acidity, gr/l, Vol%: % alcohol content

container	months of ageing	TA	pH	SO_2 (free)	SO_2 (total)	VA	Vol%	sugars gr/lt
				VILANA				
SS	3	6.07	3.41	9	87	0.43	14.3	2.1
	6	5.85	3.52	7	82	0.44	14.25	2.1
	9	5.1	3.6	6.5	68	0.58	14.25	2.1
	12	4.8	3.58	6	57	0.56	14.25	2.1
SO	3	6.07	3.47	7	92	0.46	14.3	2.1
	6	6.07	3.48	9.5	107	0.49	14.3	2.1
	9	5.7	3.52	9	102	0.49	14.3	2.1
	12	5.07	3.52	8.5	97	0.52	14.3	2.1
AO	3	6.07	3.47	8	94	0.53	14.3	2.1
	6	6.03	3.47	7.7	86	0.52	14.3	2.1
	9	5.4	3.54	9	66	0.53	14.35	2.1
	12	5.05	3.54	8.2	68	0.56	14.35	2.1
FO	3	6.03	3.48	10	86	0.54	14.35	2.1
	6	5.92	3.49	9	89	0.53	14.4	2.1
	9	5.45	3.54	8	80	0.52	14.45	2.1
	12	5.05	3.54	8	77	0.56	14.45	2.1
Ac	3	6.07	3.43	7	85	0.52	14.4	2.1
	6	5.99	3.48	11	94	0.51	14.4	2.1
	9	5.7	3.5	9	87	0.51	14.5	2.1
	12	5.1	3.51	9	86	0.51	14.5	2.1
				DAFNI				
SS	3	5.25	3.35	13.5	107.5	0.4	12.75	1.05
	6	5.1	3.43	6.5	117	0.46	12.75	1.05
	9	4.8	3.45	6.5	117	0.45	12.75	1.05
SO	3	4.57	3.44	11	89.5	0.31	12.7	1.05
	6	4.1	3.57	13.5	123	0.41	12.7	1.05
	9	4	3.57	14	192	0.58	12.65	1.05
	12	4	3.57	11	145	0.57	12.65	1.05
AO	3	5.17	3.37	7.7	108	0.34	12.75	1.05
	6	4.32	3.53	9.6	112	0.53	12.75	1.05
	9	4.1	3.54	8.5	104	0.52	12.8	1.05
	12	4.1	3.54	7	96	0.52	12.8	1.05
FO	3	4.95	3.37	11.5	110	0.31	12.75	1.05
	6	4.3	3.54	9.6	107.5	0.48	12.8	1.05
	9	4.1	3.55	7.5	102	0.47	12.85	1.05
	12	4.1	3.55	7	96.8	0.5	12.85	1.05
Ac	3	5.1	3.33	13.5	115	0.32	12.75	1.05

container	months of ageing	TA	pH	SO_2 (free)	SO_2 (total)	VA	Vol%	sugars gr/lt
	6	5.25	3.38	9.6	106.8	0.49	12.8	1.05
	9	4.65	3.42	8.3	101	0.48	12.85	1.05
	12	4.55	3.43	6	95	0.48	12.8	1.05
				KOTSIFALI				
	6	4.95	3.47	27	83	0.46	13.6	2.16
	9	4.95	3.45	35	93	0.47	13.6	2.16
	12	4.85	3.45	22	67	0.47	13.6	2.16
SO	3	4.95	3.45	18	88	0.51	13.6	2.16
	6	4.8	3.5	28	93	0.55	13.55	2.16
	9	4.8	3.55	13	88	0.69	13.65	2.16
	12	4.87	3.55	10.24	80	0.68	13.65	2.16
AO	3	4.95	3.46	32.5	89	0.52	13.6	2.16
	6	4.85	3.52	29	89	0.51	13.6	2.16
	9	4.75	3.56	14	72	0.51	13.65	2.16
	12	4.77	3.55	11	68	0.53	13.65	2.16
FO	3	4.95	3.45	31.5	93.4	0.53	13.6	2.16
	6	4.90	3.49	27.5	82.5	0.55	13.6	2.16
	9	4.95	3.51	17	72	0.53	13.7	2.16
	12	5	3.51	11	67	0.53	13.7	2.16
Ac	3	4.87	3.48	23	90	0.53	13.6	2.16
	6	4.85	3.53	18.5	90	0.53	13.6	2.16
	9	4.85	3.56	16.5	79	0.53	13.65	2.16
	12	4.95	3.56	11.5	74	0.64	13.65	2.16
Ch	3	4.95	3.47	20	103	0.63	13.6	2.16
	6	4.95	3.5	18	92	0.58	13.55	2.16
	9	4.95	3.51	16	78	0.57	12.7	2.16
	12	5	3.51	12	77	0.59	12.7	2.16
				MANDILARI				
	6	5.05	3.42	24	79	0.29	12.95	1.56
	9	5.05	3.45	19	69	0.32	12.95	1.56
	12	5	3.45	16	67	0.35	12.95	1.56
SO	3	5.15	3.41	20	71	0.31	12.9	1.56
	6	5.05	3.43	23	76	0.3	12.95	1.56
	9	5	3.45	22	65	0.3	13	1.56
	12	5	3.45	19	63	0.32	13	1.56
AO	3	5.20	3.38	22.5	73	0.3	12.9	1.56
	6	5.17	3.41	28	88	0.33	12.9	1.56
	9	5.15	3.44	23	72	0.33	13	1.56
	12	5.15	3.44	19	66	0.35	13	1.56
FO	3	5.17	3.40	19.5	68	0.3	12.95	1.56
	6	5.1	3.42	26	76	0.33	13.05	1.56

container	months of ageing	TA	pH	SO_2 (free)	SO_2 (total)	VA	Vol%	sugars gr/lt
	9	5.15	3.44	17	63	0.33	13	1.56
	12	5.15	3.44	15	61	0.35	13.6	1.56
Ac	3	5.15	3.41	19.67	67	0.3	12.95	1.56
	6	5.1	3.42	25	74	0.3	13	1.56
	9	5.1	3.44	14	59	0.31	13.1	1.56
	12	5.12	3.44	13.5	58	0.35	13.1	1.56
Ch	3	5.17	3.41	21.75	73	0.3	12.95	1.56
	6	5.05	3.44	23	74	0.32	12.95	1.56
	9	5.1	3.45	18.5	74	0.34	12.95	1.56
	12	5.3	3.45	16.64	60	0.45	12.95	1.56

2.2. Influence of the phenolic substances hydroxytyrosol and oleuropein on wine oxidation induced by acetic acid bacteria.

The aim of the experiment was to test the effect that hydroxytyrosol and oleuropein, have against the oxidation of wine induced by acetic acid bacteria (AAB). Under the influence of acetic acid bacteria, acetic acid is produced increasing volatile acidity in wine. Oleuropein and its phenolic derivative, hydroxytyrol, possess bacteriostatic properties. For this reason, volatile acidity was determined. Total acidity of wines was monitored through time, mainly as an indicator of the possible wine spoilage (during the growth of acetic acid bacteria in wine volatile as well as total acidity, increases).

As wines made in wineries almost always contain sulphur dioxide as an additive, red and white dry wines, as described in Table 2.3 were selected from local, amateur winemakers, with the aim of ensuring the absence of sulphites as additives in them, which are responsible for preventing wine oxidation. Commercial wines, produced in wineries, where not used, as sulphur dioxide is almost always used as additive during vinification procedures.

Acetic acid bacteria (AAB) must was added in wines in order to ensure the oxidation of ethanol to acetic acid and therefore, to increase volatile acidity. The bacteria were added in the form of vinegar grape must, bought from the UACH (Union of Agricultural Cooparatives of Heraklion)

vinegar production factory. Prior to use, AAB must, was incubated for 48h at 30° C, in a Rostfrei, Edelstahl, Memert waterbath, to activate the bacterial populations.

Table 2.3 Wines used in the experiments. Code names B1, B2, B3, B4, B5 will be used to refer to each wine

Wine	Grape varieties	Type of wine
B1	Kotsifali, Mandilari, Mourverde	Red, dry wine
B2	Kotsifali, Mandilari	Red, dry wine
B3	Vilana	White, dry wine
B4	Vilana, Thrapsathiri	White, dry wine
B5	Vilana, Mosxato	White wine

Experiments with each wine were carried out employing the treatments that are shown in Table 2.4.

All samples were stored in 250ml erlenmeyer flasks, in a chamber with constant temperature of 30°C. Aeration of samples, to ensure contact of the aerobic bacteria with oxygen, was done by using agitation at 300 r.p.m.

Wine treatments - as well as time of measurements - were differentiated. They were adjusted from one wine experiment to the other, according to the obtained results in order to determine the level of the tested substances that might have an effect on volatile acidity and thus on the production of acetic acid in wine by the acetic acid bacteria. Table 2.5 are shows the time points that volatile and titratable acidity of each wine were measured.

Table 2.4 Treatments and final concentration of additives in wines. A volume of 250ml of wine was used in each replication (three replications per wine sample). Wine B3 was not treated with AAB, only with the antibacterial substances.

Wine	abbreviation	Wine treatment
B1	C1	None (negative control)
	AAB	Acetic acid bacteria (positive control)
	H0.5	Hydroxytyrol 0.5 mg/l + 0.4% AAB
	H1	Hydroxytyrol 1 mg/l + 0.4% AAB
	OL2	Oleuropein 2 mg/l + 0.4% AAB
	OL200	Oleuropein 20 mg/l + 0.4% AAB
B2	C2	None (negative control)
	AAB	Acetic acid bacteria 200ppm (positive control)
	OL0.1	Oleuropein 0.1 mg/l + 200ppm AAB
	OL0.4	Oleuropein 0.4 mg/l + 200ppm AAB
B3	C3	None
	E	EtOH 10%
	H1	Hydroxytyrol 1 mg/l
	H2	Hydroxytyrol 2 mg/l
	OL100	Oleuropein 100 mg/l
	OL500	Oleuropein 500 mg/l
B4	C4	None (negative control)
	AAB	Acetic acid bacteria (positive control)
	H0.5	Hydroxytyrol 0.5 mg/l + 0.4% AAB
	H1	Hydroxytyrol 1 mg/l + 0.4% AAB
	OL50	Oleuropein 100 mg/l + 0.4% AAB
	OL400	Oleuropein 100 mg/l + 0.4% AAB
	OL800	Oleuropein 100 mg/l + 0.4% AAB

Wine	abbreviation	Wine treatment
B5	AAB	1,2 % AAB
	H1	Hydroxytyrol 1 mg/l + 1,2 % AAB
	OL1	Oleuropein 1 mg/l + 1,2 % AAB
	OL2	Oleuropein 2 mg/l + 1,2 % AAB

Table 2.5 Time points of measurements of volatile and titratable acidity of wines

Tested wine	Titratable acidity	Volatile acidity
B1	0 days, 2 days, 5 days, 1 week, 10 days, 1 month	7 weeks
B2	0 days, 1 week, 2 weeks, 3 weeks	7 weeks
B3	-	0 months, 1 month, 2 months
B4	0 days, 1 week, 2 weeks, 3 weeks	5 weeks
B5	0 days, 1 week, 1 month	1 month

Titratable (total) acidity was monitored through time before and after the addition of additives in wines. Titratable acidity was measured for indication of the possible increase of volatile acidity. Approximately one month later, volatile acidity was measured. For titratable acidity, 10 ml of wine samples were titrated with 0,1N NaOH. 10 drops of Bromothymol blue indicator were added to the 10ml wine samples prior to titration, for the determination of the endpoint of the reaction. Total (titratable) acidity (TA) was determined using the equation:

$TA= V_{NaOH}/V_{sample} *7.5.$.

Volatile acidity analysis was made by steam distillation of 20 ml of wine, collecting 250 ml of distillate. Titration followed using 0.1N sodium hydroxide solution. Phenolophthalein was used as indicator (methodology according to OIV, Compendium of International Methods of Analyses). A DE-1626 J.P. Selecta steam distillator was used for that purpose. Volatile acidity (VA) was determined in gr/l of acetic acid, following the equation $VA = V_{NaOH} / V_{sample}*6$.

In B1wine, colour intensity (I) and hue (T) were measured, by absorption of 1ml of sample at WL420, WL520 and WL620nm using a Hitachi U-2000 spectrophotometer (I=Abs420 + Abs520 + Abs620, T= Abs 420/ Abs 520).

2.3 Phenolic content and antioxidant activity of wine with natural phenolic additives.

Olive oil mills waste water and grape pomace wineries residues are very rich in phenolic content and substances possessing antioxidant activities. Extracts were obtained from olive oil mill and winery residues and were used in several concentrations at the end of vinification and before adding the wines to stainless steel tanks, in replacement for sulphites. Grape pomace (GP) of Mandilari cultivar, obtained by Miliarakis winery in Heraklion area, was previously used to obtain a semi solid extract reach in polyphenols. A semi - solid residue obtained from Olive oil mill waste water treated with resins was also used in the assay (OMW).

Phenolic content and antioxidant activity of the obtained wines were determined. Vilana grapes were used for the production of wine in Miliarakis winery. The obtained wines and the extract concentration used are described in table 2.6.

Table 2.6 Vilana wines produced with addition of natural polyphenolic extracts as a replacement for sulphites and the treatments performed. Vilana grapes were used for the vinification of each wine. GP: grape pomace extract, OMW: olive oil mill waste water residue.

Wine	Treatment
C	Control
V1	GP 5gr/100l
V2	GP, 7.5gr/100l
O1	OMW 55%, 0.875gr/100l
O2	OMW 55%, 2.67gr/100l
O3	OMW 95%, 2.7gr/100l

Antioxidant activity of sulphites in the form of SO_2, OMR and GP were determined using the TEAC method as previously described. Antioxidant activity of sulphites was determined. The amount of the natural extracts used in each treatment was determined based on the antioxidant activity of sulphites. Amounts of OMR and GP were added in wines, in levels that would lead to at least an equal antioxidant activity to sulphites.

2.4 Statistical analysis

For the statistical analysis of the results one-way analysis of variance (ANOVA) was used followed by Duncan's multiple range tests ($p = 0.05$) in order to determine significant differences between the tested samples. The correlation of antioxidant activity and phenolic content of wines was determined by using Pearson product-moment correlation coefficient measure. The statistical analysis and handling of data was performed using SPSS (SPSS statistics, version 21) and PCA, PLS analysis were performed using JMP 11. The graphs were produced using Microsoft Excel 2007.

CHAPTER THREE

RESULTS

3.1 Total phenolic content, phenolic fingerprints and antioxidant activity of Vilana, Dafni, Kotsifali and Mandilari wines.

The effect of type of barrel on Vilana, Dafni, Kotsifali and Mandilari wine total phenolic content and antioxidant activity was determined in order to monitor the changes occurring during ageing and to determine the effect of barrel type on the above characteristics.

Phenolic concentration and total antioxidant activity of Vilana, Dafni, Kotsifali and Mandilari wines were determined after two different vinifications in 2012 and 2013. After each vinification, total phenolic content and total antioxidant activity were determined directly after vinification and after 3, 6, 9 and 12 month ageing in the different containers.

Phenolic fingerprints of the tested wines were taken in the mid-ir region of the spectra. The observed changes in the spectra of each wine in different containers were monitored during ageing.

3.1.1 Total phenolic content of Vilana, Dafni, Kotsifali and Mandilari wines during ageing in different containers

3.1.1.1 Results of the first year of vinification (2012)

A. Total phenolic content directly after vinification

Total phenolic content (TPC) of single variety wines produced by Vilana, Dafni, Kotsifali and Mandilari grapes was determined directly after vinification. The total phenolic content of each wine is shown in Table 3.1. Phenolic content of red wines was approximately 3-7 times higher than phenolic content of white wines.

Table 3.1 Total phenolic content (TPC) of wines. Results are expressed in mg/l gallic acid.

WINE	TPC*
Vilana	349.7
Dafni	352.0
Kotsifali	1115.5
Mandilari	2383.6

*mean values

B. Total phenolic content of Vilana wines during ageing.

Figure 3.1 shows total phenolic content of Vilana wine in each type of container directly after vinification and after 3, 6, 9 and 12 month period of ageing. Different letters within containers represent statistical differences in total phenolic content of wine within each container (Duncan's multiple rang tests, p = 0.05).

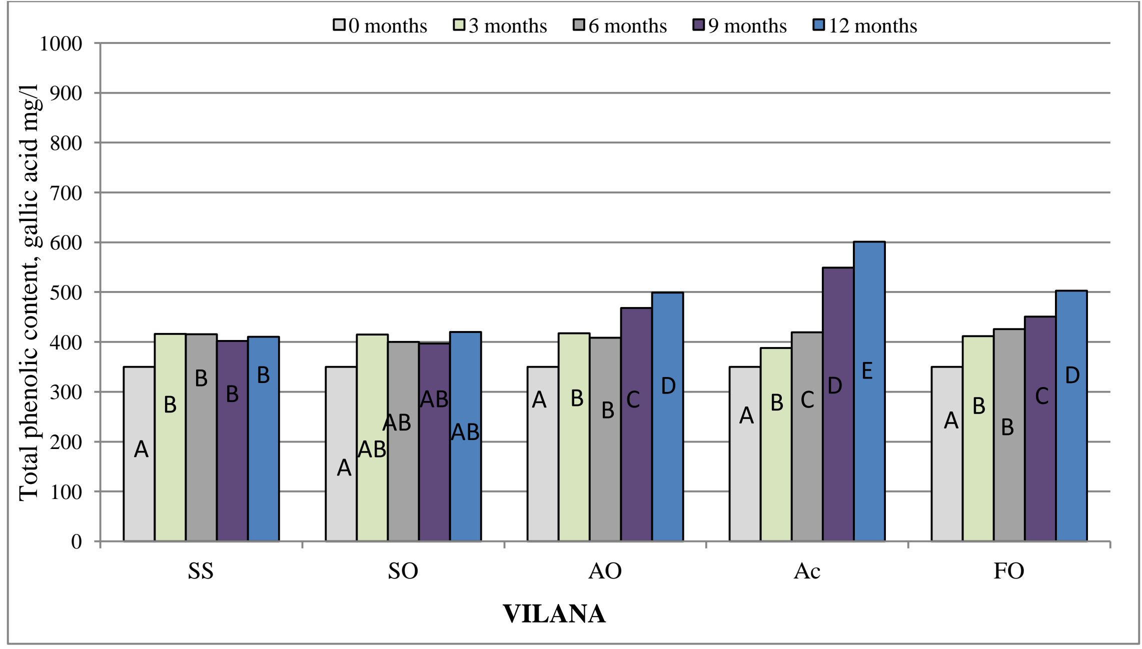


Figure 3.1 Changes in total phenolic content of Vilana wine ageing in SS, SO, AO, Ac and FO containers, through time. Measurements were taken directly after vinification and after 3, 6, 9 and 12 months ageing period in the containers. Different letters in columns represent statistical

differences within each container. SS: stainless steel container, SO: stainless steel with oenosticks, AO: American oak barrel, Ac: acacia barrel, FO: French oak barrel.

Minor changes were observed in stainless steel containers with and without oenosticks through ageing; phenolic content after a slight increase in the first months of ageing, remained almost stable. In contrast, wooden barrels showed a significant increase in the phenolic content. The observed differences within each wooden barrel type were statistically significant during ageing (ANOVA, p = 0.05).

The differences in total phenolic content, comparing the different containers after three months of ageing was not statistically significant. However, after 6 and 9 and 12 months of ageing phenolic content showed a significant difference (ANOVA, p = 0.05).

TPC increased in all containers after 12 month ageing, reaching in acacia barrels significantly higher TPC than that in any other container (Figure 3.2) (Duncan's multiple range tests, p = 0.05). Compared to the TPC directly after vinification wines wine in acacia barrel had the highest increase (72%), followed by French oak (43.9%) and American oak barrels (42.7%).

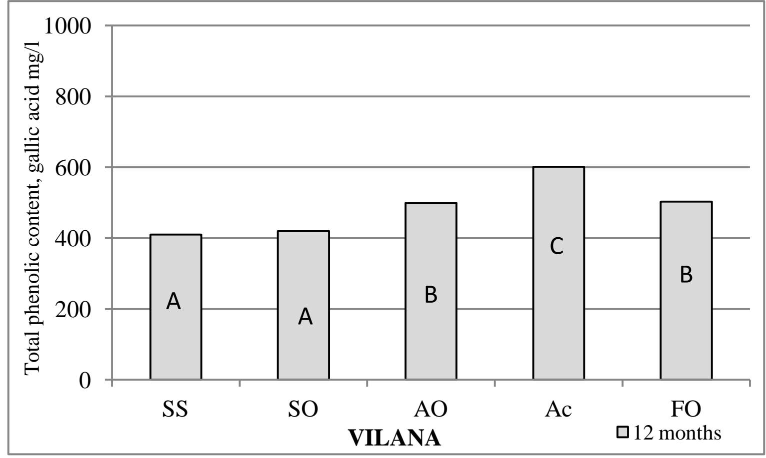


Figure 3.2 Total phenolic content of Vilana wine ageing in SS, SO, AO, Ac and FO containers, after 12 months of ageing in different containers. Different letters represent statistical differences between containers. SS: Stainless steel container, SO: stainless steel with oenosticks, AO: American oak barrel, Ac: acacia barrel, FO: French oak barrel.

C. Total phenolic content of Dafni wines during ageing

In Figure 3.3 changes in total phenolic content during ageing of Dafni wine in different containers are displayed. Different letters within the containers represent statistical differences within containers (Duncan, p = 0.05).

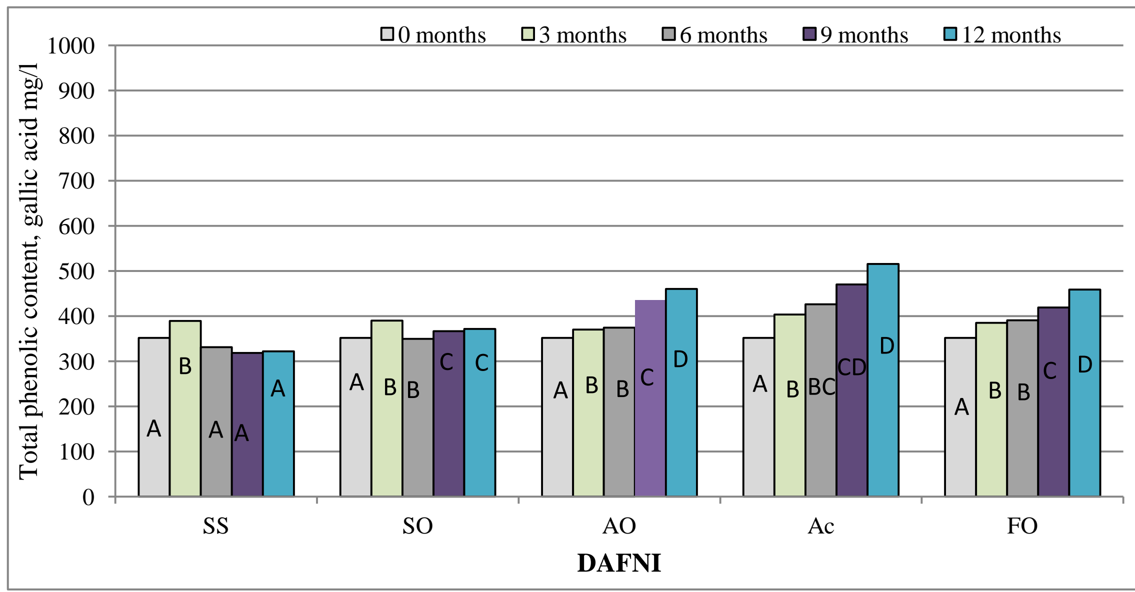


Figure 3.3 Changes in total phenolic content of Dafni wine ageing in SS, SO, AO, Ac and FO containers, through time. Measurements were taken directly after vinification and after 3, 6, 9 and 12 months ageing period in the containers. Different letters in columns represent statistical differences within each container. SS: stainless steel container, SO: stainless steel with oenosticks, AO: American oak barrel, Ac: acacia barrel, FO: French oak barrel.

Slight changes were observed within stainless steel with and without oenosticks containers through ageing. Total phenolic content increased constantly through time in American oak, acacia and French oak barrels. TPC in acacia barrel was significantly higher from any other container after 6 months of ageing. (Duncan, p = 0.05).

After 12 months of ageing, total phenolic content had increased by 46.5% in acacia, by 30.7% in American oak, and by 30.3% in French oak barrels (Figure 3.4). Compared to wine in stainless steel containers, total phenolic content in acacia barrels, was 60.1% higher.

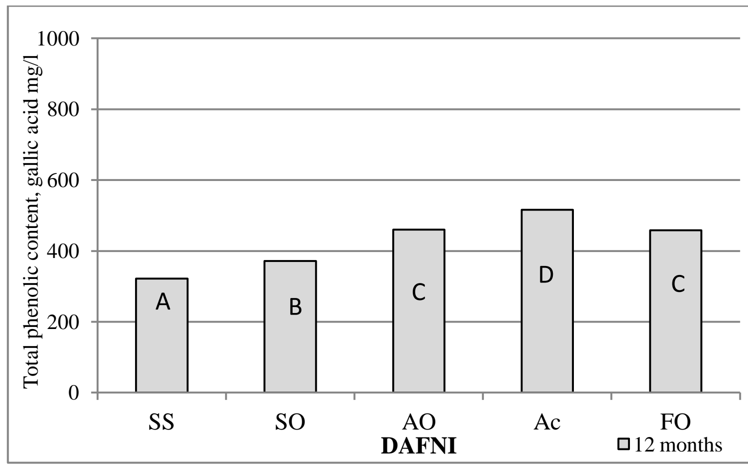


Figure 3.4 Total phenolic content of Dafni wine ageing in SS, SO, AO, Ac and FO containers, through after 12 months of ageing in different containers. Different letters represent statistical differences between containers (Duncan, p = 0.05). SS: Stainless steel container, SO: stainless steel with oenosticks, AO: American oak barrel, Ac: acacia barrel, FO: French oak barrel.

D. Total phenolic content of Kotsifali wines during ageing

Changes in total phenolic content during ageing of Kotsifali in different containers are shown in Figure 3.5.

Total phenolic content, during the first 6 months of ageing within each container, was significantly lower than that observed in the beginning of the experiment with the exception of wine in chestnut barrel. The lowest value was observed in stainless steel container at 6 months of ageing, being 31.9% lower than that in the beginning of the experiment. TPC increased during the last 6 months of ageing, in all the containers.

Comparing the phenolic content between different containers at 3, 6 and 9 months of ageing, phenolic content in chestnut barrel, was constantly statistically significant different than in the rest of the containers.

After 12 months of ageing, phenolic content, compared to the initial one was found to be 51.8% increased in chestnut barrel and 36.4% increased in acacia barrel. Results of Duncan's multiple range tests (p = 0.05) between containers at 12 months of ageing are shown in Figure 3.6. Different letters in columns indicate statistical differences amongst the containers.

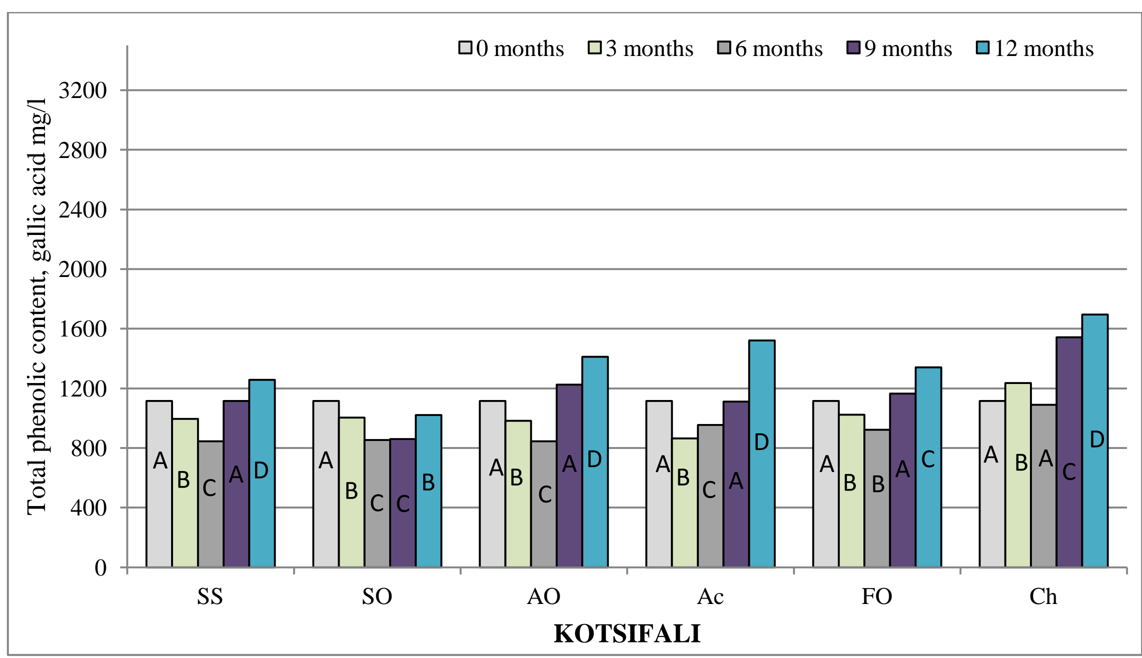


Figure 3.5 Changes in total phenolic content of Kotsifali wine ageing in SS, SO, AO, Ac, FO and Ch containers, through time. Measurements were taken directly after vinification and after 3, 6, 9 and 12 months ageing period in the containers. Different letters in columns represent statistical differences within each container. SS: stainless steel container, SO: stainless steel with oenosticks, AO: American oak barrel, Ac: acacia barrel, FO: French oak barrel, Ch: chestnut barrel.

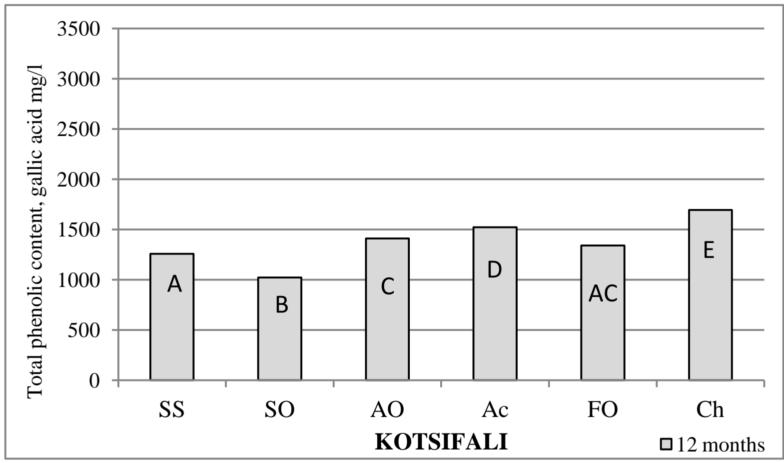


Figure 3.6 Total phenolic content (TPC) of Kotsifali wines in different containers at 12 months of ageing. Different letters in columns represent statistical differences amongst the containers. SS: Stainless steel container, SO: stainless steel with oenosticks, AO: American oak barrel, Ac: acacia barrel, FO: French oak barrel, Ch: chestnut barrel.

E. Total phenolic content of Mandilari wines during ageing

Changes in total phenolic content of Mandilari wine during ageing in different containers are shown in Figure 3.7.

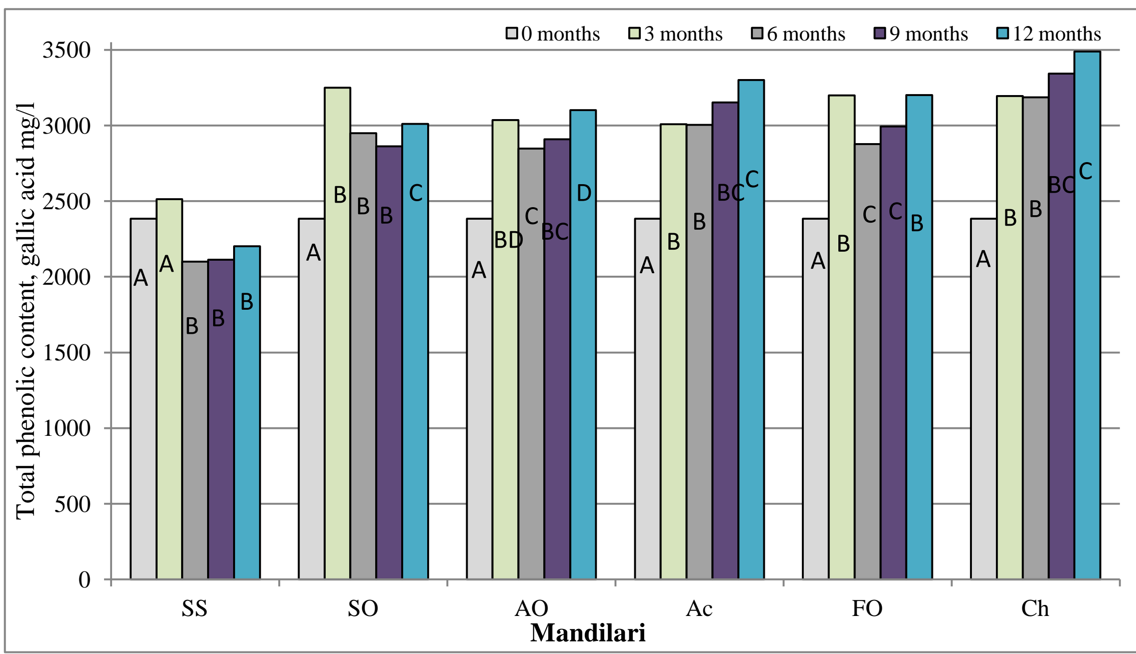


Figure 3.7 Changes in total phenolic content of Mandilari wine ageing in SS, SO, AO, Ac, FO and Ch containers, through time. Measurements were taken directly after vinification and after 3, 6, 9 and 12 months ageing period in the containers. Different letters in columns represent statistical differences within each container. SS: stainless steel container, SO: stainless steel with oenosticks, AO: American oak barrel, Ac: acacia barrel, FO: French oak barrel, Ch: chestnut barrel.

With the exception of stainless steel container, phenolic content in each container increased through ageing; the highest increase in each container occurred during the first three months of ageing, varying from 5.9% in stainless steel container to 34.9% in French oak barrel. One-way analysis of variance ($p = 0.05$) revealed statistically significant differences in total phenolic content amongst the containers at 3, 6, 9 and 12 months of ageing in containers. It was observed that after the 3rd month, phenolic content in chestnut barrel was constantly significant higher from the rest of the containers (Duncan, $p = 0.05$).

Statistically significant differences between the containers after 12 months of ageing, are shown in Figure 3.8 (p = 0.05). Different letters in columns indicate differences amongst the containers. Total phenolic content in Mandilari was found increased in most of the containers after 12 months of ageing (compared to phenolic concentration directly after vinification. The highest total phenolic content was observed in wine ageing in chestnut barrel. The observed increase since the begging of the experiment in Chestnut, Acacia, French oak and American oak barrels and barrels and stainless steel with oenosticks container was 46.9%, 39.1%, 34.9%, 30.7% and 29% respectively. In the case of stainless steel container a slight decrease (5.8%) was observed.

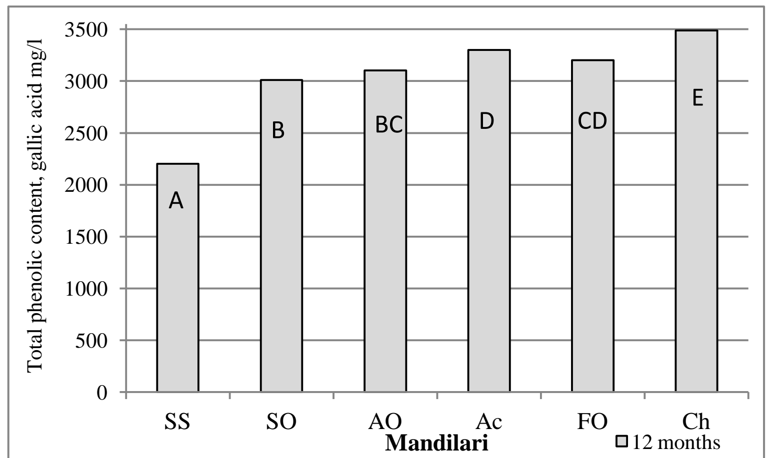


Figure 3.8 Total phenolic content (TPC) of Mandilari wines in different containers at 12 months of ageing. Different letters represents statistical differences amongst the containers. SS: stainless steel container, SO: stainless steel with oenosticks, AO: American oak barrel, Ac: acacia barrel, FO: French oak barrel, Ch: chestnut barrel.

3.1.1.2 Results of the second year of vinification (2013)

A. Phenolic content of wines directly after vinification

Total phenolic content (TPC) of the tested wines was determined directly after vinification. The results are shown in Table 3.2. Phenolic content of red wines was approximately 2-9 times higher than that determined in white wines.

Table 3.2 Total phenolic content (TPC) of wines directly after vinification. Results are expressed in mg/l gallic acid.

WINE	TPC*
Vilana	425.9
Dafni	288.6
Kotsifali	1022.7
Mandilari	2786.4

*Mean values

B. Total phenolic content of Vilana wines during ageing

Figure 3.9 represents total phenolic content of Vilana wine in each container during ageing. The different letters within containers reveals significant differences amongst TPC of each measurement point (Duncan's multiple range tests, p = 0.05).

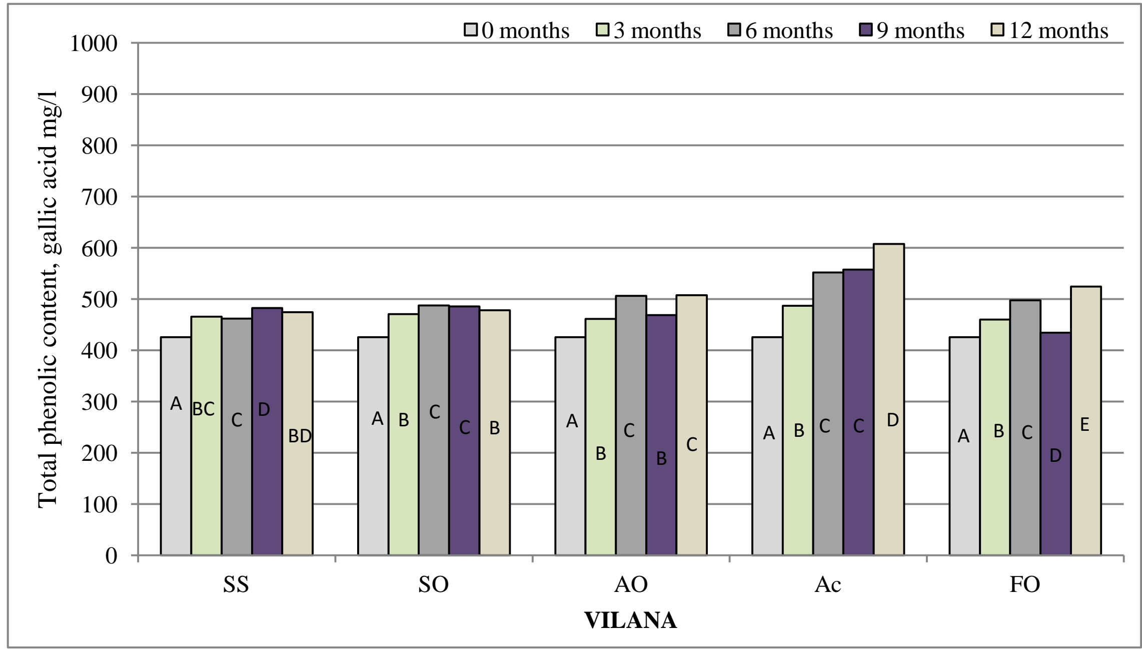


Figure 3.9 Changes in total phenolic content of Vilana wine ageing in SS, SO, AO, Ac and FO containers, through time. Measurements were taken directly after vinification and after 3, 6, 9 and 12 months ageing period in the containers. Different letters in columns represent statistical

differences within each container. SS: stainless steel container, SO: stainless steel with oenosticks, AO: American oak barrel, Ac: acacia barrel, FO: French oak barrel.

Phenolic content (TPC) increased in all the containers; the biggest differences were observed in wooden barrels – especially in acacia barrel. TPC of wine in American oak and in French oak barrel after nine months of ageing was found to be significant lower than TPC observed at 6 and at 12 months of ageing in each container (Duncan, $p = 0.05$).

Comparison between the different containers after 3, 6, 9 and 12 months, revealed that phenolic content in acacia barrels was constantly statistically significant higher than any other container. After 3 months of ageing, comparing the phenolic content of the different containers, except from acacia barrel, there were no any statistically significant differences. Comparing phenolic content in stainless steel with and without oenosticks containers through ageing, showed only small differences between them.

Total phenolic content of wine in acacia barrel was the highest amongst all containers after 12 months of ageing (Figure 3.10), displaying higher total phenolic content than any other container, since the 6th month of ageing. After 12 months of ageing TPC in acacia barrel, was 42.6% higher than the initial one. Statistical differences in phenolic content at 12 months of ageing between containers are indicated with different letters in columns of Figure 3.10 (Duncan, $p = 0.05$).

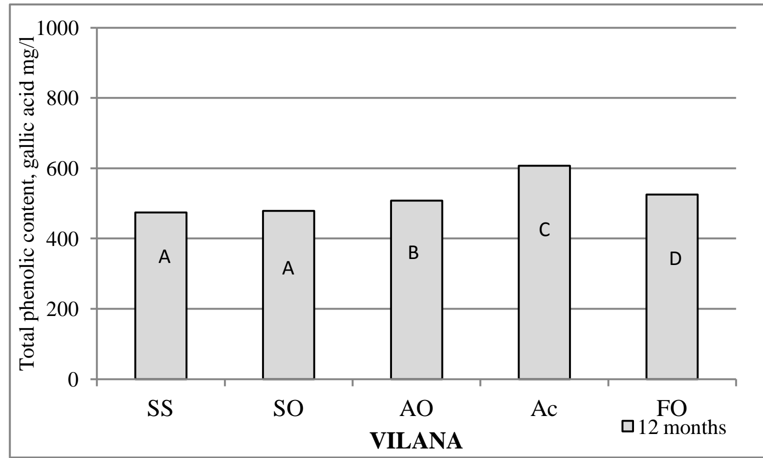


Figure 3.10 Total phenolic content of Vilana wine ageing in SS, SO, AO, Ac and FO containers after 12 months of ageing. Different letters in columns indicate statistically significant differences. SS: stainless steel container, SO: stainless steel with oenosticks, AO: American oak barrel, Ac: acacia barrel, FO: French oak barrel.

C. Total phenolic content of Dafni wines during ageing

Changes in total phenolic content of Dafni wine in each container are shown in Figure 3.11. Different letters within columns represent statistically significant differences (Duncan, p = 0.05).

One-way analysis of variance for Dafni in stainless steel with and without oenosticks containers revealed that, although not very intense, differences in total phenolic content of each wine during ageing were statistically significant (p = 0.05). The major differences in TPC were observed in phenolic content of wines in acacia barrel, increasing constantly since the beginning of the experiment. During the first 3 months of ageing TPC was statistically significant increased only in acacia barrels, increasing constantly through time.

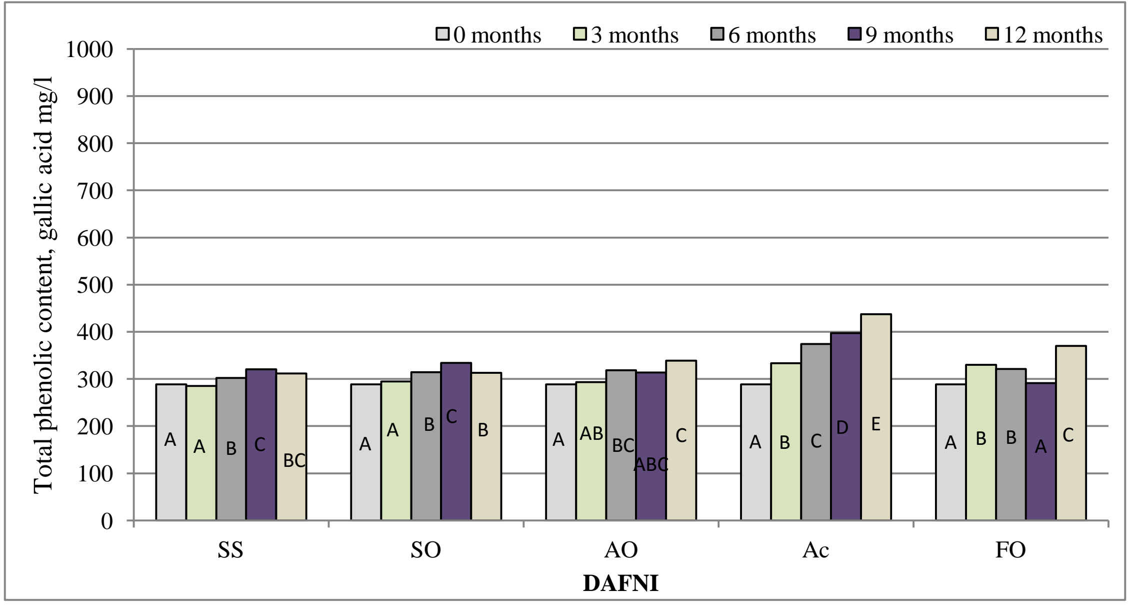


Figure 3.11 Changes in total phenolic content of Dafni wine ageing in SS, SO, AO, Ac and FO containers, through time. Measurements were taken directly after vinification and after 3, 6, 9 and 12 months of ageing in the containers. Different letters in columns represent statistical differences within each container. SS: stainless steel container, SO: stainless steel with oenosticks, AO: American oak barrel, Ac: acacia barrel, FO: French oak barrel.

Comparing the different containers through ageing, similarly to Vilana wines, phenolic content in acacia barrels was constantly statistically significant higher than that observed in any other container. In all containers, total phenolic content reached the highest levels after 12 months of ageing (Figure 3.11). In comparison with phenolic content of wine directly after vinification, total phenolic content was 51.5% higher in acacia barrels and 28.4% higher in French oak barrels. Similarly to Vilana wine, Dafni in FO barrels after 9 months of ageing had significant lower TPC than that after 6 and 12 months of ageing (Duncan, p = 0.05).

Differences in phenolic content of wines at 12 months of ageing between containers are shown in Figure 3.12 (Duncan, p = 0.05).

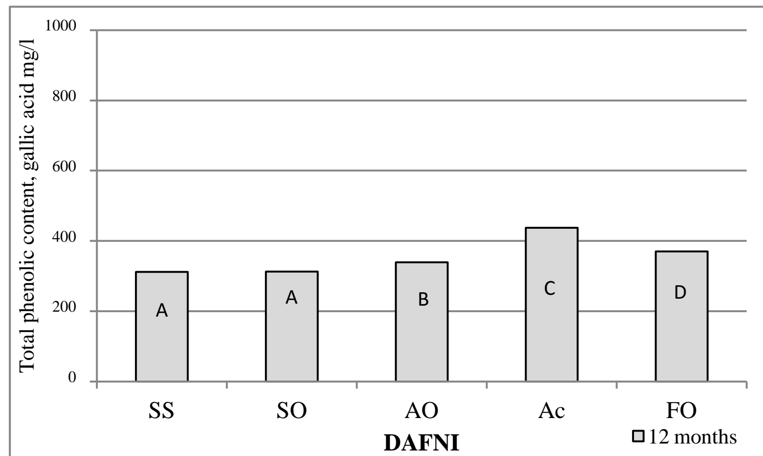


Figure 3.12 Total phenolic content of Dafni wine ageing in SS, SO, AO, Ac and FO containers after 12 months of ageing. Different letters in columns indicate statistically significant differences. SS: stainless steel container, SO: stainless steel with oenosticks, AO: American oak barrel, Ac: acacia barrel, FO: French oak barrel.

D. Total phenolic content of Kotsifali wines

Changes in total phenolic content of Kotsifali wines ageing for 12 months in different containers are shown in Figure 3.13. In general, in stainless steel and stainless steel with oenosticks containers no or minor changes were observed during ageing. Phenolic content in all wooden barrels increased during ageing. The highest increase in all the wooden barrels was observed during the last 3 months of ageing. In acacia and chestnut barrels, phenolic content increased constantly through ageing, whereas in American and French oak barrel, a decrease was observed from the 6th to 9th month of ageing.

Total phenolic content in Kotsifali wines increased after 12 months in containers by 41.9% in chestnut barrel, 37% in acacia, 33.4% in American oak and 21.7% in French oak barrels. As shown in Figure 3.14 phenolic content was not differentiated in stainless steel with and without oenosticks containers, on the contrary to phenolic content of wines in wooden barrels (Duncan, p = 0.05).

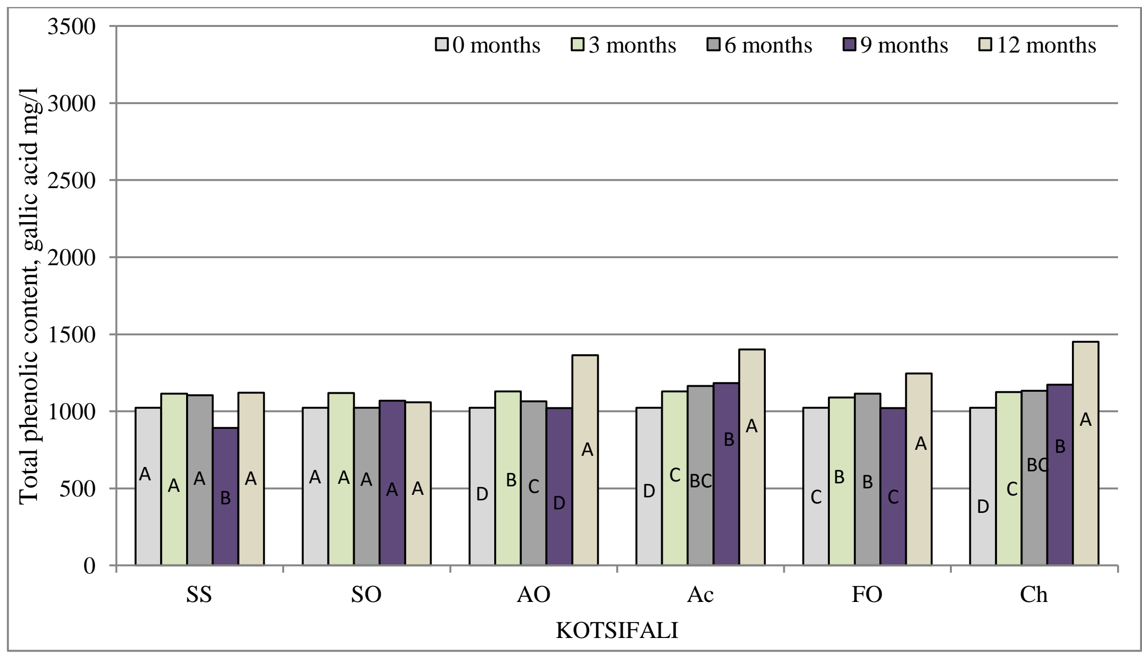


Figure 3.13 Changes in total phenolic content of Kotsifali wine ageing in SS, SO, AO, Ac and FO containers, through time. Measurements were taken directly after vinification and after 3, 6, 9 and 12 months ageing period in the containers. Different letters in columns represent statistical differences within each container. SS: stainless steel container, SO: stainless steel with oenosticks, AO: American oak barrel, Ac: acacia barrel, FO: French oak barrel, Ch: chestnut barrel.

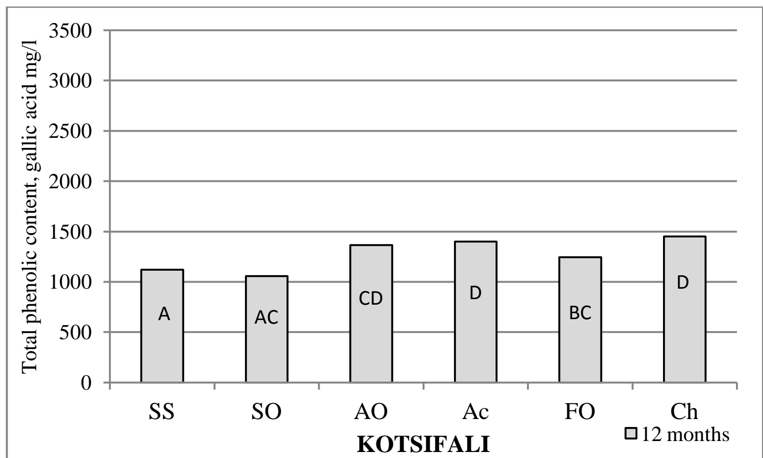


Figure 3.14 Total phenolic content of Kotsifali wine ageing in SS, SO, AO, Ac and FO containers, after 12 months of ageing in the containers. SS: Stainless steel container, SO: stainless steel with oenosticks, AO: American oak barrel, Ac: acacia barrel, FO: French oak barrel, Ch: chestnut barrel.

E. Total phenolic content of Mandilari wines

Changes in total phenolic content of Mandilari wine ageing in different containers is shown in Figure 3.15.

In general, phenolic content increased during the first 6 months of ageing. In most of them the increase took place from the 3rd to 6th month of ageing. A statistically significant decrease was observed in total phenolic content after 9 months of ageing in American oak, French oak and stainless steel container, reaching the initial levels. During the 12 month ageing, changes were most intense in chestnut, acacia and French oak barrel.

Total phenolic content increased from 17.8% in chestnut barrel, to 4.4% in stainless steel with oenosticks container after 12 months of ageing, compared to total phenolic content directly after vinification. Statistical differences in total phenolic content between the containers are indicated with different letters in Figure 3.16 (Duncan, p = 0.05). Compared with phenolic content of wine in stainless steel container, phenolic content was 12.9% higher in wine in chestnut barrel.

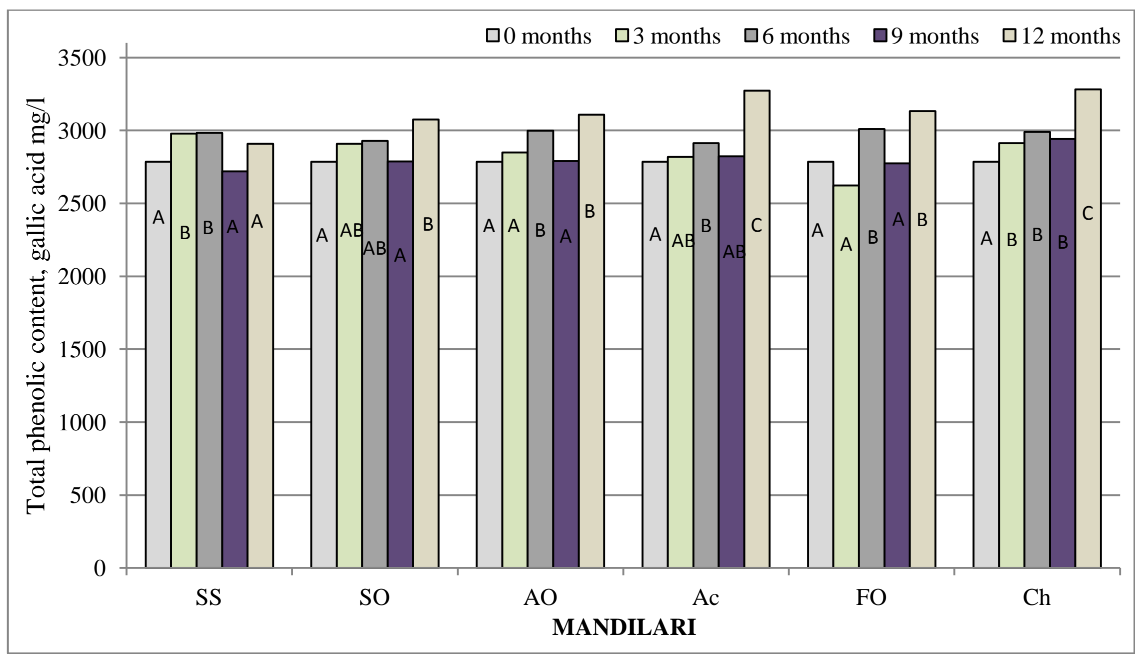


Figure 3.15 Changes in total phenolic content of Mandilari wine ageing in SS, SO, AO, Ac and FO containers, through time. Measurements were taken directly after vinification and after 3, 6, 9 and 12 month ageing in the containers. Different letters in columns represent statistically significant differences within each container. SS: stainless steel container, SO: stainless steel with oenosticks, AO: American oak barrel, Ac: acacia barrel, FO: French oak barrel, Ch: chestnut barrel.

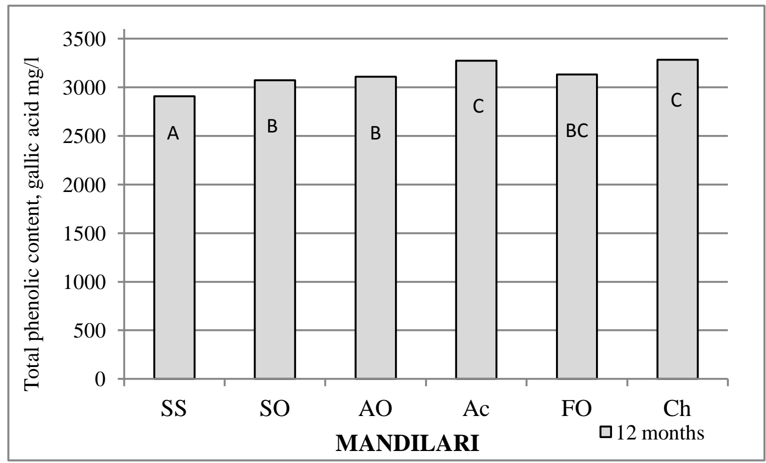


Figure 3.16 Total phenolic content of Mandilari wine ageing in SS, SO, AO, Ac, FO and Ac containers, after 12 months of ageing in the containers. Different letters in columns represent statistically significant differences between the containers. SS: Stainless steel container, SO: stainless steel with oenosticks, AO: American oak barrel, Ac: acacia barrel, FO: French oak barrel, Ch: chestnut barrel.

The results of ANOVA for the determination of significant differences in TPC between the containers and during ageing are summarised in Table 3.3 and |Table 3.4.

Table 3.3: Comparison of the phenolic content of wines between the different containers after 3, 6, 9 and 12 months of ageing. Results of one-way ANOVA (significance level p = 0.05).

	1st vinification	2nd vinification
	VILANA	
3 months	NS	S
6 months	S	S
9 months	S	S
12 months	S	S
	DAFNI	
3 months	NS	S
6 months	S	S
9 months	S	S
12 months	S	S
	KOTSIFALI	
3 months	S	NS
6 months	S	S
9 months	S	S
12 months	S	S
	MANDILARI	
3 months	S	S
6 months	S	NS
9 months	S	S
12 months	S	S

Table 3.4: Comparison of the phenolic content of wines within each container during ageing. Results of one-way ANOVA (significance level p = 0.05).

	1st vinification	2nd vinification
	VILANA	
SS	S	S
SO	NS	S
AO	S	S
Ac	S	S
FO	S	S
	DAFNI	
SS	S	S
SO	S	S
AO	S	S
Ac	S	S
FO	S	S
	KOTSIFALI	
SS	S	S
SO	S	NS
AO	S	S
Ac	S	S
FO	S	S
Ch	S	S
	MANDILARI	
SS	S	S
SO	S	S
AO	S	S
Ac	S	S
FO	S	S
Ch	S	S

3.1.2 Total antioxidant activity (TEAC) of Vilana, Dafni, Kotsifali and Mandilari wines

3.1.2.1 Results of the first year vinification (2012)

A. Total antioxidant activity (TEAC) of wines directly after vinification

Total antioxidant activity of Vilana, Dafni, Kotsifali and Mandilari wines was determined directly after vinification. Total antioxidant activity of red wines, Kotsifali and Mandilari directly after vinification, was found approximately 9-17 times greater than that of white wines, Vilana and Dafni. Total antioxidant activity of wines directly after vinification (0 months) is shown in Table 3.5, in mM of Trolox.

Table 3.5 Total antioxidant activity (TEAC) of first vinification wines. Results are expressed in mM of Trolox.

WINE	TEAC*
Vilana	1.138
Dafni	1.569
Kotsifali	9.815
Mandilari	19.280

*Mean values

B. Antioxidant activity (TEAC) of Vilana wines during ageing

Changes in total antioxidant activity (TEAC) of Vilana ageing are shown it Figure 3.17. Different letters in columns reveal statistically significant differences within each container during ageing (Duncan's multiple range tests, p = 0.05).

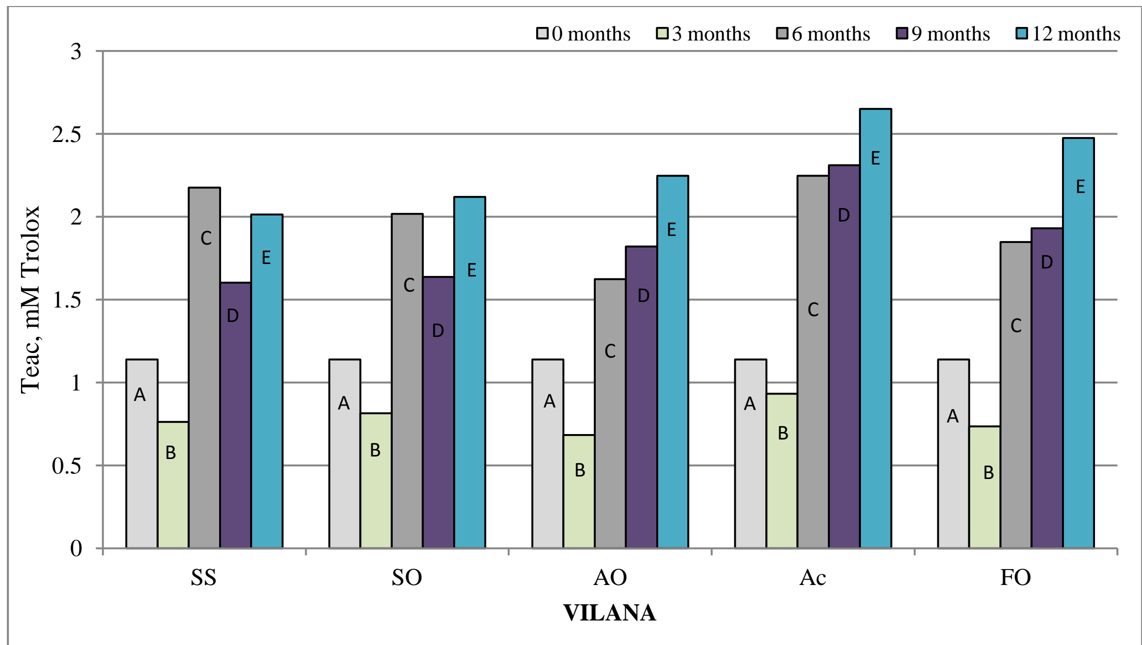


Figure 3.17 Changes in total antioxidant activity of Vilana wine ageing in SS, SO, AO, Ac and FO containers, through time. Measurements were taken directly after vinification and after 3, 6, 9 and 12 months ageing period in the containers. Different letters in columns represent statistical differences within each container. SS: stainless steel container, SO: stainless steel with oenosticks, AO: American oak barrel, Ac: acacia barrel, FO: French oak barrel.

In all containers, the antioxidant activity increased compared to antioxidant activity directly after vinification, with the exception of wine at 3 months of ageing, when it decreased significantly. The decrease varied from 21.3% in acacia to 43% in American oak barrel. The highest level in antioxidant activity was observed in Acacia barrel, followed by French oak barrel. Even though antioxidant activity in American oak barrel at 6 months of ageing was higher than the initial, it was the lowest observed compared to the rest of the containers, including stainless steel with and without oenosticks.

Comparing different containers, differences in antioxidant activity between them, apart from 3 months of ageing, were statistically significant at each time of measurement (Duncan p = 0.05).

Vilana in all containers had the highest total antioxidant activity after 12 months of ageing compared to the initial one. Acacia barrel and French oak barrel had the greatest increase amongst all the containers (132 % in acacia and 117 % in French oak barrel). Stainless steel with oenosticks container, when compared to stainless steel container had a slight but also statistical significant increase in antioxidant activity after twelve months of ageing (p =0.05). In comparison to antioxidant activity in stainless steel container, antioxidant activity in Acacia and French oak barrels was 31.6% and 23.1% higher respectively. Statistically significant differences between the containers are shown in Figure 3.18 (Duncan, p = 0.05).

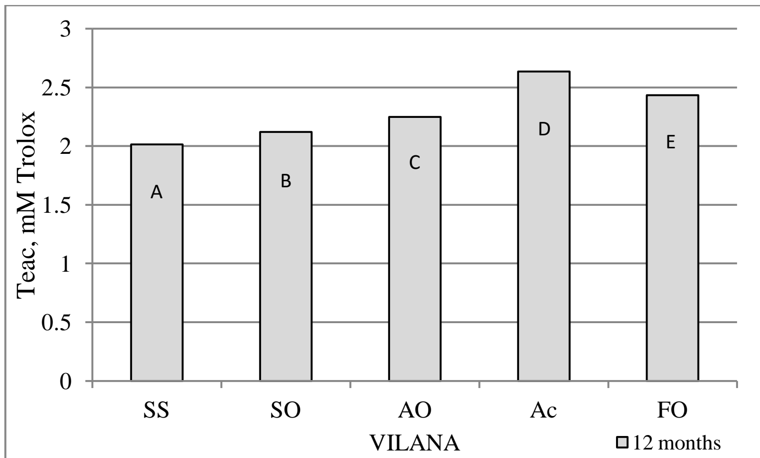


Figure 3.18 Total antioxidant activity of Vilana wine, after 12 month ageing in SS, SO, AO, Ac and FO containers. Different letters within containers, represent statistical differences between containers. SS: Stainless steel container, SO: stainless steel with oenosticks, AO: American oak barrel, Ac: acacia barrel, FO: French oak barrel.

C. Total antioxidant activity (TEAC) of Dafni wines during ageing

Changes in total antioxidant activity (TEAC) of Dafni ageing in different containers are shown it Figure 3.19.

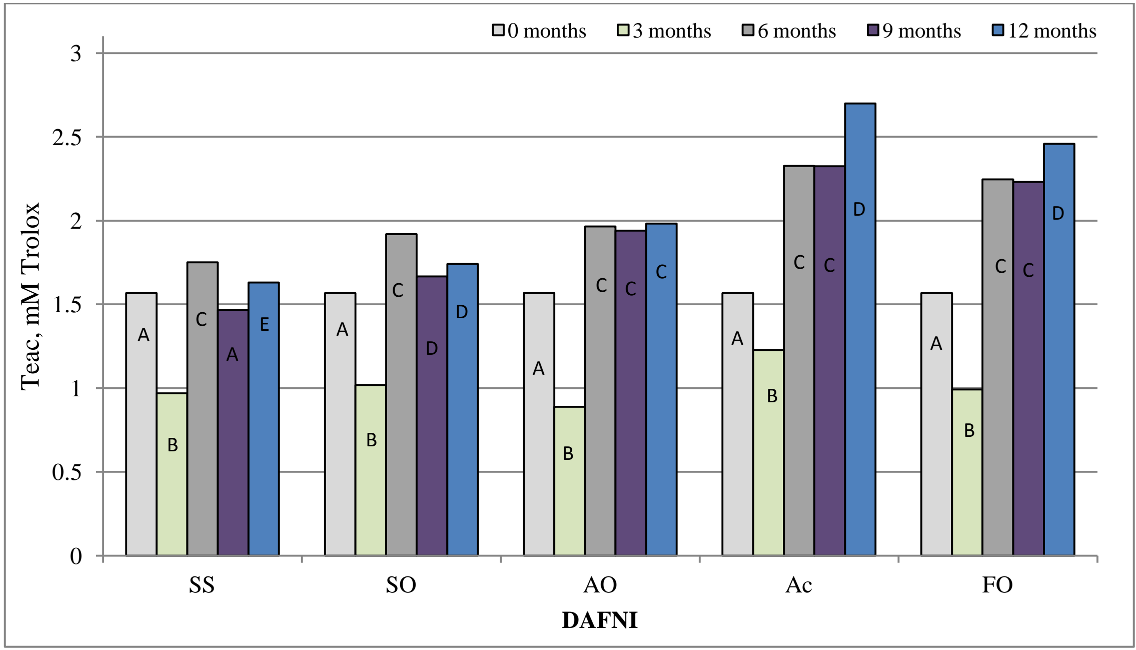


Figure 3.19 Changes in total antioxidant activity of Dafni wine ageing in SS, SO, AO, Ac and FO containers, through time. Measurements were taken directly after vinification and after 3, 6, 9 and 12 months ageing period in the containers. Different letters in columns represent statistical differences within each container. SS: stainless steel container, SO: stainless steel with oenosticks, AO: American oak barrel, Ac: acacia barrel, FO: French oak barrel.

Same to Vilana wine, a statistically significant decrease was observed in antioxidant activity of wines in all the containers, at 3 months of ageing (Figure 3.19). Total antioxidant activity increased significantly afterwards, reaching levels higher than the begging of the experiment. Compared to antioxidant activity at 6 months of ageing, at the end of ageing antioxidant activity increased only in acacia and French oak barrels whereas in stainless steel with and without oenosticks containers was found significant lower. The highest levels of antioxidant activity were observed in acacia and French oak barrels. The differences in antioxidant activity between the containers, after 12 months of ageing, are shown in Figure 3.20 ($p = 0.05$).

The greatest increase in TEAC, compared to antioxidant activity directly after vinification, was observed in wine in acacia barrels (70% increase) followed by wine in French oak barrels (56.6% increase).

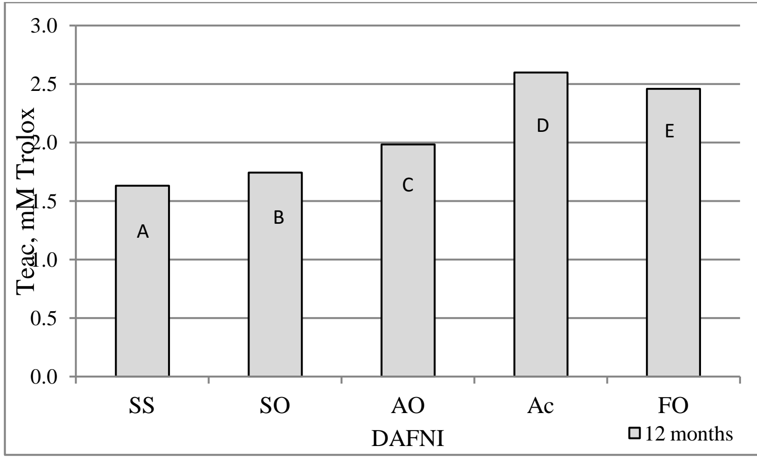


Figure 3.20 Total antioxidant activity of Dafni after 12 months of ageing in containers. Different letters represent statistical differences between containers. SS: Stainless steel container, SO: stainless steel with oenosticks, AO: American oak barrel, Ac: acacia barrel, FO: French oak barrel, Ch: chestnut barrel.

D. Total antioxidant activity (TEAC) of Kotsifali wines

Antioxidant activity of Kotsifali during ageing and is shown in Figure 3.21. The level of antioxidant activity during ageing was, in most of the cases, lower than that observed in the beginning of the experiment. The only exception found was in wine ageing in chestnut barrel, having since the 6th month of ageing higher antioxidant activity than that observed in the beginning of the experiment. After the 3rd month of ageing no changes occurred in antioxidant activity of wines in American oak and stainless steel with and without oenosticks containers, whereas in acacia, French oak and chestnut barrels a constant increase was observed from the 3rd to 9th month of ageing. Antioxidant activity of wine in chestnut barrels, was constantly statistically significant higher that that observed in any other container. (Duncan, $p = 0.05$).

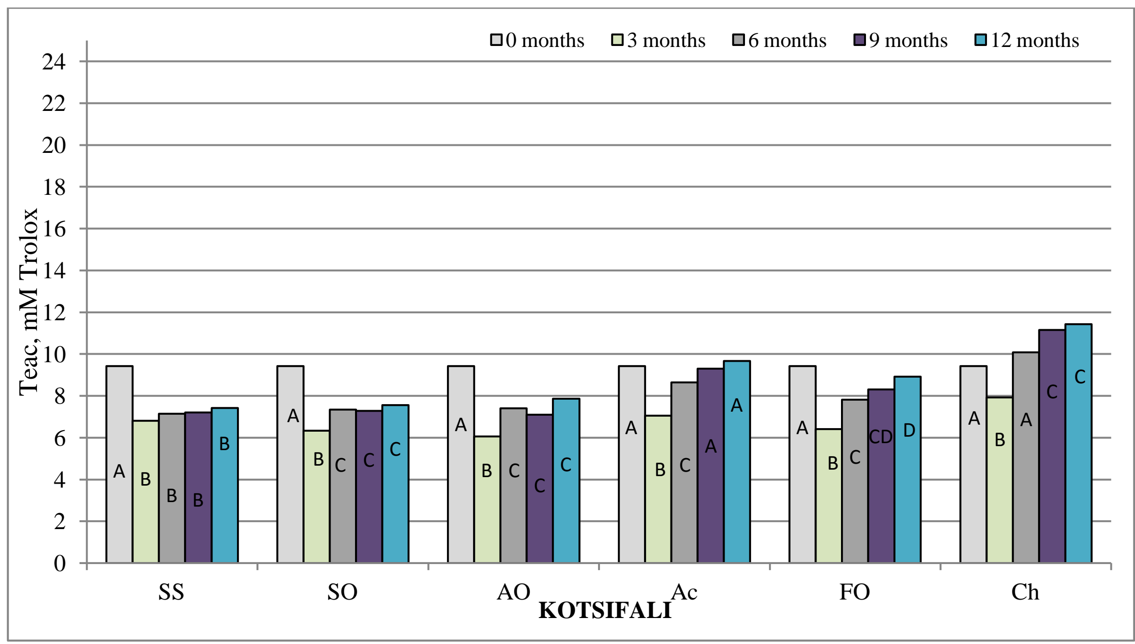


Figure 3.21 Changes in total antioxidant activity (TEAC) of Kotsifali wine ageing in SS, SO, AO, Ac, FO and Ch containers, through time. Measurements were taken directly after vinification and after 3, 6, 9 and 12 months ageing period in the containers. Different letters in columns represent statistical differences within each container. SS: stainless steel container. SO: stainless steel with oenosticks, AO: American oak barrel, Ac: acacia barrel, FO: French oak barrel, Ch: chestnut barrel.

In Figure 3.22 is shown antioxidant activity of Kotsifali wine in the different containers after 12 months of ageing. Compared to total antioxidant activity directly after vinification, TEAC decreased in the cases of wines in stainless steel container, stainless steel with oenosticks container, American oak and French oak barrels, and increased only in chestnut barrel (15% higher than in the beginning of the experiment). The greatest decrease was observed in wine in stainless steel container (15.2% lower). After 3 months of ageing in containers, antioxidant activity decreased in wines in all containers varying from 38.9% in American oak barrel to 27.4% in chestnut barrel.

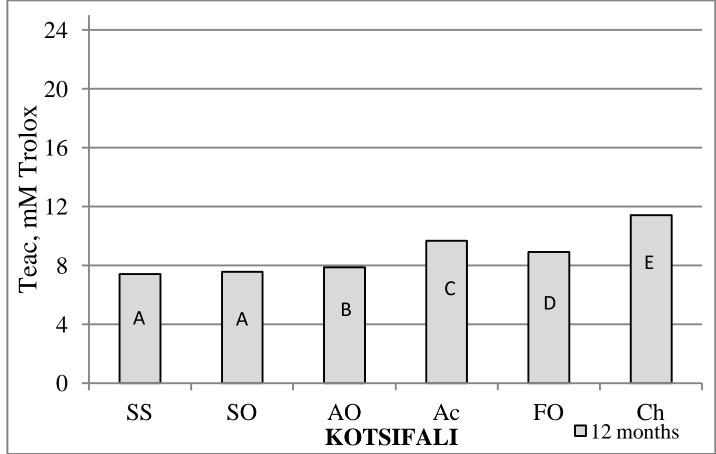


Figure 3.22 Total antioxidant activity of Kotsifali after 12 months of ageing in containers. . Different letters in columns represent statistical differences between containers. SS: Stainless steel container, SO: stainless steel with oenosticks, AO: American oak barrel, Ac: acacia barrel, FO: French oak barrel, Ch: chestnut barrel.

E. Total antioxidant activity (TEAC) of Mandilari wines

Changes in total antioxidant activity of Mandilari wine ageing in different containers for 12 months are shown in Figure 3.23. Antioxidant activity of wines in all containers decreased during the first three months of ageing but afterwards an increase was observed leading to similar (in stainless steel, stainless steel with oenosticks and American oak barrel) or even higher (in chestnut, acacia and French oak barrels) antioxidant activity than the initial.

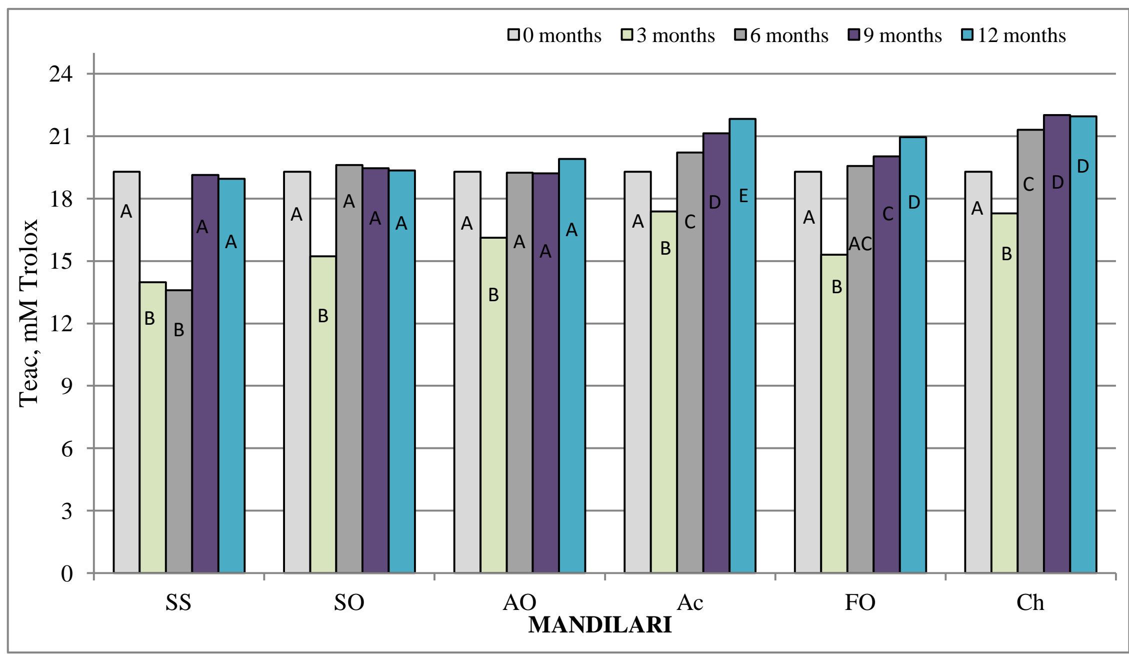


Figure 3.23 Changes in total antioxidant activity of Mandilari wine ageing in SS, SO, AO, Ac, FO and Ch containers, through time. Measurements were taken directly after vinification and after 3, 6, 9 and 12 months ageing period in the containers. Different letters in columns represent statistical differences within each container. SS: stainless steel container, SO: stainless steel with oenosticks, AO: American oak barrel, Ac: acacia barrel, FO: French oak barrel, Ch: chestnut barrel.

Comparing the different containers, antioxidant activity of wines in Chestnut and acacia barrels was always statistically significant higher than the rest of the containers ($p = 0.05$). Antioxidant activity of wine in French oak barrel was significantly lower than that observed in chestnut and acacia barrels.

Compared to total antioxidant activity directly after vinification total antioxidant activity increased by 13.8% in wine in chestnut barrel after 12 months of ageing (Figure 3.24).

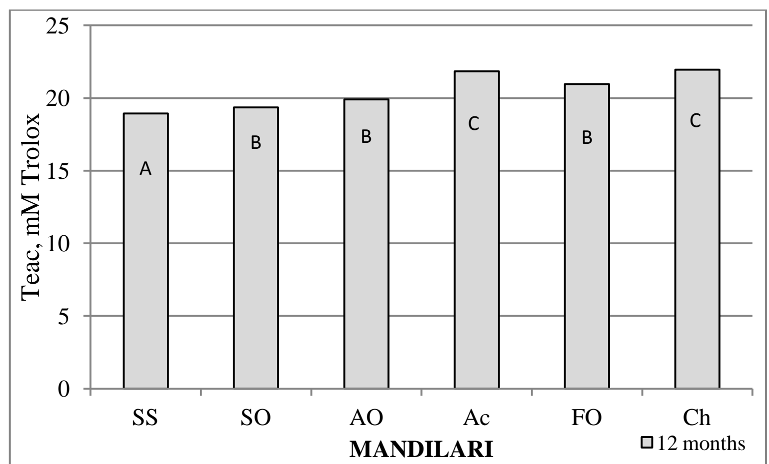


Figure 3.24 Total antioxidant activity of Mandilari in different containers after 12 months of ageing. Different letters represent statistical differences between the containers. SS: stainless steel container, SO: stainless steel with oenosticks, AO: American oak barrel, Ac: acacia barrel, FO: French oak barrel, Ch: chestnut barrel.

Total antioxidant activity of wines decreased after 3 months of ageing in all containers (Figure 3.25). The highest antioxidant activity was observed in chestnut barrel followed by acacia barrel.

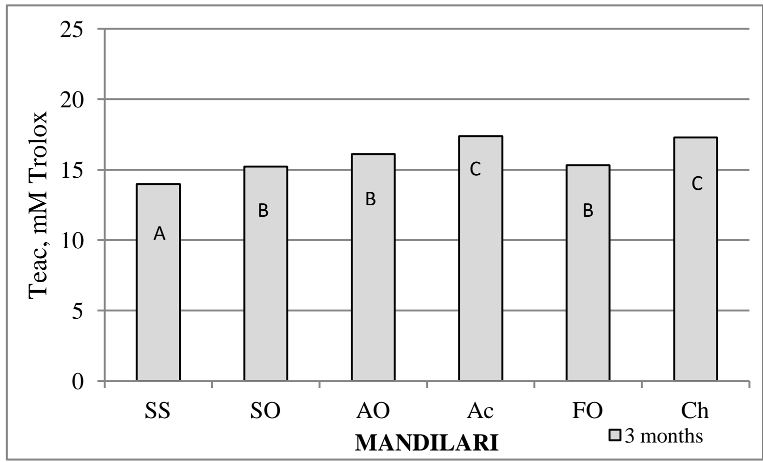


Figure 3.25 Total antioxidant activity of Mandilari in different containers after 3 months of ageing. Different letters in columns represent statistical differences between containers. SS: Stainless steel container, SO: stainless steel with oenosticks, AO: American oak barrel, Ac: acacia barrel, FO: French oak barrel, Ch: chestnut barrel.

3.1.2.2 Results of the second year of vinification (2013)

A. Total antioxidant activity (TEAC) of wines, directly after vinification

Total antioxidant activity directly after vinification, was approximately 6-12 times higher in red wines than that observed in white wines. TEAC directly after vinification (0 months) is shown in Table 3.6, in mM of Trolox.

Table 3.6 Total antioxidant activity (TEAC) of second vinification wines. Results are expressed in mM of Trolox.

WINE	TEAC*
Vilana	2.011293
Dafni	1.374699
Kotsifali	8.353361
Mandilari	17.42835

*Mean values

B. Total antioxidant activity (TEAC) of Vilana wines during ageing

Changes in total antioxidant activity of Vilana wines occurred during ageing for 12 months in different containers, as shown in Figure 3.26.

Antioxidant activity of wines decreased in the first three months of ageing regardless the container. After the 6th month of ageing antioxidant activity increased again resulting in all the containers in higher levels than those observed initially. Wines French oak barrel had the highest antioxidant activity at the end of the ageing period.

At 3 months of ageing, a decrease in total antioxidant activity was observed in all containers. The decrease varied from 66.2% in French oak barrel to 29.3% in acacia barrel (Figure 3.28), (Duncan, $p = 0.5$). After 12 months of ageing in the containers (Figure 3.27), total antioxidant activity of Vilana wine increased 43.7% in French oak and 35.2% % in American oak barrels compared to antioxidant activity of wine directly after vinification. However, in the last three

months of ageing in containers, antioxidant activity increased in all containers, apart from wine in acacia barrel where it decreased significantly (Figure 3.26).

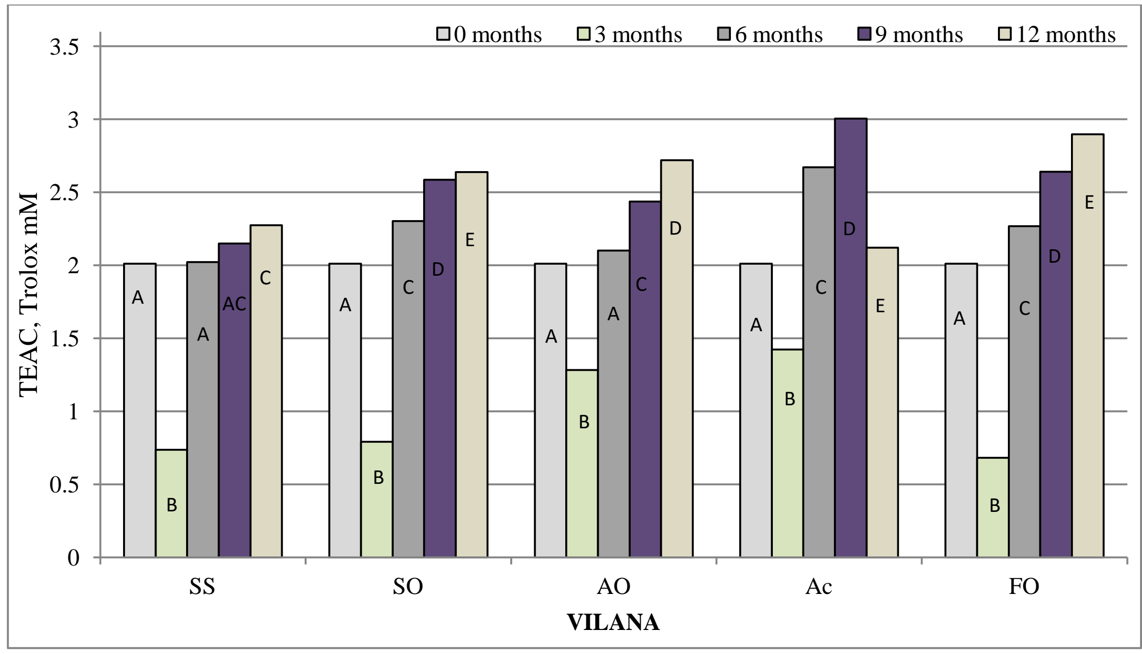


Figure 3.26 Total antioxidant activity of Vilana wine ageing in SS, SO, AO, Ac and FO containers, during 12 months of ageing in the containers. Different letters in columns represent statistical differences within each container. SS: stainless steel container, SO: stainless steel with oenosticks, AO: American oak barrel, Ac: acacia barrel, FO: French oak barrel.

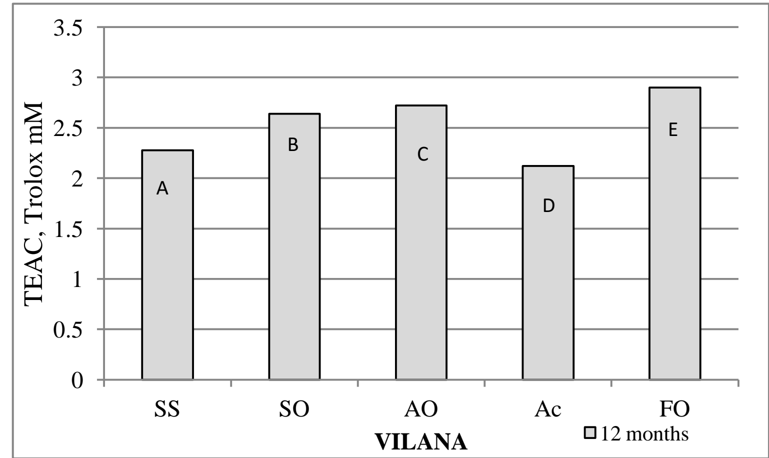


Figure 3.27 Total antioxidant activity of Vilana wine ageing in SS, SO, AO, Ac and FO containers, after 12 months of ageing in the containers. Different letters in columns represent statistical differences between the containers. SS: stainless steel container, SO: stainless steel with oenosticks, AO: American oak barrel, Ac: acacia barrel, FO: French oak barrel.

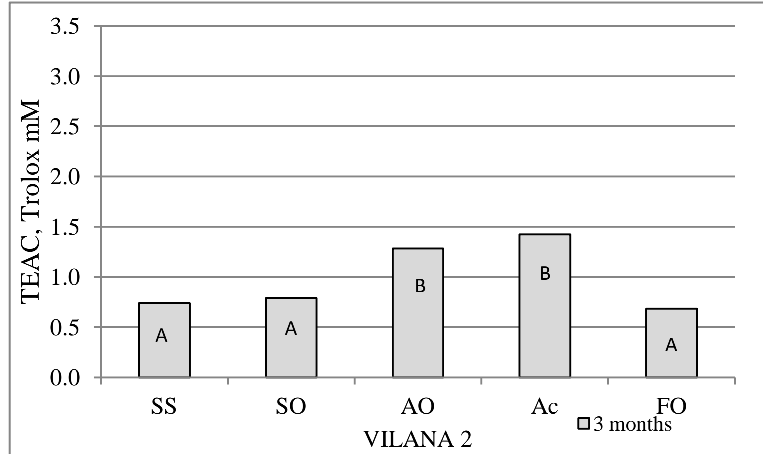


Figure 3.28 Total antioxidant activity of Vilana wine ageing in SS, SO, AO, Ac and FO containers, after 3 months of ageing in the containers. Different letters in columns represent statistical differences between containers. SS: Stainless steel container, SO: stainless steel with oenosticks, AO: American oak barrel, Ac: acacia barrel, FO: French oak barrel.

C. Total antioxidant activity (TEAC) of Dafni wines during ageing

Changes in total antioxidant activity of Dafni wines ageing in the different containers are shown in Figure 3.29 (Duncan, $p = 0.05$).

After a decrease observed in the first three months of ageing, antioxidant activity increased in all the containers. Minor changes were observed in stainless steel with and without oenosticks containers, whereas in wooden barrels, it was increasing constantly until the 12th month of ageing. Wine in acacia barrel, had the highest antioxidant activity, followed by French oak barrel.

Comparison between containers revealed that antioxidant activity of wines was statistically significant differentiated after the 6th month of ageing. Wine in acacia barrel had significantly higher antioxidant activity than wine in any other container. Total antioxidant activity decreased significantly after 3 months of ageing in containers (Duncan, $p = 0.05$).

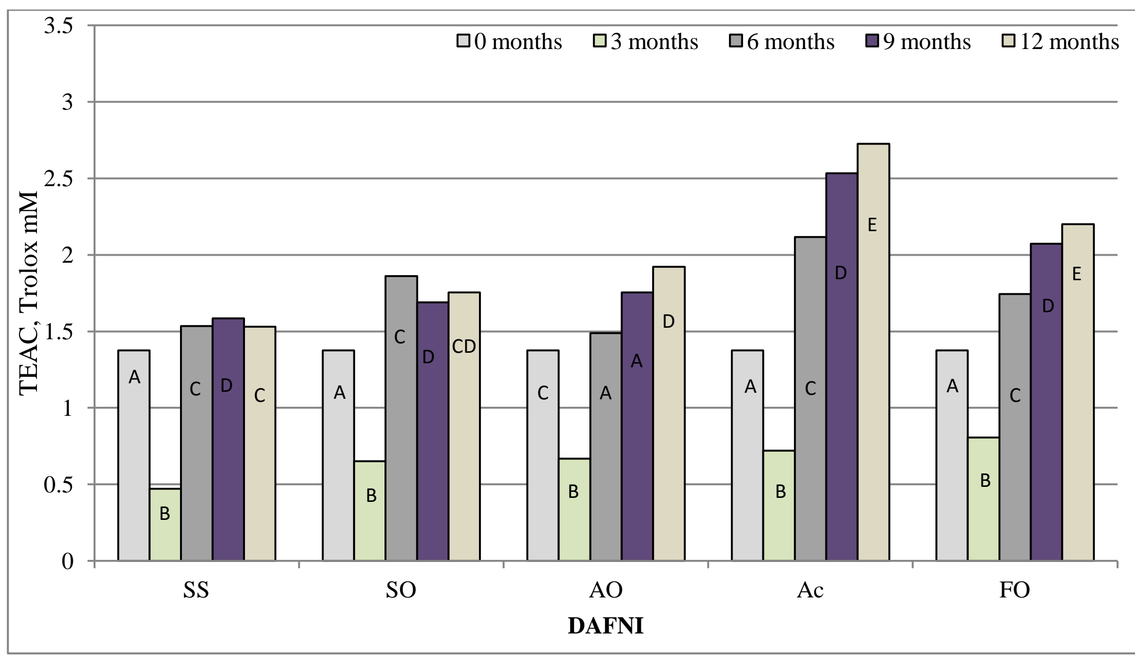


Figure 3.29 Total antioxidant activity of Dafni wine ageing in SS, SO, AO, Ac and FO containers, during 12 months of ageing in the containers. Different letters in columns represent statistical differences within each container. SS: stainless steel container, SO: stainless steel with oenosticks, AO: American oak barrel, Ac: acacia barrel, FO: French oak barrel.

Total antioxidant activity of Dafni increased in all barrels after 12 months of ageing. Compared to antioxidant activity directly after vinification wine ageing in acacia barrel displayed the major increase amongst all containers (96.2%). Changes in total antioxidant activity of wines in the different containers during 12 months of ageing are shown in Figure 3.29. Statistical differences within containers are shown with different letters in columns (Duncan's multiple range tests, $p = 0.05$).

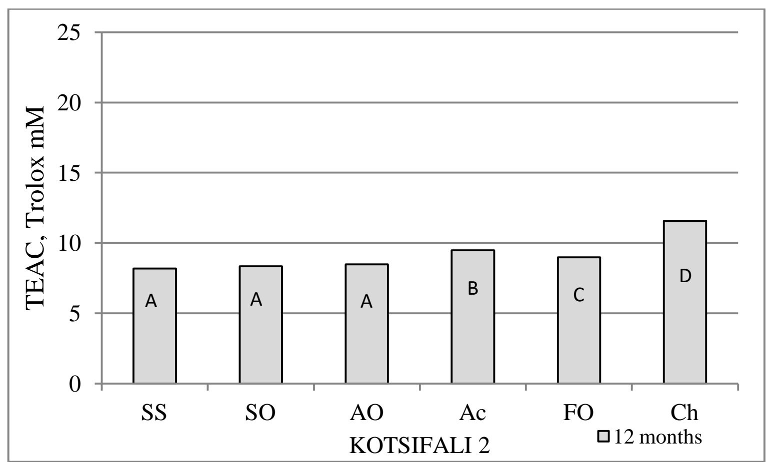


Figure 3.30 Total antioxidant activity of Dafni wine ageing in SS, SO, AO, Ac and FO containers, after 12 months of ageing in the containers. Different letters in columns represent statistical differences between the containers. SS: Stainless steel container, SO: stainless steel with oenosticks, AO: American oak barrel, Ac: acacia barrel, FO: French oak barrel.

D. Total antioxidant activity (TEAC) of Kotsifali wines

Changes in total antioxidant activity of Kotsifali wines ageing in the different containers are shown in Figure 3.31. Minor changes were observed in stainless steel with and without oenosticks containers and American oak barrel Phenolic content in chestnut barrel increased constantly, whereas the major increase in acacia and French oak was observed at 9 months of ageing.

Total antioxidant activity of Kotsifali wine ageing in different containers, compared to antioxidant activity directly after vinification, increased 36.1% in chestnut and 13.5% in acacia barrels (Figure 3.31).

Statistically significant differences (p= 0.05) were reported after twelve months of ageing as shown in Figure 3.32. Antioxidant activity, in comparison to antioxidant activity in stainless steel container, was found 41.2% higher in chestnut barrel.

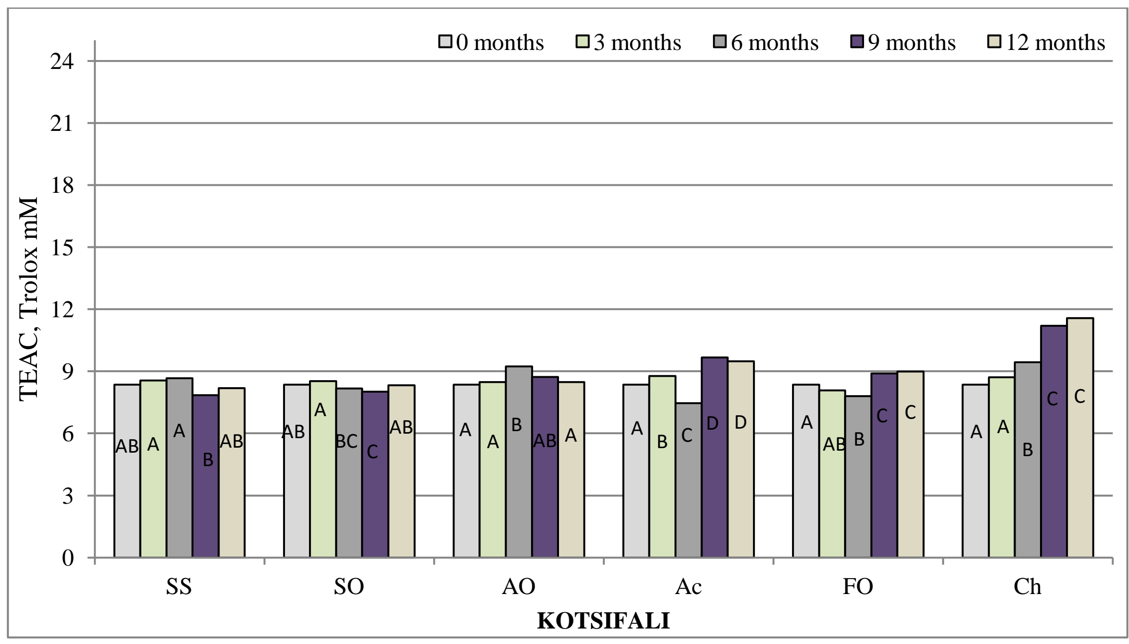


Figure 3.31 Total antioxidant activity of Kotsifali wine ageing in SS, SO, AO, Ac, FO and Ch containers, during 12 months of ageing in the containers. Different letters in columns represent statistical differences within each container. SS: Stainless steel container, SO: stainless steel with oenosticks, AO: American oak barrel, Ac: acacia barrel, FO: French oak barrel, Ch: chestnut barrel.

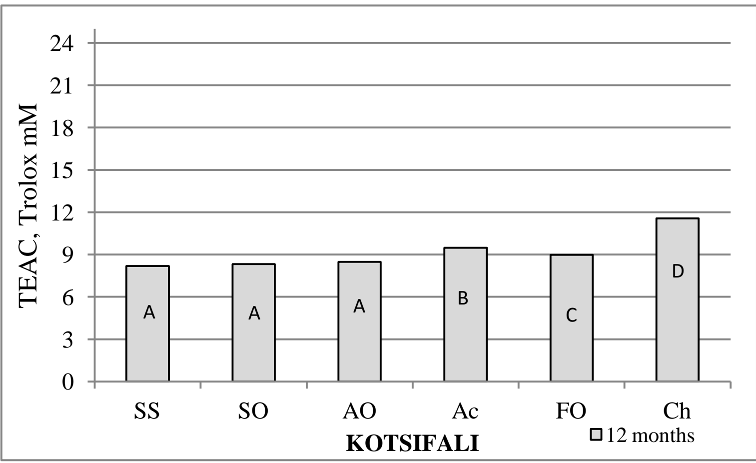


Figure 3.32 Total antioxidant activity of Kotsifali wine ageing in SS, SO, AO, Ac, FO and Ch containers, after 12 months of ageing in the containers. Different letters in columns represent statistical differences between containers. SS: stainless steel container, SO: stainless steel with oenosticks, AO: American oak barrel, Ac: acacia barrel, FO: French oak barrel, Ch: chestnut barrel.

E. Total antioxidant activity (TEAC) of Mandilari wines

Changes in total antioxidant activity of Mandilari wine during ageing for 12 months in different containers are shown in Figure 3.33. Different letters in columns indicate statistically significant differences within containers (p = 0.05).

Antioxidant activity decreased significantly in the first three months of ageing in all the containers. An increase followed leading to even higher antioxidant activity at 6 months of ageing than that determined directly after vinification. Total antioxidant activity of wines after 12 months of ageing, compared to total antioxidant activity directly after vinification increased from 16.1% in stainless steel container to 29.7% in French oak barrels. Statistically significant differences between the containers are shown with different letters in columns of Figure 3.34 (p = 0.05).

In all containers, total antioxidant activity decreased after 3 months of ageing in containers compared to total antioxidant activity directly after vinification. No statistically significant differences were observed between the containers (ANOVA, p = 0.05).

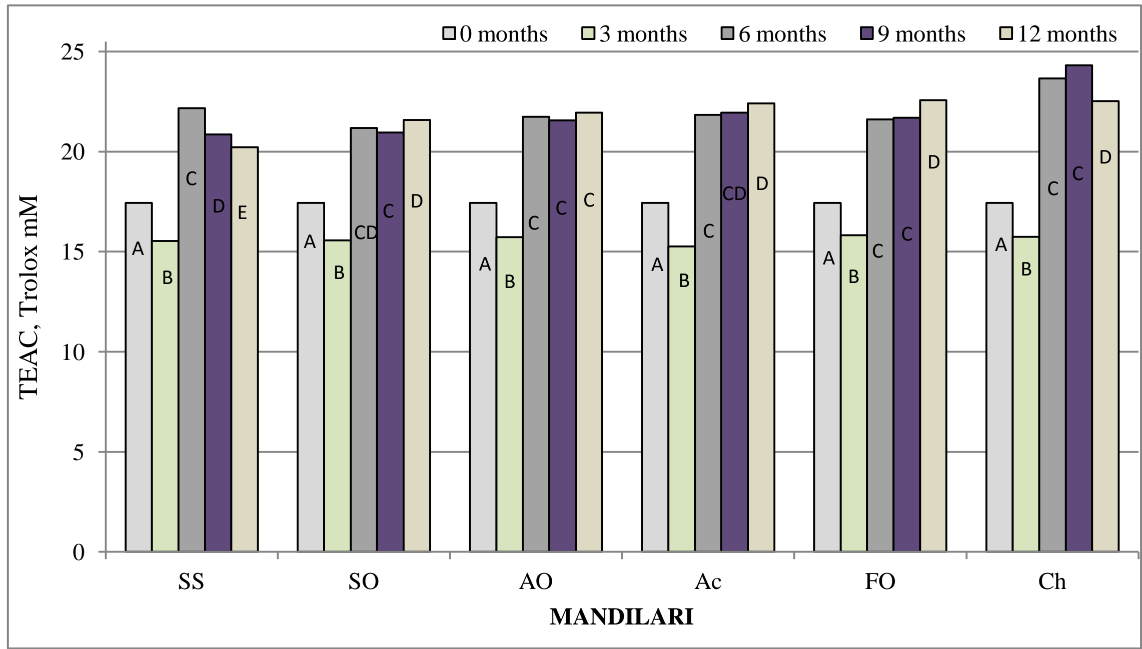


Figure 3.33 Total antioxidant activity of Mandilari wine ageing in SS, SO, AO, Ac, FO and Ch containers, during 12 months of ageing in the containers. Different letters in columns represent statistical differences within each container. SS: stainless steel container, SO: stainless steel

with oenosticks, AO: American oak barrel, Ac: acacia barrel, FO: French oak barrel, Ch: chestnut barrel.

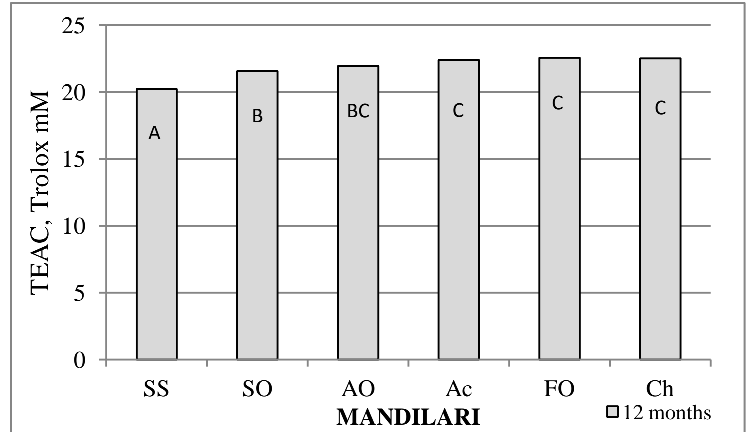


Figure 3.34 Total antioxidant activity of Mandilari wine ageing in SS, SO, AO, Ac, FO and Ch containers, after 12 months of ageing in the containers. Different letters in columns represent statistically significant differences between the containers. SS: Stainless steel container, SO: stainless steel with oenosticks, AO: American oak barrel, Ac: acacia barrel, FO: French oak barrel, Ch: chestnut barrel.

The results of ANOVA for the determination of significant differences in TEAC between the containers and during ageing are summarised in Table 3.7 and in Table 3.8.

Table 3.7 Results of one-way ANOVA (significance level p = 0.05). Comparison of the antioxidant activity of wines within each container during ageing.

	1st vinification	2nd vinification
	VILANA	
SS	S	S
SO	S	S
AO	S	S
Ac	S	S
FO	S	S
	DAFNI	
SS	S	S
SO	S	S
AO	S	S
Ac	S	S
FO	S	S
	KOTSIFALI	
SS	S	NS
SO	S	S
AO	S	S
Ac	S	S
FO	S	S
Ch	S	S
	MANDILARI	
SS	S	S
SO	S	S
AO	S	S
Ac	S	S
FO	S	S
Ch	S	S

Table 3.8 Results of one-way ANOVA (significance level p = 0.05). Comparison of the antioxidant activity of wines in the different containers at 3, 6, 9 and 12 months of ageing.

	1st vinification	2nd vinification
	VILANA	
3 months	S	NS
6 months	S	S
9 months	S	S
12 months	S	S
	DAFNI	
3 months	NS	NS
6 months	S	S
9 months	S	S
12 months	S	S
	KOTSIFALI	
3 months	S	NS
6 months	S	S
9 months	S	S
12 months	S	S
	MANDILARI	
3 months	S	NS
6 months	S	S
9 months	S	S
12 months	S	S

3.1.3 Correlation between total antioxidant activity and total phenolic content of wines

3.1.3.1 Results of the first vinification year (2012)

Pearson product-moment correlation coefficient calculation of antioxidant activity (Teac) and total phenolic content (TPC) revealed a significant positive correlation (at level 0,01) between total phenolic content and antioxidant activity of wines ($r = 0.948$, $DF = 264$, $p < 0,001$) (Figure 3.35).

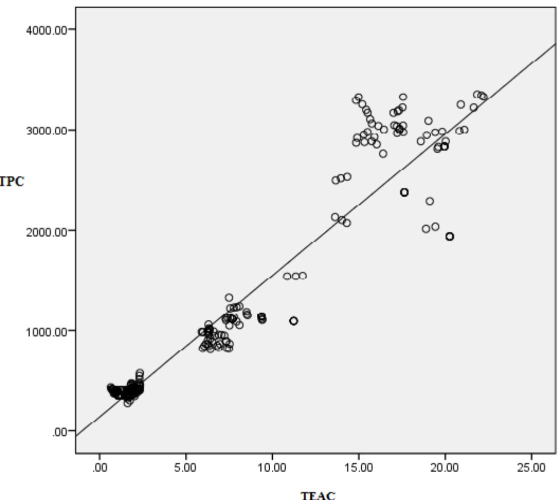


Figure 3.35 Correlation between total phenolic content (TPC) and total antioxidant activity (TEAC) of first vinification wines (Pearson product-moment correlation coefficient calculation).

3.1.3.2 Results of the second vinification year (2013)

Pearson product-moment correlation coefficient calculation of antioxidant activity (Teac) and total phenolic content (TPC) revealed a significant positive correlation (at level 0,01) between total phenolic content and antioxidant activity ($r = 0.980$, $DF = 248$, $p < 0,001$) of wines (Figure 3.36).

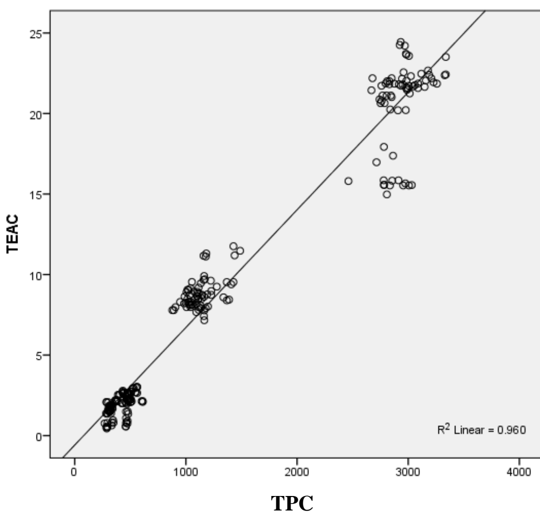


Figure 3.36 Correlation between total phenolic content (TPC) and total antioxidant activity (TEAC) of 2nd vinification wines (Pearson product-moment correlation coefficient calculation).

Pearson product-moment correlation coefficient calculation of antioxidant activity (Teac) and total phenolic content (TPC) revealed positive correlations (at level 0,01) between total phenolic content and antioxidant activity of white wines (r = 0.583, *DF*= 278) and of red wines of both vinifications (r = 0.944, *DF*= 334) (Figure 3.37). Correlation was much more intense in red wines than in white wines.

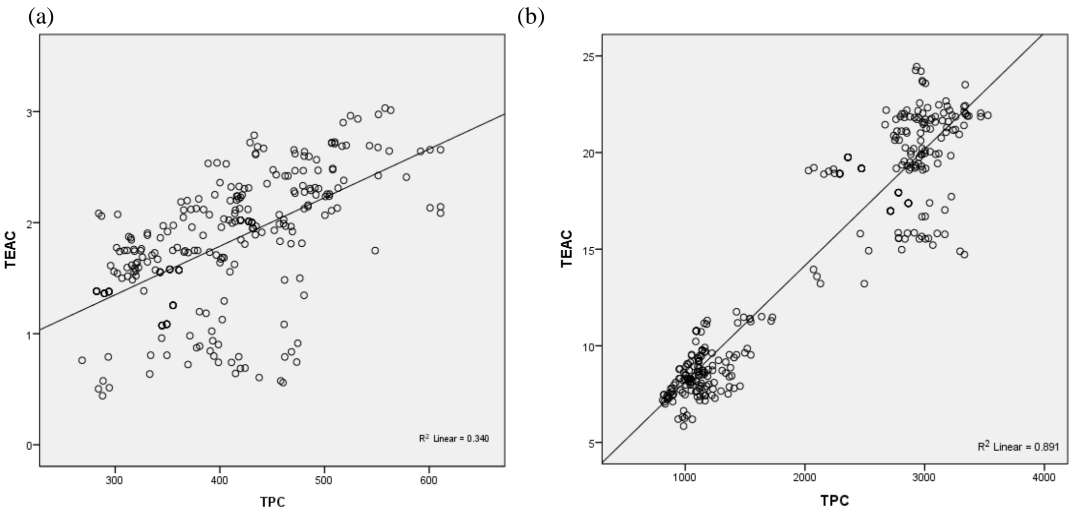


Figure 3.37 Correlation between total phenolic content (TPC) and total antioxidant activity (TEAC) of (a) white wines and (b) red wines (Pearson product-moment correlation coefficient calculation). Results refer to both vinifications.

3.1.3 Phenolic fingerprints of extracts obtained from Vilana, Dafni, Kotsifali and Mandilari wines.

Phenolic extracts were obtained by Solid Phase Extraction (SPE). Absorption spectrum of each wine was obtained by attenuated total reflectance (ATR) FTIR, at mid-infrared (1800-900 cm^{-1}) (100 scans at 4 cm^{-1}).

Absorption spectra of phenolic extracts of each variety were taken directly after vinification and through ageing, and characteristic peaks at the fingerprint region were determined.

Directly after vinification, in Dafni phenolic extracts characteristic peaks were observed that are attributed to the phenol-structure at 1060, 1200, 1230, 1280, 1315, 1340, 1445 and 1520 cm^{-1} as shown in Figure 3.38. In Vilana characteristic peaks were observed attributed to the phenol-structure at 1060, 1230, 1315, 1340, 1445 and 1520 cm^{-1}, and 1110-1150 cm^{-1} (Figure 3.39). Differences between Vilana and Dafni extracts were observed at 1200 cm^{-1} and 1280 cm^{-1}. Both peaks were observed in Dafni phenolic extracts but not in Vilana. Peaks at 1110-1150 cm^{-1}, which were observed in Vilana, were not observed in Dafni extracts.

In Kotsifali phenolic extracts peaks at 1060, 1200, 1230, 1280, 1315, 1340, 1445 and 1520 cm^{-1} were observed, attributed to phenol structure. Compared to Mandilari, there was absence of a peak at 1110 cm^{-1}. A wick peak appeared at 1145 cm^{-1}(Figure 3.40). Mandilari, absorbed strongly at 1060, 1110, 1145, 1200, 1230, 1280, 1340, 1445 and 1520 cm^{-1} (Figure 3.41), wavelengths that phenolics are known to absorb. The peak at 1200 cm^{-1} appeared stronger in Mandilari than in Kotsifali.

Absorption spectra of phenolic extracts of each variety were taken through the 12 month period of ageing in containers. Overlays of typical absorption spectra, are shown in Figure 3.42, Figure 3.43, Figure 3.44 and Figure 3.45.

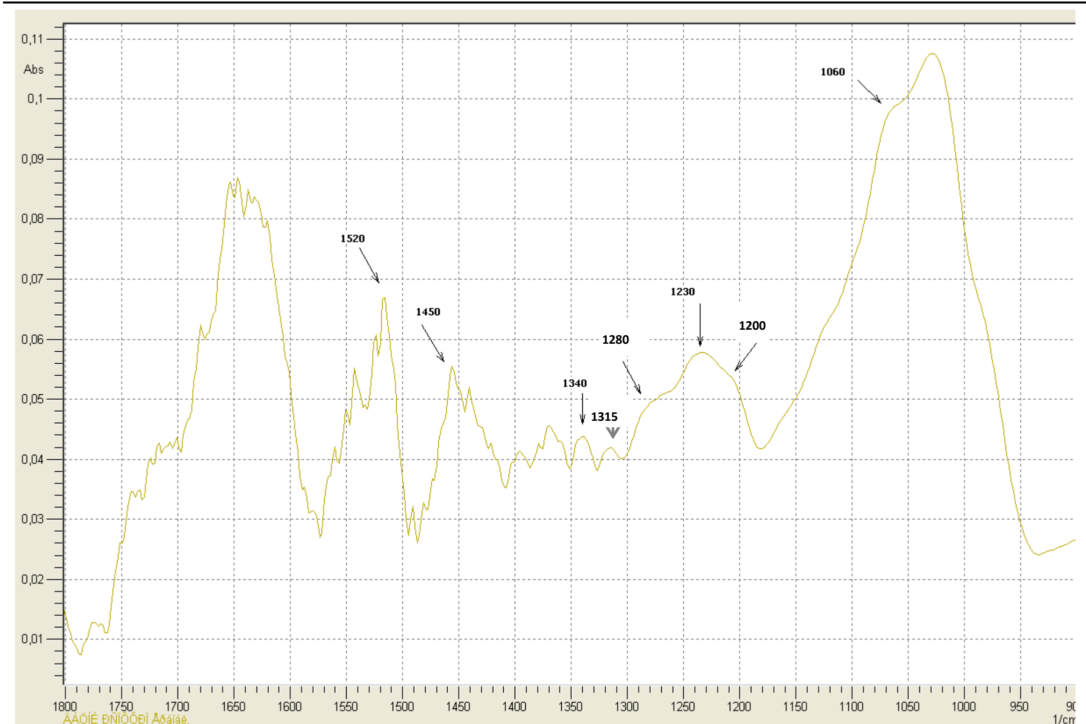


Figure 3.38 Absorption spectrum of Dafni phenolic extract directly after vinification. Arrows point out characteristic peaks attributed to Vilana wine.

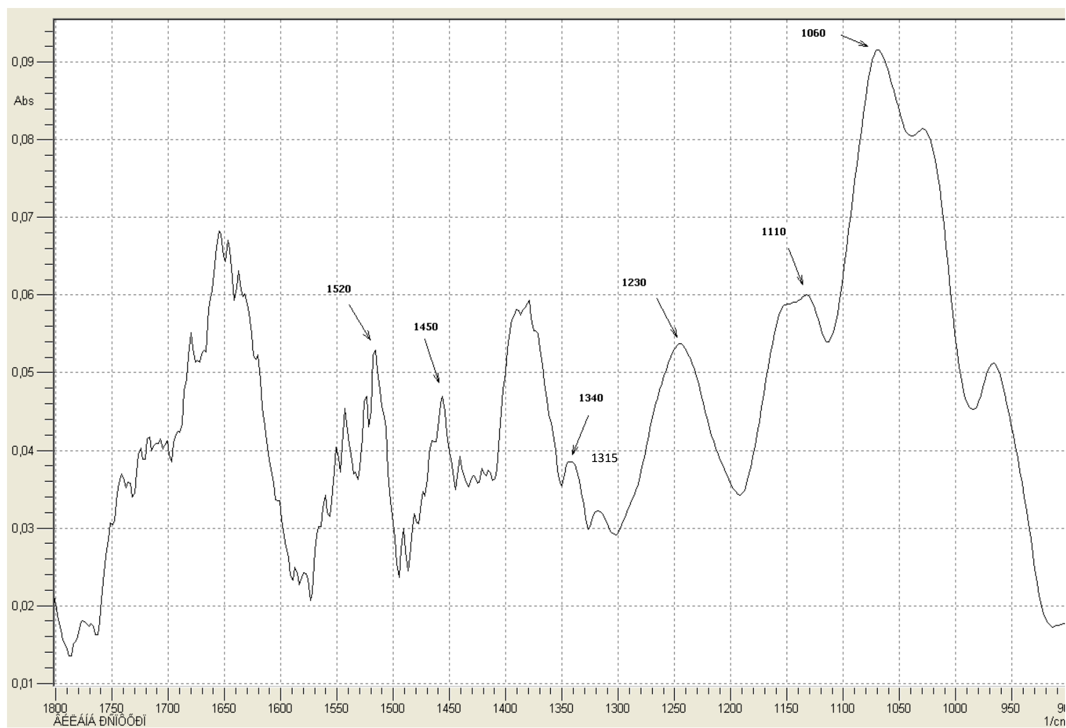


Figure 3.39 Absorption spectrum of Vilana phenolic extract directly after vinification. Arrows point out characteristic peaks of phenolic moieties.

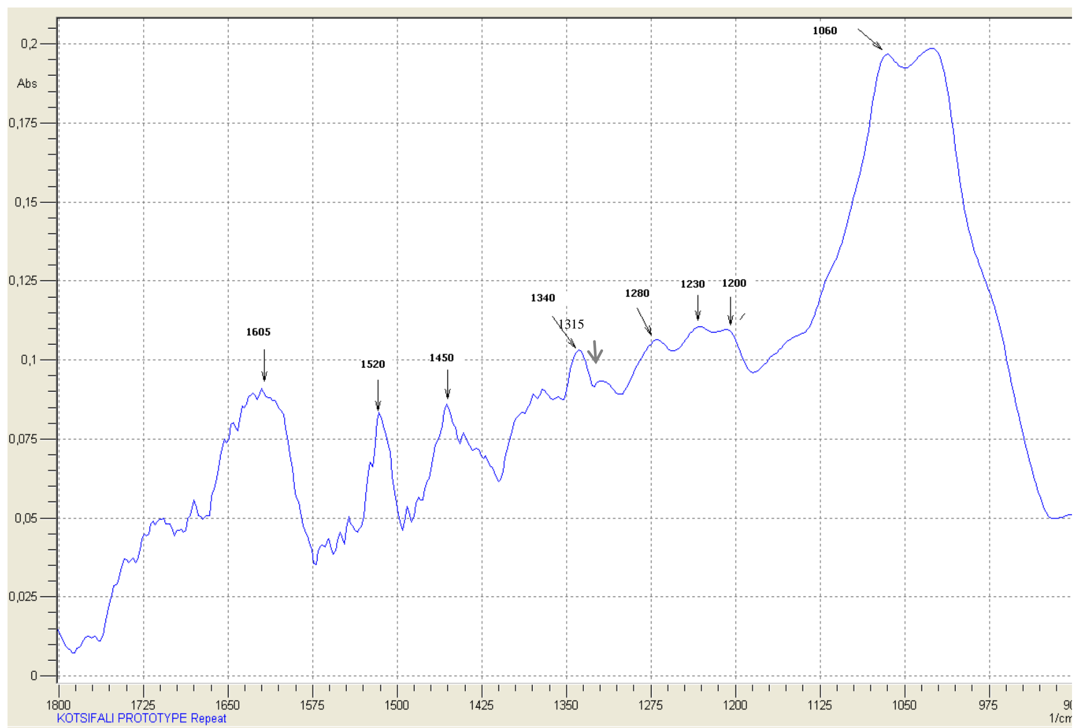


Figure 3.40 Absorption spectrum of Kotsifali phenolic extract directly after vinification. Arrows point out characteristic absorption peaks of phenolic moieties in Kotsifali wine.

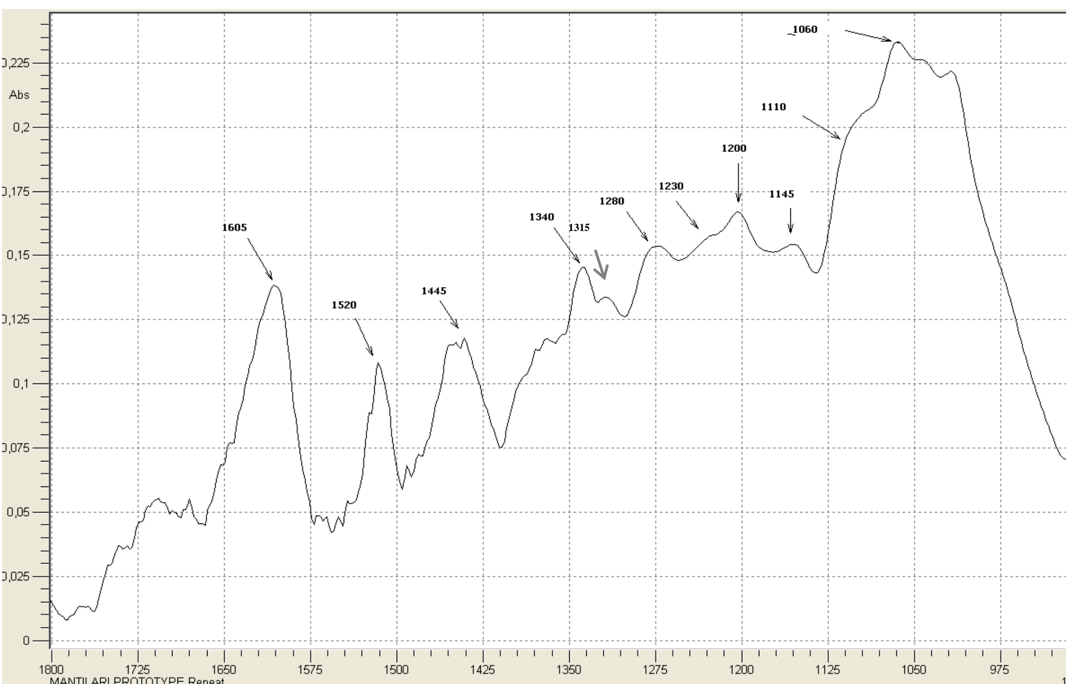


Figure 3.41 Absorption spectrum of Mandilari phenolic extract directly after vinification. Arrows point out characteristic peaks of phenolic moieties in Mandilari wine.

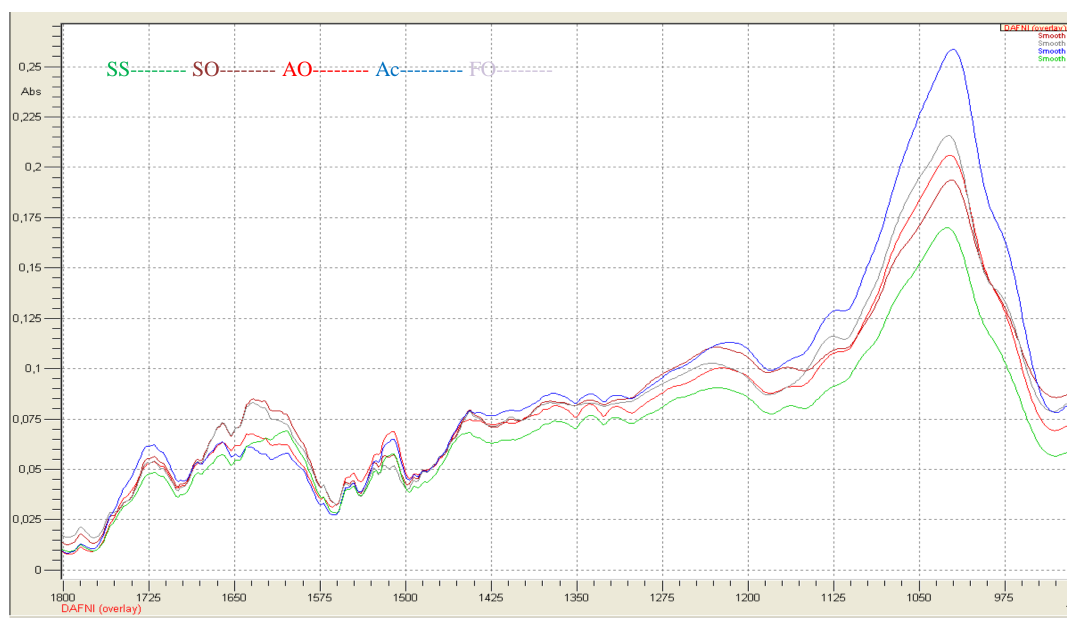


Figure 3.42 Spectra overlays of phenolic extracts of Dafni wine, after 9 months of ageing in different containers.SS: Stainless steel container, SO: stainless steel with oenosticks container, AO: American oak barrel, Ac: acacia barrel, FO: French oak barrel.

Figure 3.43 Spectra overlays of phenolic extracts of Vilana wine, after 9 months of ageing in different containers. SS: Stainless steel container, SO: stainless steel with oenosticks container, AO: American oak barrel, Ac: acacia barrel, FO: French oak barrel.

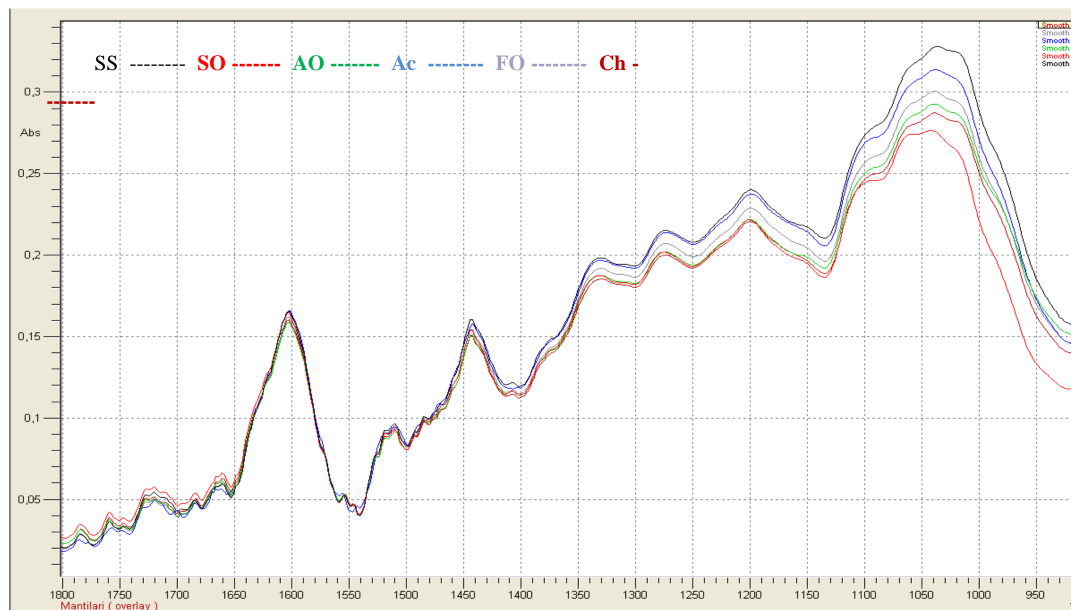


Figure 3.44 Spectra overlays of phenolic extracts of Kotsifali wine, after 9 months of ageing in different containers. SS: Stainless steel container, SO: stainless steel with oenosticks container, AO: American oak barrel, Ac: acacia barrel, FO: French oak barrel, Ch: chestnut barrel

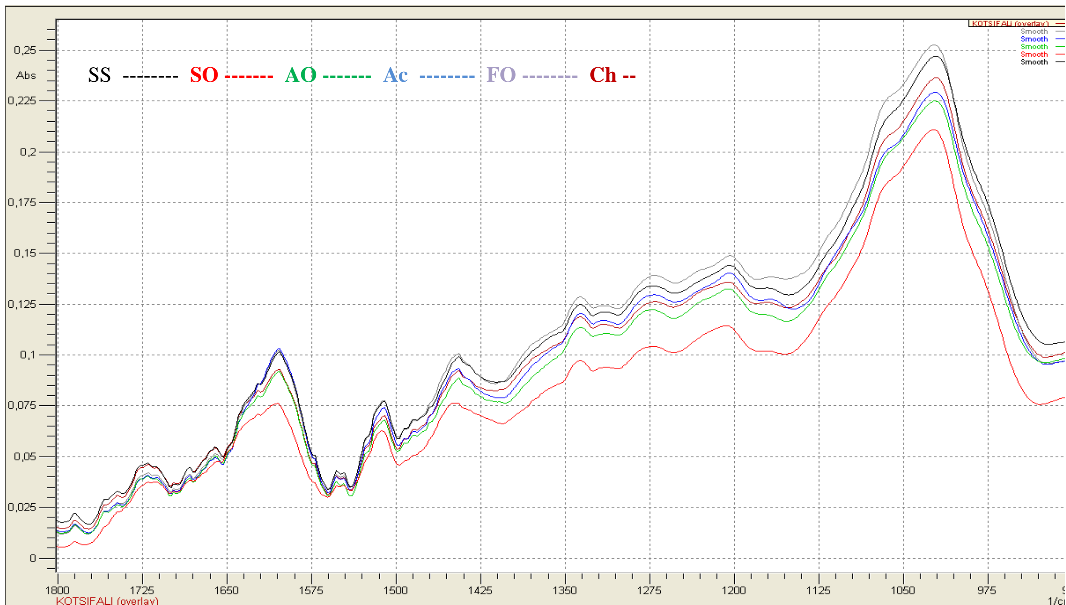


Figure 3.45 Spectra overlays of phenolic extracts of Mandilari wine, after 6 months of ageing in different containers. SS: Stainless steel container, SO: stainless steel with oenosticks container, AO: American oak barrel, Ac: acacia barrel, FO: French oak barrel, Ch: chestnut barrel.

3.1.2 Results of PCA and PLS analysis

i) PCA for the discrimination of wine varieties

The infrared spectral data from the analysis of the wine samples ageing for 3, 6, 9 and 12 months, were used to perform the principal components analysis (PCA). The first component (PC1) was the major contributor to the separation of the varieties. The sum of the first three PCs of white wines explained 97.5% of the total variance (the scores for PC1 for white wines explained 82.4% of the total variability; PC2 and PC3 explained 12.5% and 2.7% of the variability, respectively). The sum of the first three PCs of red wines explained 87.8% of the total variance.

PCA Discriminant Analysis (PCA-DA) was performed on both white and red wines. A complete differentiation (100%) of the white (Figure 3.46, Table 3.9) and red (Figure 3.47, Table 3.10) wine samples was achieved by PCA-DA, based on their FT-IR spectra. (PCA was used to derive 16 principal components that ensured that at least 99.9 % of the variability was considered by the analysis).

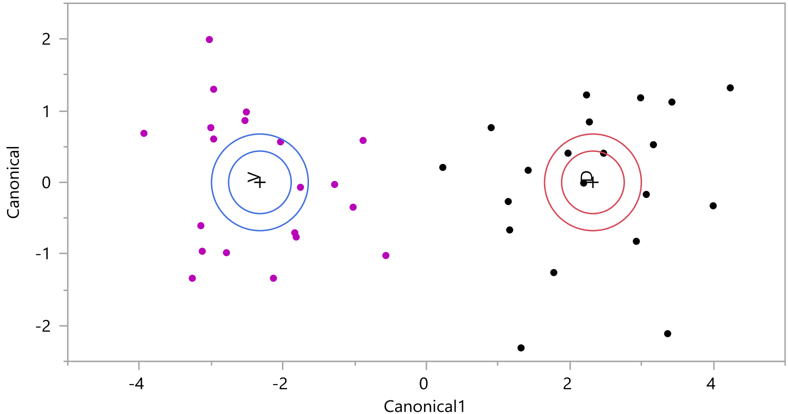


Figure 3.46 Discrimination results by PCA-DA regarding the variety of white wines.

(V: Vilana, D: Dafni)

Table 3.9 Variety classification based on phenolic fingerprints (white wines)

Number Misclassified	0
Percent Misclassified	0
-2LogLikelihood	0.813

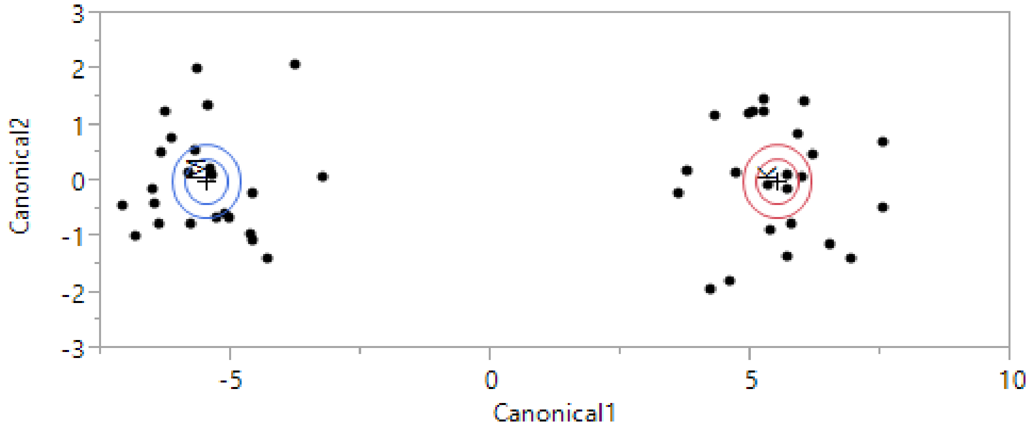


Figure 3.47 Discrimination results by PCA-DA regarding the variety of red wines.

(K: Kotsifali, M: Mandilari)

Table 3.10 Variety classification based on phenolic fingerprints (red wines)

Number Misclassified	0
Percent Misclassified	0
-2LogLikelihood	9e-16

ii) Prediction of total phenolic content by PLS models

Total phenolic content of wine was achieved based on PLS analysis. Data from the FTIR spectra were used in order to predict the TPC of wines. Cross-validation (leave-one-out technique) was used in all cases for calibration models evaluation. Eight factors were used for the quantitative analysis, explaining 92.7% of the variance (phenolic content of wines) as presented in Table 3.11. A good correlation was determined by plotting the full cross-validated PLS predicted TPC values (r = 0.98) by the PLS model (Figure 3.50).

Table 3.11 Model comparison summary

Method	**Number of factors**	**Percent Variation Explained for Cumulative Y**
NIPALS	8	92.733668

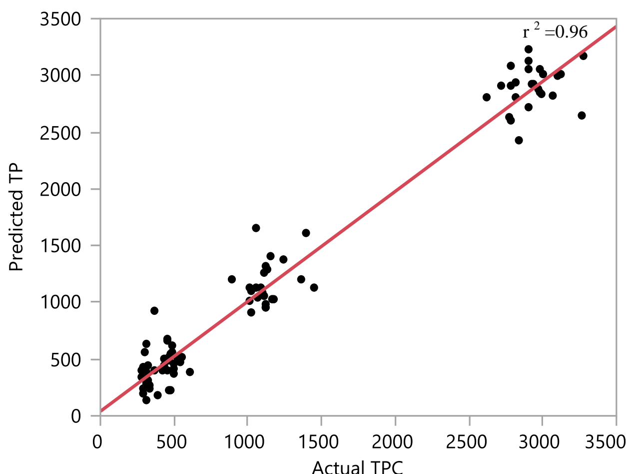


Figure 3.48 Plot of actual by predicted TPC by PLS analysis

3.2 Influence of oleuropein and hydroxytyrosol on acetic acid bacteria wine spoilage as an alternative to sulphur dioxide.

3.2.1 Changes in volatile and titratable acidity of wine B1.

The effect of AAB and different concentrations of oleuropein and hydroxytyrosol on titratable acidity of wine B1 is shown in Figure 3.49. Statistical differences in titratable acidity within treatments are stated with different letters in columns of Figure 3.49 (Duncan, p = 0.05). All treatments were incubated at 30°C. In all cases, titratable acidity increased under the employed conditions. AAB was the treatment that had the highest titratable acidity level amongst the treatments. Titratable acidity of H0.5 treatment at the last measurement was significant different from any other treatment.

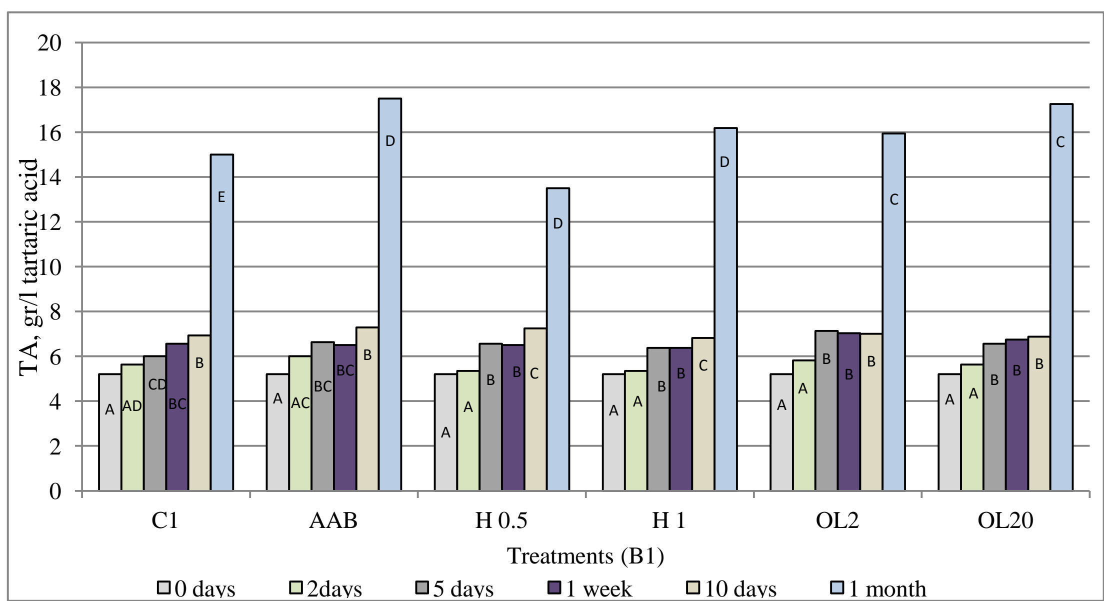


Figure 3.49 Titratable acidity (TA) of wine B1 per treatment. Total acidity was measured at the beginning of the experiment and after 2, 5 days, 1 week, 10 days and one month. All treatments were incubated at 30°C. Statistical differences within treatments are indicated with different letters in columns; Wine treatments: C1: Positive control, AAB: acetic acid bacteria, H0.5: hydroxytyrosol 0.5mg/l + acetic acid bacteria, H1: hydroxytyrosol 1mg/l + acetic acid bacteria, OL2: oleuropein 2 mg/l +acetic acid bacteria, OL20: oleuropein 20mg/l + acetic acid bacteria.

Figure 3.50 displays the levels of volatile acidity of each the treatment, after seven weeks incubation period at 30°C. Control and H0.5 treatments had significant differences with AAB treatment. Compared to control, VA in AAB treatment was 66.5% higher. The rest of treatments displayed smaller increase than AAB, varying from 24% in H0.5 treatment to 34% in H1 treatment.

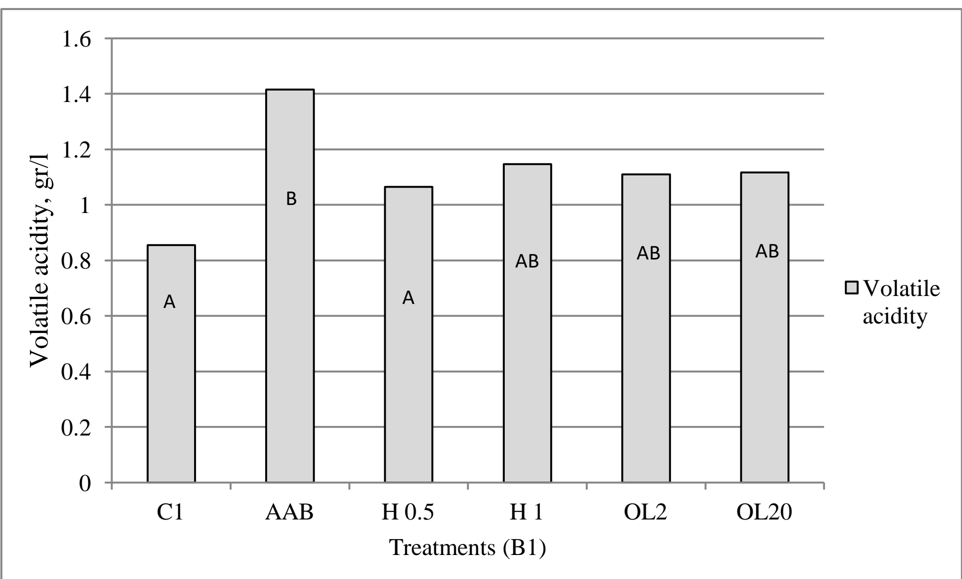


Figure 3.50 Volatile acidity of wine B1 after 7 week incubation period at 30° C. The different letters in columns indicate significant differences of volatile acidity between treatments; Wine treatments: C1: Positive control, AAB: acetic acid bacteria, H0.5: hydroxytyrosol 0.5mg/l + acetic acid bacteria, H1: hydroxytyrosol 1mg/l + acetic acid bacteria, OL2: oleuropein 2mg/l +acetic acid bacteria, OL20: oleuropein 20mg/l + acetic acid bacteria.

Changes in intensity (I) and hue (T) of the wine through tine are shown in Table 3.12. The increase in density reflects the changes occurring due to wine oxidation. In all treatments, density increased through time, demonstrating more brownish– orange colour in wine, an effect of wine spoilage.

Table 3.12 Changes in colour characteristics of wine B1 in time.

WINE B1	0 days		2 days		1 month		7 weeks	
Treatment	I	T	I	T	I	T	I	T
C1	4.801	0.984	5.590	1.027	8.172	1.008	8.806	1.000
AAB	5.101	0.976	5.510	1.017	8.110	1.011	8.978	1.010
H0.5	5.458	0.991	5.605	1.021	8.08	1.024	8.946	1.013
H1	4.993	0.983	5.958	1.018	7.337	1.101	8.941	1.000
OL2	5.006	0.985	5.623	1.029	7.542	1.000	8.862	1.006
OL20	5.040	0.989	5.601	1.028	7.568	1.000	8.851	1.006

3.2.2 Changes in volatile and titratable acidity of wine B2.

Wine B2 was treated with acetic acid bacteria, oleuropein 0.1mg/l and oleuropein 0.4mg/l. Titratable acidity increased during incubation for 3 weeks at 30° C. Wine treated with acetic acid bacteria had reached the highest level in titratable acidity amongst treatments at the end of the experiment (Figure 3.51).

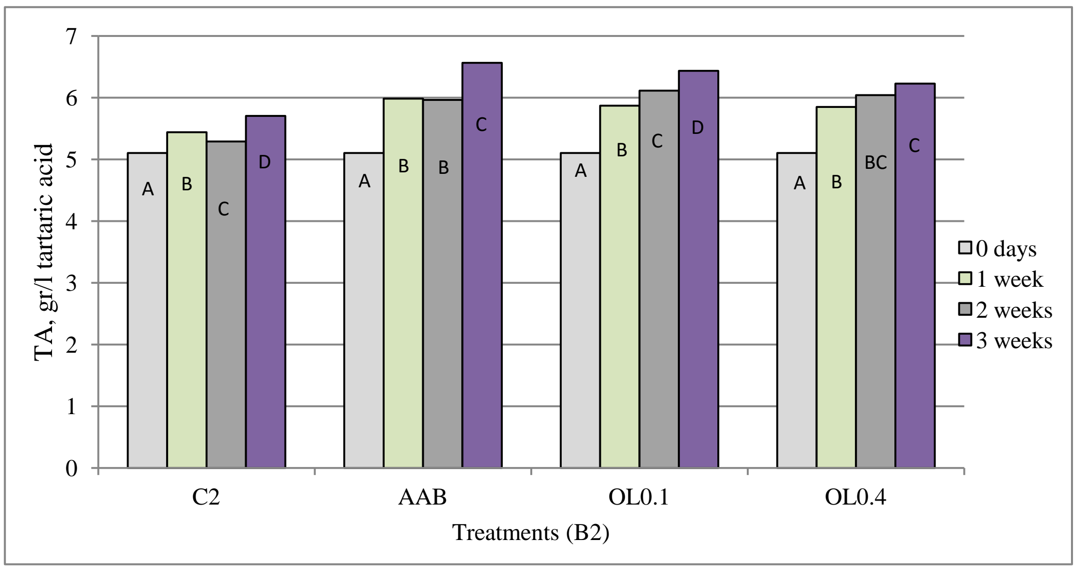


Figure 3.51 Titratable acidity of B2 wine 1, 2, and 3 weeks after addition of oleuropein 0.1mg/l and oleuropein 0.4mg/l. Titratable acidity increased during that period. AAB treatment was the one with the biggest increase after 3 weeks in 30°C; Wine treatments: C1: Positive control, AAB: acetic acid bacteria, OL0.1: oleuropein 0.1mg/l +acetic acid bacteria, OL0.4: oleuropein 0.4mg/l + acetic acid bacteria.

In all treatments titratable acidity increased compared to acidity of wine at the beginning of the essay ranging from 29.1% in acetic acid bacteria treatment to 12.2% in positive control.

Volatile acidity of wine B2 treated with AAB, OL0.1 and OL0.4 increased during the experiment as shown in Figure 3.52. After 7 weeks of incubation volatile acidity reached the highest levels in OL0.04 treatment. After 7 weeks of incubation at 30°C compared to C2 treatment (positive control), AAB treatment displayed 53%, OL0.1 50% and OL0.04 57% increased volatile acidity.

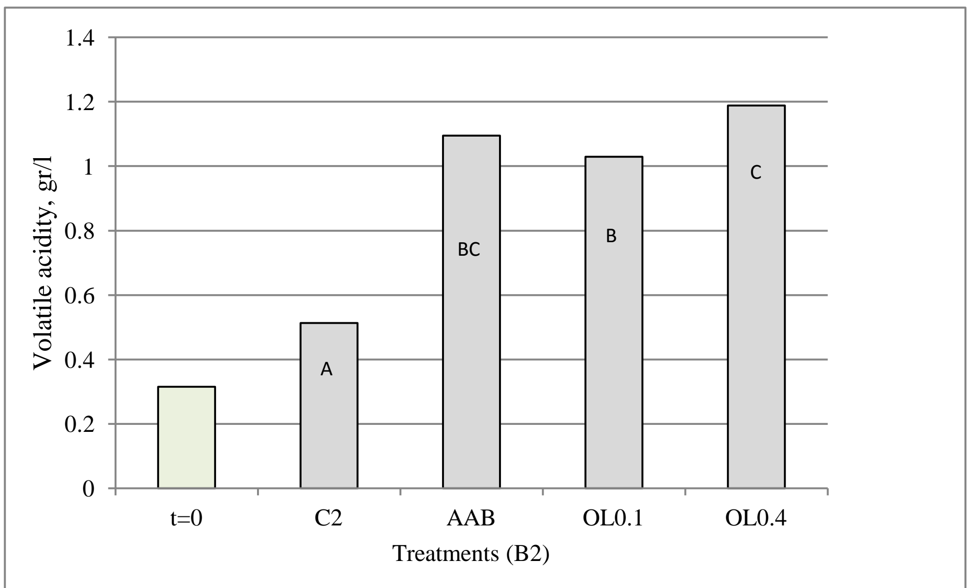


Figure 3.52 Volatile acidity of wine B2, 0 days (t=0) and 7 weeks after the beginning of the experiments. Samples were stored at 30°C during the experiment. Statistical differences are indicate with different letters in columns ($p = 0.05$); Wine treatments: C2: Positive control, AAB: acetic acid bacteria, OL0.1: oleuropein 0.1mg/l +acetic acid bacteria, OL0.4: oleuropein 0.4mg/l + acetic acid bacteria; t=0: volatile acidity at the beginning of the experiment).

3.2.3 Changes in volatile acidity of wine B3.

Wine treated with hydroxytyrosol 1mg/l had increased volatile acidity during a 3 month period in a lesser extent than the rest of the treatments (Figure 3.53). Acetic acid bacteria were not added in any of the treatments allowing wine’s existing population of acetic acid bacteria to

increase in order to monitor the effect the additives would have on volatile acidity. Statistically significant differences within treatments are shown in Figure 3.64 (p = 0.05).

Statistically significant differences of volatile acidity between the different treatments, 3 months after the begging of the experiment, are shown in Figure 3.54. Statistically significant differences are indicated by different letters in the columns (Duncan, p = 0.05).

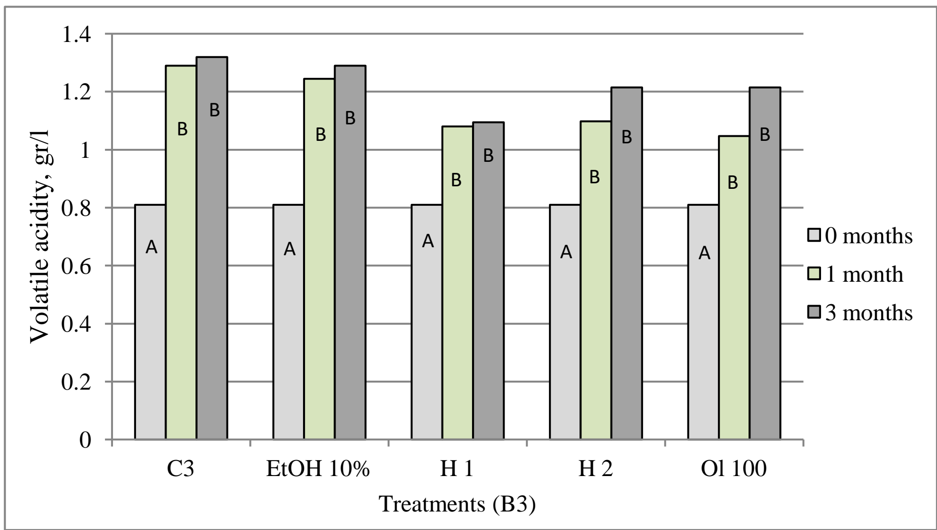


Figure 3.53 Volatile acidity of wine B3. All samples were incubated at 30° C throughout the experiment. Samples were stored at 30°C during the experiment; Wine treatments: C3: Positive control, EtOH 10%: ethanol 10%, H1: hydroxytyrosol 1mg/l, H2: hydroxytyrosol 2mg/l, Ol100: oleuropein 100mg/l.

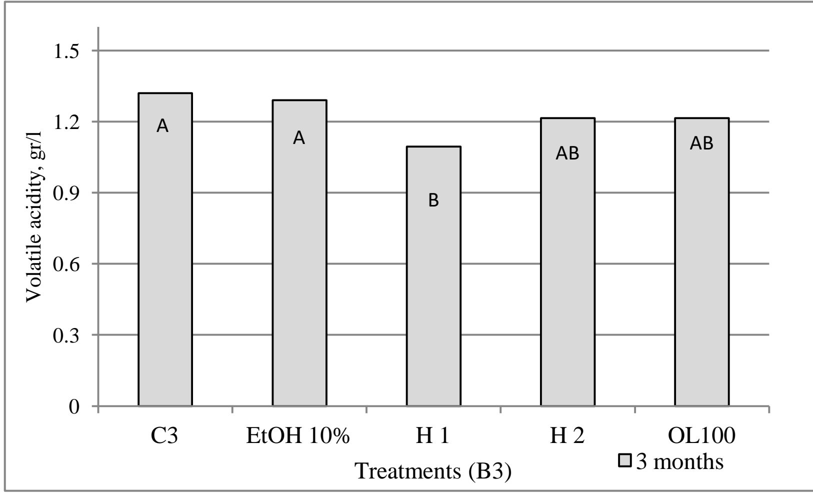


Figure 3.54 Volatile acidity of wine B3, 3 months after the beginning of the experiment. All samples were incubated at 30° C. Statistical differences, are indicated by different letters in

columns; Wine treatments: C3: Positive control, EtOH 10%: ethanol 10%, H1: hydroxytyrosol 1mg/l, H2: hydroxytyrosol 2mg/l, OL100: oleuropein 100mg/l.

3.2.4 Changes in volatile and titratable acidity of wine B4

Amongst all treatments in wine B4, hydroxytyrosol 0.5gr/l showed the lowest increase in volatile acidity amongst all the treatments – positive control excluded (Figure 3.55). In all cases, treated wine with AAB and hydroxytyrosol or oleuropein had increased volatile acidity compared to control.

Oleuropein treatments in concentration 400mg/l and 800mg/l had very similar results to AAB treatment. Oleuropein 50mg/l treatment had similar effect to hydroxytyrosol 1mg/l treatment.

Statistically significant differences of volatile acidity between the different treatments, 5 weeks after the begging of the experiment, are shown in Figure 3.56 (p = 0.05)

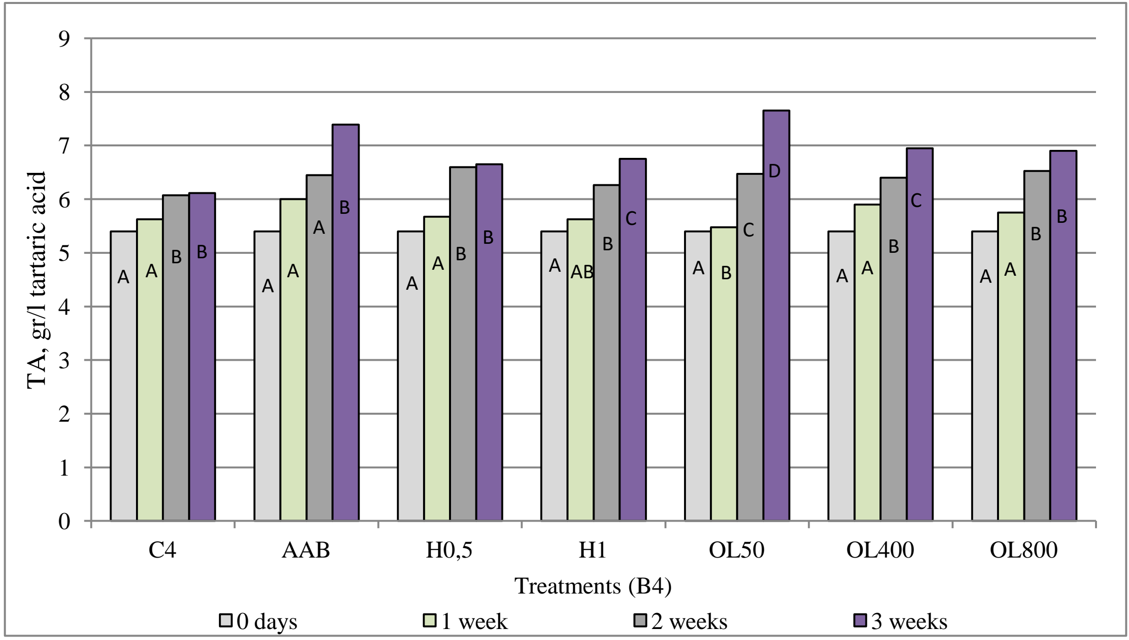


Figure 3.55 Titratable acidity of wine B4 5 weeks since the beginning of the essay. All samples were incubated at 30° C. Statistical differences, are indicated by different letters in columns; Wine treatments: C4: Positive control AAB: acetic acid bacteria, H0.5: hydroxytyrosol 0.5mg/l + acetic acid bacteria, H1: hydroxytyrosol 1mg/l + acetic acid bacteria, OL50: oleuropein 50mg/l + acetic acid bacteria, OL400: oleuropein 400mg/l + acetic acid bacteria; OL800: oleuropein 800mg/l + acetic acid bacteria.

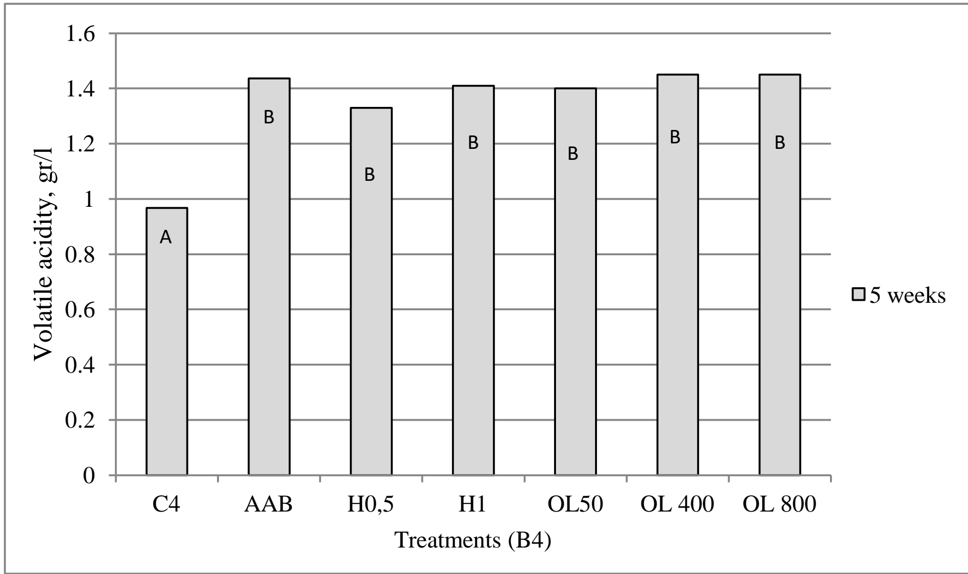


Figure 3.56 Volatile of wine B4 5 weeks since the beginning of the essay. All samples were incubated at 30° C. Statistical differences, are indicated by different letters in columns; Wine treatments: C4: Positive control AAB: acetic acid bacteria, H0.5: hydroxytyrosol 0.5mg/l + acetic acid bacteria, H1: hydroxytyrosol 1mg/l + acetic acid bacteria, OL50: oleuropein 50mg/l + acetic acid bacteria, OL400: oleuropein 400mg/l + acetic acid bacteria, OL800: oleuropein 800mg/l + acetic acid bacteria.

3.2.5 Changes in volatile and titratable acidity of wine B5

Wine B5 was treated with acetic acid bacteria, hydroxytyrosol 1mg/l + acetic acid bacteria (H1), oleuropein 1mg/l + acetic acid bacteria (OL1) and oleuropein 2mg/l + acetic acid bacteria (OL2). Throughout incubation at 30° C for one month, titratable acidity was measured. At the end of the experiment volatile acidity of each treatment was determined.

In all treatments of wine B5 titratable acidity increased during a three period time (Figure 3.57). At the end of the experiment titratable acidity was significant lower in H1 treatment than the rest of the treatments (Duncan, $p = 0.05$).

Volatile acidity was significantly different level in hydroxytyrosol 1mg/l treatment, compared to AAB and oleuropein mg/l and 2 mg/l (Figure 3.58). Hydroxytyrosol 1mg/l treatment exhibited 50.7% lower volatile acidity than AAB treatment. Treatment with oleuropein 2mg/l was found

to have the highest volatile acidity after one month period, displaying significant differences than the rest of the treatments. (Duncanp = 0.05).

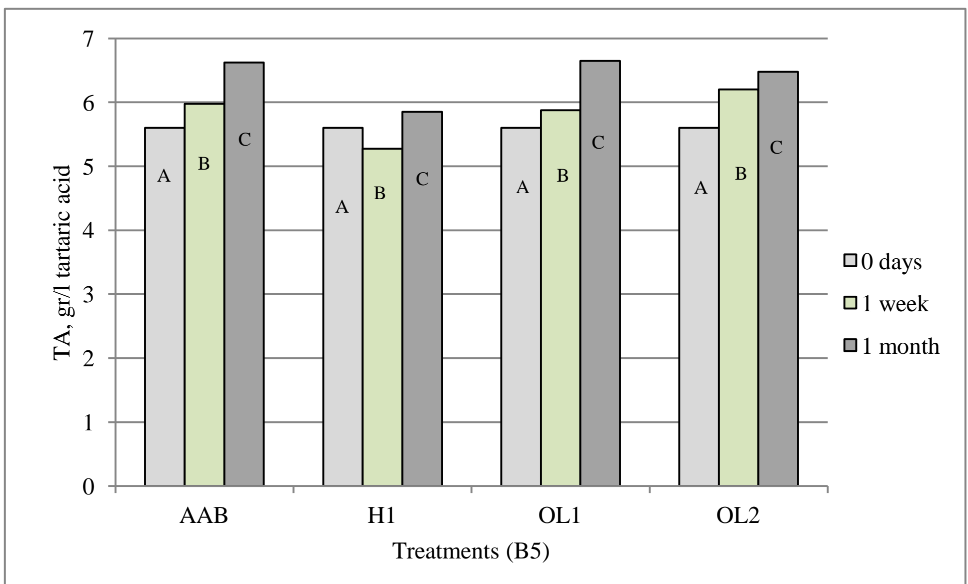


Figure 3.57 Titratable acidity of wine B5 1 month since the beginning of the experiment. All samples were incubated at 30° C. Statistical differences, are indicated by different letters in columns; Wine treatments: AAB: acetic acid bacteria, H1: hydroxytyrosol 1mg/l + acetic acid bacteria, OL1: oleuropein 1mg/l + acetic acid bacteria, OL2: oleuropein 2mg/l + acetic acid bacteria.

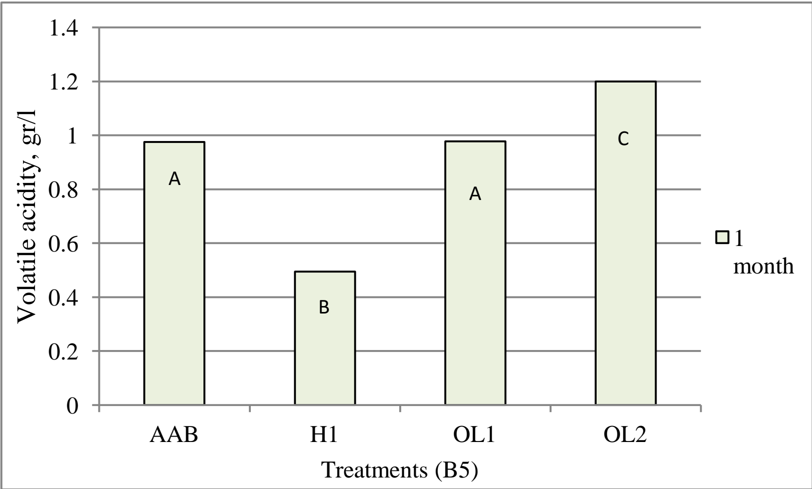


Figure 3.58 Titratable acidity of wine B5 1 month since the beginning of the experiment. All samples were incubated at 30° C. Statistical differences, are indicated by different letters in columns (Duncan’s multiple range tests, p = 0.05). Wine treatments: AAB: acetic acid bacteria,

H1: hydroxytyrosol 1mg/l + acetic acid bacteria, OL1: oleuropein 1mg/l+ acetic acid bacteria, OL2: oleuropein 2mg/l + acetic acid bacteria.

3.3 Total phenolic content and total antioxidant activity of Vilana wine with natural phenolic extracts as additives instead of sulphites.

Vilana wines were vinificated using GP extract and OMW residue in different concentrations as a replacement of sulphites during vinification.

Differences in total phenolic content of Vilana wine amongst the treatments are shown in Figure 3.59. Total phenolic content was 16.3% higher than positive control (C1) in V2 wine followed by O3 (15.6 % higher). Significantly statistical differences are shown in Figure 3.61

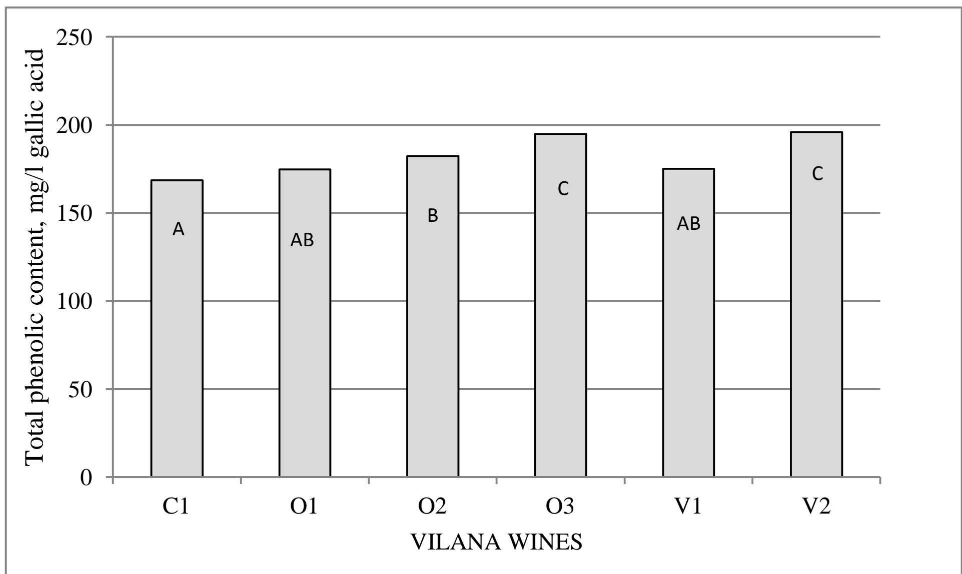


Figure 3.59 Total phenolic content of Vilana wines directly after vinification. Different letters indicate statistical significant differences amongst the treatments. C1: control, O1: OMR 55%, 0.875 gr/100l, O2: OMR 55%, 2.67 gr/100l, O3: OMR 95%, 2.7gr/100l, V1: GP 5 gr/100l, V2: GP, 7.5 gr/100l. (GP: grape pomace extract, OMW: olive oil mill waste water residue).

Total antioxidant activity of the wines obtained by the different vinifications of Vilana is shown in Figure 3.60. Total antioxidant activity was 96% higher than positive control (C1) in V2 wine followed by V1 (47 % higher)and O3 (39 % higher) wines.

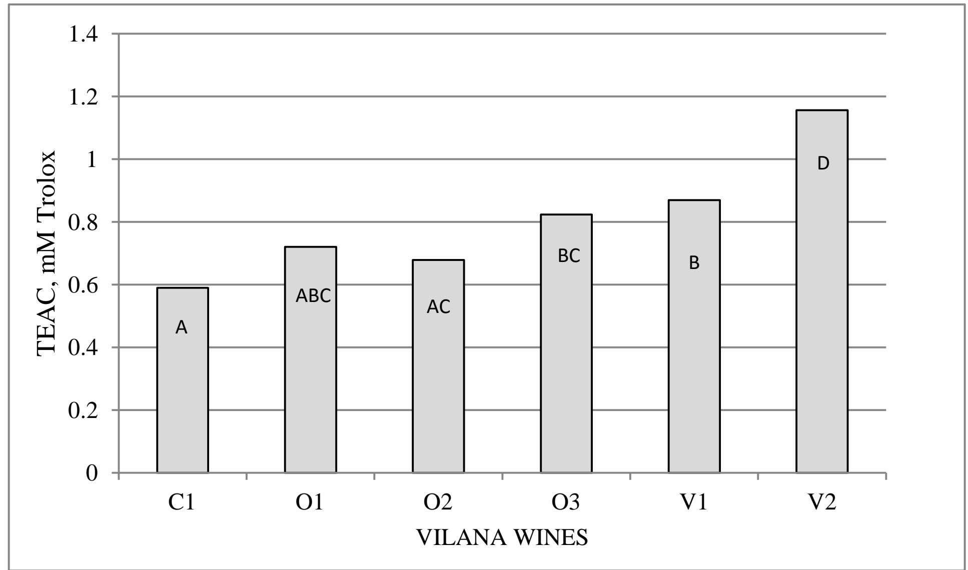


Figure 3.60 Total antioxidant activity of the obtained Vilana wines directly after vinification. Different letters indicate statistical significant differences amongst the treatments. C1: control, O1: OMR 55%, 0.875 gr/100l, O2: OMR 55%, 2.67 gr/100l, O3: OMR 95%, 2.7gr/100l, V1: GP 5 gr/100l , V2: GP, 7.5 gr/100l (GP: grape pomace extract, OMW: olive oil mill waste water residue).

CHAPTER FOUR

DISCUSSION, CONCLUSIONS AND FURTHER WORK

4.1 Total phenolic content, antioxidant activity and phenolic fingerprint of four Greek grape varieties.

4.1.1 Total phenolic content of Vilana, Dafni, Kotsifali and Mandilari wines. Effect of different containers during ageing.

Phenolic content and antioxidant activity of Vilana, Dafni, Kotsifali and Mandilari single grape varieties wines was determined and their evolution during ageing for 12 months in different containers was monitored.

There are no previous data concerning phenolic content and antioxidant activity in Dafni and Kotsifali wine. Any information concerning Kotsifali wines is mainly about wines that are a combination of Kotsifali and Mandilari. No previous work has been reported concerning changes in total phenolic content and antioxidant activity of the tested wines during one year of ageing in different containers. Furthermore, there are some limitations in the ability to make direct comparisons with data concerning wine phenolics and antioxidant activity. In general, the most of the studies on phenolic content have been carried out either in already commercial bottled wines or in wines undergoing ageing, using glass bottles as ageing medium rather than different containers. In the second case, in most of the researches wines were bottled directly after vinification and phenolic content was determined usually after 6, 12 or even more months. Very commonly in tests made in glass bottles there were no data concerning ageing conditions, whether wines had previously been aged in wooden barrels and if so, for how long.

The red wines tested are commonly aged in oak barrels for at least 6 months or 12 months. As far as white wines are concerned, ageing in barrels in not commonly employed. However, lately the use of wooden barrels in white wine has been moderately used, in order to favour aroma and flavour.

According to our results, white wine phenolic content is generally 3-5 times less than red wine content, an expected result as red wines contain more phenolic substances than white wines due to higher phenolic content of red grape berries and longer maceration time during vinification. In Table 4.1 reported phenolic content of commercial wines by several authors are shown, demonstrating the levels and differences between the phenolic contyent of red and white wines.

Phenolic content and antioxidant activity of Vilana wine is higher than Dafni wine regardless of ageing. Similarly, phenolic content and antioxidant activity of Mandilari wine is higher than Kotsifali wine regardless of ageing. Mandilari wine, is famous in Greece for its deep red colour, a characteristic owned to Mandilari grapes high phenolic content and to Mandilari grape seeds high tannin content, as reported by Mylona et al. (2013). Furthermore, according to Kallithraka et al. (2006), Mandilari is one of the richest Greek varieties in phenolic content ranking 3rd out of 20, in tests run in 20 different wines mainly of Greek origin, supporting our results that indicated high phenolic content of Mandilari wine.

Table 4.1 Examples of reported total phenolic content levels (mg/l) in red and white wines.

TPC	Vintage, container, origin/variety	Author
	Red wines	
1019.2-2446.1	1996-1997, bottled	Sánchez-Moreno et al. (1998)
1800-3340	1987-1992, bottled, California wines	Frankel et al. (1995)
1827	2005, bottled, Portuguese wines	Paixao et al. (2007)
2334-3340	Cabernet Sauvignon	Frankel et al. (1995)
1800, 2133	Merlot	Frankel et al. (1995)
1458-2938	2003, 2004, bottled, Thailand wines	(Woraratphoka et al., 2007)
	White wines	
178-486	1996-1997, bottled	Sánchez-Moreno et al. (1998)
165-331	1990-1992, bottled, California wines	Frankel et al. (1995)
282-434	2005, bottled , Portuguese wines	Paixao et al. (2007)
306-845	2004, bottled, Thailand wines	(Woraratphoka et al., 2007)
240, 259	Chardonay, bottled	Paixao et al. (2007)

Makris et al. (2003), reported phenolic content in three commercial bottled Vilana wines of 223.6 mg/l, 311.6 mg/l and 347.6 mg/l. The three tested Vilana wines were of the same vintage (1999). However, as factors such as grape maturity and vinification procedures affect the phenolic content of wines, differences in their results can subsequently be observed. No information was given about whether the wines were aged in barrels or not prior to bottling - a factor that can also affect phenolic content - and for how long the tested wines were bottled.

Mylona et al. (2013) reported a total phenolic content of Mavrokountoura wine (a Mandilari clone, growing in the island of Evoia) of 2.165mg/l directly after vinification (before oak ageing), less in content than that observed in our experiments. Our mean value of the two vinifications directly after vinification was 2786.36mg/l. Kallithraka et al. (2006) reported a phenolic content of 2917.7mg/l of Mandilari wine (island of Paros origin) a few months after

vinification and Kallithraka et al. (2011) reported phenolic content varying from 2764.3mg/l to 3256.4mg/l in bottled Mandilari wines (vintage 2007). Differences in phenolic content of wines can be explained by the dependency of the phenolic content on the grape berry maturity, cultivar conditions and origin, vinification procedures etc.

According to our results phenolic content of wines varied amongst the containers during ageing, indicating the effect that the ageing medium can have on the phenolic content. Wooden barrels had the major influence on wines. Contribution of barrels to total phenolic concentration was greater in white wines, Vilana and Dafni in comparison to red wines, Kotsifali and Mandilari as the extraction of phenolic substances into wines, attributed in a greater extent to their final total phenolic content.

In Vilana and Dafni wines, up until the 6th month of ageing, a slight increase was observed in phenolic content in all containers. Afterwards, until the 12 month of ageing, the increase becomes more intense in wooden barrels reaching the highest content in wine in acacia barrel. Minor differences were observed between wines in American and French oak barrels. Phenolic content increased in stainless steel containers with and without oenosticks in Vilana but to a much lesser extent than in wooden barrels during ageing for 12 months. This increase might be explained by possible transformations of phenolics in wine that may have lead to revealing and therefore measuring under the employed method, higher phenolic content. In Dafni, similar changes were observed in stainless steel with oenosticks container; in stainless steel without oenosticks phenolic content decreased through ageing.

In red wines, the effect of barrels in phenolic content was more intense in Kotsifali wine rather than in Mandilari. In both varieties, the highest phenolic content was observed at the end of the ageing period in wine in Chestnut barrel, followed by wine in Acacia barrel. In Kotsifali wine, phenolics were higher in American oak than in French oak barrels. The exact opposite was observed in Mandilari as phenolic content was higher in French oak than in American oak barrels.

Contrary to our results, Fernández de Simón et al. (2003) reported that polyphenol content in wine ageing in French oak and American oak barrels decreased after 12 month ageing (red, Tempranillo wine). The decrease was higher in French oak than in American oak (by 16.5% in French oak and 10% in American oak). When low molecular weight phenolics were monitored, significantly lower concentrations of ellagic acid (the phenolic substance that is mainly extracted from wooden barrels) flavonols and flavanols were observed in American oak than in French oak barrel.

de Beer et al. (2005) in experiments carried out in red and white wines aged for 12 months in bottles, observed that the phenolic content as well as the antioxidant activity of wines, decreased during ageing and proposed that changes in individual phenolic compounds should be monitored in order to understand the effect of the phenolic composition of wines on their antioxidant activity.

Wines aged in chestnut barrels, were richer in phenolic compounds and tannins compared to wines aged in French oak barrels according to Gambuti et al. (2010). These results agree with our findings, as both in Kotsifali and Mandilari wines phenolic content was higher in chestnut barrels than in French oak barrels.

Alañón et al. (2013) reported that in wine aged for 6 months in chestnut barrels, ellagic acid and gallic acid concentrations increased greatly during ageing on the contrary, anthocyanin concentration decreased. Moreover, the major decrease in anthocyanin concentration was observed in the first three months of ageing in chestnut barrels. They also mentioned that wood components such as ellagic acid and ellagitannins may play a role in the decrease of the content of anthocyanins. This supports our results giving a possible explanation to the observed decrease in antioxidant activity of our wines during the first three months of ageing and the following increase as well. The decrease in antioxidant activity might be explained by oxidation of anthocyanins and conjunction with tannins. Hydroxycinammic acids extracted from the barrels might further induce anthocyanin decrease in wines.

High gallic acid concentration in wine contributes to bitterness and astringency, affecting negatively wine taste. Furthermore as stated by Chira et al. (2014), ellagitannins attribute a smooth and velvety astringency to wines but only at low threshold concentrations, varying from 0.9-2.8μmol/l.

However, ellagitannins concentrations may become higher than these values, a characteristic that should be considered especially in the case of wooden barrels that may donate though extraction high ellagitannins' concentration in wine, effecting negatively their organoleptic properties. Extensive extraction of ellagitannins in Vilana, Dafni, Kotsifali and Mandilari wines from wooden barrels may result in a negative effect on their quality especially in wines ageing in chestnut barrels, known for their high content in polyphenols that can be attributed to wine (De Rosso et al., 2009), pointing out the fact that many factors must be taken under consideration to determine the best medium for ageing of each wine.

According to De Rosso et al. (2009) acacia barrels are more suitable for long term ageing than chestnut and oak barrels due to higher content of oxidazable polyphenols that indicates that acacia wood is the less oxidative environment.

As reported by Zafrilla et al. (2003) minor changes were observed in the phenolic content of white Airen wine during 7 month of ageing in bottles, whereas phenolic content of Monastrell wine (red wine) decreased during the same period of time. These results, compared to wines aged for 6 months in stainless steel containers (agree in general with our findings as far as Dafni, Kotsifali and Mandilari wines are concerned (in Vilana wine, total phenolic content increased during 6 months of ageing).

(Castellari et al., 2001), agreeing with our results, observed a significant increase in total phenolic content in Sangiovese wine (red wine) ageing in chestnut barrel at the end of ageing period, being at the same time significantly higher to that ageing in French oak barrel (ageing period: 320 days). In both barrels, phenolic content increased during ageing. Compared to the initial phenolic content, a reduction was observed after 320 days in stainless steel container. Similar results were found in Kotsifali wine. Castellari et al. (2001) also found significant differences in phenolic content amongst wines in barrels and wine ageing in stainless steel container. They stated that phenolic substances extracted from chestnut barrels, were more tannic in chestnut wood that in oak barrels and were not compensated by the polymerization reactions occurring during ageing.

Gómez-Plaza et al. (2000), reported statistically significant changes in the phenolic content of tested Monastrell wines during a 12 month, ageing in bottles, period. Phenolic content varied between 1006.66-1305.75mg/l during ageing. The highest content in phenolic substances was observed at 3 month ageing. At the end of the essay (12 months) they also observed that phenolic content was higher (but not statistically significant different than the initial).

4.1.2 Total antioxidant activity of Vilana, Dafni, Kotsifali and Mandilari wines. Effect of different containers during ageing

Total antioxidant activity of Vilana, Dafni, Kotsifali and Mandilari wine was determined. As expected, antioxidant activity in red wines was much higher than in white wines, due to the higher phenolic content of red wines. Great differences were observed in antioxidant activity of Kotsifali (9.82mM and 8.35M of Trolox) and Mandilari wine (17.43mM and 19.28 of Trolox), also attributed to differences in their phenolic content. Total antioxidant activity of all wines tested increased during ageing in wooden barrels. Changes in antioxidant activity after 12 months of ageing was greater in white wines than in red wines, possibly due to the contribution of wine phenolics which also exhibit a markedly increase during ageing.

In Dafni and Vilana, the highest antioxidant activity was observed in Acacia barrel, followed by French oak and American oak barrels. It was interesting that regardless of the container, a significant decrease in total antioxidant activity occurred after three months of ageing, followed by such an increase in the next months that the antioxidant activity in most of the studied wines reached the initial levels. The same was observed in antioxidant activity of Kotsifali (1st vinification) and Mandilari wines. Changes in the phenolic composition of wines such as formation of new tannin molecules, oxidation and polymerization of anthocyanins to more complex molecules, are possible transformations affecting antioxidant activity. Furthermore, according to Psarra et al. (2002) the antiradical activity of white wines may also derive from synergistic phenomena between individual polyphenols.

De Beer et al. (2003) determined total phenolic content and antioxidant activity (Teac method) of bottled red (Cabermet Souvignon, Merlot, Shiraz) and white wines (Sauvignon blanc, Chardonnay, Chenin blanc, Colombard), stating approximately 8 times higher phenolic content of red wines than of white wines. According to the results, phenolic content and antioxidant activity in red wines varied between 2016-2498.8mg/l and 13.177-15.757mM of Trolox respectively, whereas in white wines phenolic content varied between 242.0-292.7mg/l and antioxidant activity between 0.8-1.06mM of Trolox. In the same assay, lower values in antioxidant activity were observed when the determination was carried out using the DPPH method. The above results, demonstrate differences in phenolic content and antioxidant activity between red and wine wines, supporting the existing (although expected) differences in phenolic content and antioxidant activity of the Vilana, Dafni, Kotsifali and Mandilari wines tested in our assay, since both antioxidant activity and phenolic content were also higher in red wines than in white. According to our results phenolic content is approximately 5 times higher in red wines than in white wines, mainly due to the higher level of white wines phenolics than that observed by De Beer et al. (2003) (our mean value in white wines was 446 mg/l).

(Zafrilla et al., 2003), contrary to our results, reported that antioxidant activity in red but also in white wines stored for 7 months in bottles was not significantly differentiated. However, they observed a decrease in antioxidant activity of Monastrell red wine, at 6 month of storage which was regained after the 7th month of storage.

(De Rosso et al., 2009) reported that ageing of wine in chestnut and oak barrels for 12 months lead to an increase in flavonoid content, whereas ageing in acacia barrel lead to decrease after six months of ageing followed by an increase at levels higher than the beginning of the experiments. In all barrels, anthocyanin content decreased constantly throughout ageing, but was not affected by the barrel type. Also, Ivanova et al. (2012) reported intensive decrease of

total anthocyanins in Vrenac wines after 2 months of bottle ageing, whereas total phenolic content remained almost stable for an even longer ageing period.

These results supports the suggestion that a possible decrease in anthocyanin content either by oxidation or transformation, (reported by many researchers) a major contributor to wine antioxidant activity, might be responsible for the observed decrease in antioxidant activity of our tested wines, during the first months of ageing. del Álamo Sanza et al. (2004) reported that red wine ageing for 12 months in barrels (French, Hungarian and American oak barrels) lead to a smaller decrease in anthocyanin content than wine ageing in containers with oak chips, decreasing in all cases after the 3rd month of ageing. Del Alamo Sanza et al. (2004) observed that anthocyanin content decreased during ageing, the highest decrease was in stainless steel, followed by sticks and wine ageing in American, French and Hungarian oak. According to Zafrilla et al. (2003), a high loss in anthocyanin concentration was observed during 7 months of ageing (88% decrease in 7 months, 76% decrease in the first three months). According to them, hydroxycinnamic acids' and flavonols' concentration did not have any significant variations during ageing. Furthermore, anthocyanins and other complex compounds contribute to the formation of tannins. Tannins are very effective antioxidants, being highly polymerized pigments and have many phenolic hydroxyl groups. In fact, according to Hagerman et al. (1998); Zafrilla et al. (2003), tannins are 15−30 times more effective at quenching free peroxyl radicals than simple phenolics. As a consequence alterations in tannin content may also have affected antioxidant activity of the wines tested in out experiments.

Furthermore according to Zúñiga et al. (2014) apart from anthocyanins, gallic acid is a substance highly correlated with antioxidant activity. Comparatively high concentrations occurring as previously mentioned in wine ageing in wooden barrels, can affect their antioxidant activity.

Rivero-Pe?rez et al. (2007) reported that antioxidant activity, is strongly related to the phenolic structure apart from the phenolic content. De Beer et al., 2005 in experiments carried out in red and white wines ageing for 12 months in bottles, observed a decrease in the phenolic content as well as in the antioxidant activity of wines during aging in bottles and proposed that changes in individual phenolic compounds should be monitored in order to understand the effect of the phenolic composition of wines to their antioxidant activity. This conclusion concerns our results as well - thus wines ageing in wooden barrels as well. In order to fully understand how phenolics affect antioxidant activity, phenolic content is not sufficient enough; an investigation on changes on the phenolic composition in wine must be made.

In general, as far as antioxidant activity is concerned and aiming health benefits resulting for moderate wine consumption, acacia barrels and chestnut barrels are the containers showing the

most promising results as they result in higher levels of both phenolic content and antioxidant activity. Furthermore, ageing for at least 6 months increases antioxidant activity. Nevertheless, this is a usual procedure, especially for red wines as red wines are usually aged for at least 12 months in wooden barrels.

4.1.3 Correlation between total antioxidant activity and phenolic content.

Correlation between total antioxidant activity and phenolic concentration of wines was studied. In the literature, most authors concluded that there is a positive correlation between total antioxidant activity and phenolic concentration (Paixao et al., 2007; Staško et al., 2008). According to our results, antioxidant activity correlates to phenolic content but more markedly in red than in white wines. The lower correlation observed in white wines is possibly affected by higher contribution of other substances present in wines such as sulphur dioxide, to antioxidant activity. White wines have lower phenolic concentration, however, as previously mentioned, some fractions of them have high antioxidant activity that despite their low concentrations, make them able of affecting the final antioxidant activity. The extraction of phenolic substances form wooden barrels also affects their antioxidant activity, either by their presence in wines or by their precipitation in reactions responsible for the formation of new antioxidants.

In disagreement to our results, no relation between antioxidant activity and phenolic content of red and white wines was reported by Zafrilla, Morillas et al. (2003).

4.2 General conclusions on antioxidant activity and phenolic content of wines

The existing information on wine phenolic content can be a little conflicting mainly due to the many factors that contributes to and affect phenolic content. Apart from grape variety, factors such as grape maturity, cultivar conditions, vinification procedures and ageing media as well as temperature can play a great role in wine characteristics such as phenolic content, colour, taste and antioxidant activity. Wooden barrels can play a significant role in wines phenolic content affecting it through the compounds that are extracted into wine and their interaction with wine compounds. The addition of oak sticks or chips in containers is a cheap alteration of wooden barrels, lately used in wine ageing. According to our results they do not result in as high phenolic content and antioxidant activity as in acacia, French oak and chestnut barrels.

However, they were more effective than stainless steel containers and also, were quite close to American oak barrel effect. Furthermore as far as antioxidant activity is concerned, any results obtained by different researches are difficult to compare due to the differences in the methods used that make direct comparison of antioxidant activity almost impossible.

The aim of this study was to determine the phenolic content and antioxidant activity of the four Greek wines and the influence different containers have on them during ageing. Acacia barrel for white wine and chestnut barrel followed by acacia barrel for red wines seemed to have the best behaviour as far as the above characteristics are concerned, resulting in wines with higher phenolic content and antioxidant activity. Wooden barrels, such as acacia and chestnut barrels, are responsible for the extraction of different type and different amount of phenolic substances - such as hydrolysable tannins - into wine, affecting in this way apart from the phenolic content, the antioxidant activity of wines, as well

The most common practice for red wine ageing nowadays are oak barrels. Further analysis must be made to determine whether Kotsifali and Mandilari wines gain, apart from higher antioxidant activity and phenolic content, better organoleptic characteristics, making acacia and chestnut barrels a better alternative for ageing. The same goes for the white wines Vilana and Dafni; white wine ageing in wooden barrels for a long time is not that common, however interesting organoleptic characteristics can be gained though wooden barrel ageing, as long as wood characteristics do not overlap the wines organoleptic characteristics.

In order to recommend the most appropriate type of barrel for each variety, factors such as taste, aromas etc, should be considered as well to achieve the best possible result. The optimum interaction between wood and wine should be achieved in order to result not only in wines with optimum health benefits (high in antioxidant potential) but also with most the desirable organoleptic characteristics for the consumers.

4.3 Phenolic fingerprint of Vilana, Dafni, Kotsifali and Mandilari wines

Phenolic fingerprints of Vilana, Dafni, Kotsifali, and Mandilari wines were obtained in the mid infrared region by means of FTIR-ATR, for the first time. Authentification and discrimination of wine varieties using their phenolic fingerprints in the mid infrared region has been recently used for red wines (Edelmann et al., 2001; Tarantilis et al., 2008). In this work, apart from red wines discrimination of two Greek white wines was also achieved using their phenolic fingerprints.

Phenolic fingerprint was found to be characteristic for each wine used in the experiment allowing discrimination amongst Vilana, Dafni, Kotsifali and Mandilari wines. Based on our knowledge, discrimination of white wines has not been made based on their phenolic fingerprint using FTIR-ATR. Although white wines contain fewer phenolic substances than red wines, discrimination of Vilana and Dafni directly after vinification was easily achieved, observing characteristic differences in absorption spectra of their phenolic extracts.

Red wine extracts (Kotsifali and Mandilari) had also differences in their phenolic fingerprint as expected. The major difference through ageing was observed at 1110 cm^{-1}, where only Mandilari gave a characteristic peak.

In general, the method with some modification can be also used for quantification of total phenolics. As reported by Silva et al. (2014) FTIR-ATR can be used for a screening of phenolic content of wines and using the right methodologies, the technique can give a rough estimation for their antioxidant activity. In most cases, absorption spectra of phenolic extracts obtained in our experiments indicated differences in the phenolic concentration of wines but more intensive analysis must be carried out to make to such a determination. Phenolic fingerprint was determined aiming phenolic qualification of wines. Even minor differences in the volume of the extracts that do not affect phenolic fingerprint in terms of qualification can affect it in terms of quantification.

4.4 Effect of hydroxytyrosol and oleuropein on volatile acidity of wines

The effect of hydroxytyrosol and oleuropein in volatile acidity of wines as a cause of spoilage wine induced by acetic acid bacteria was determined. In most tests, in hydroxytyrosol treatments minor increases in volatile acidity were observed. Volatile acidity of control as well as acetic acid bacteria treatment was increased indicating wine spoilage in both cases. Comparing treatments to control, hydroxytyrosol displayed a smaller increase amongst the treatments. The differences between hydroxytyrosol and control were still significant. It appeared that hydroxytyrosol was more effective at low concentrations. Treatment with 0.5mg/l hydroxytyrosol gave better results than treatment with 1mg/l of hydroxytyrosol.

Hydroxytyrosol is a substance already contained in wines and therefore could be approved for use as a wine additive. Obtained results, did not suggest that it could replace sulphites in wines. Even though, a potential to regulate wine spoilage was observed, it was not sufficient enough to prevent the spoilage. Further experiments should be carried out for its use in wines as an

additive instead of sulphites, maybe in combination with other compounds, or possibly in combination with sulphites, decreasing the used sulphites amounts. However, the high cost of hydroxytyrosol is a negative factor on its use especially in common commercial wines. However, some wine consumers including those with sensitivity in sulphites can disregard wine's price in order to buy wines with certain characteristics (e.g sulphite-free wines).

Minor differences were observed between acetic acid bacteria and oleuropein treatments. It was quite interesting that in one of the wines used in the experiment oleuropein gave higher volatile acidity compared to acetic acid bacteria, which - taken in account the antibacterial characteristics of oleuropein - was not expected.

4.5 Effect of natural phenolic additives on phenolic content and antioxidant activity of Vilana wines.

Vilana wines in which grape pomace extract obtained from vineries and olive oil mill waste-water residue were used during vinification instead of sulphites, had higher phenolic content than that observed in control, increasing with concentration. Although there were no differences between them on the effect they had on phenolic content, differences existed on the antioxidant activity of wines. Grape pomace extract treated wines, resulted in wines with higher antioxidant activity, revealing that despite the similar attribution to phenolic content, the attribution of grape pomace extract to antioxidant activity was significantly higher than that of olive oil mill waste-water. A possible explanation of this observation can be the higher antioxidant activity of the phenolic substances contained in grape pomace extract. Further research could reveal if this is due to interactions with wine substances or if this is a richer resource of antioxidant substances compared to olive oil mill waste-water residues.

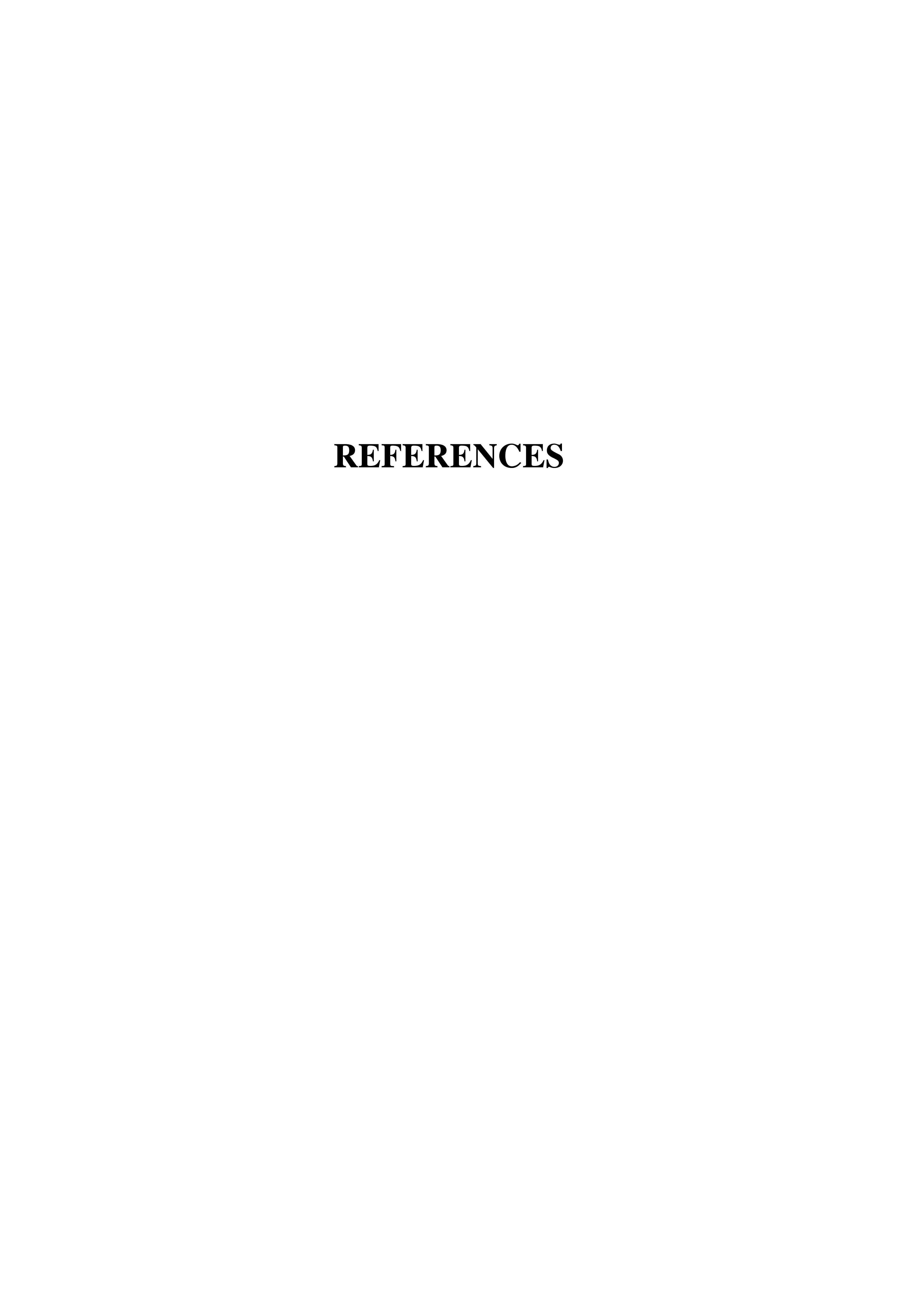
4.6 Further work

Wine quality is a combination of many factors. In order to determine the best medium for ageing of the tested wines, characteristics such as wine colour, aroma and taste (sensory analysis) must be done and definitely be taken into account to determine the optimum ageing medium for each variety. The optimum ageing period for each wine must also be determined as a combination of the above characteristics. With the same aim, antioxidant activity and phenolic content of wines will be monitored during storage for one year in bottles.

This study was carried out under an interlaboratory program with title "Evaluation and optimization of the quality factors during maturation of wines produced from Cretan red and white grape varieties for the production of high quality wines". Results concerning sensory characteristics, colour development, anthocyanin and tannin and other wines components content and changes obtained by both classical and modern techniques and methods will be combined in order to evaluate the conditions favouring the tested wines quality.

Vilana, Dafni, Kotsifali and Mandilari wines' phenolic fingerprint was determined directly after vinification for their discrimination and changes occurring during ageing in containers were monitored. Phenolic fingerprint of wines can be characteristic allowing the discrimination of wines of different vintages, origin etc. Investigation of differences or similarities driven out by the two different vinifications of wines, ageing in containers and afterwards stored for one year in bottles will be made. Libraries based on the phenolic fingerprints of the wines should be used to investigate whether the ageing medium of the wines give distinctive characteristics to wine's phenolic fingerprint or not.

Volatile fingerprints of wines can be taken to investigate the possible prediction of volatile acidity by means of FTIR.



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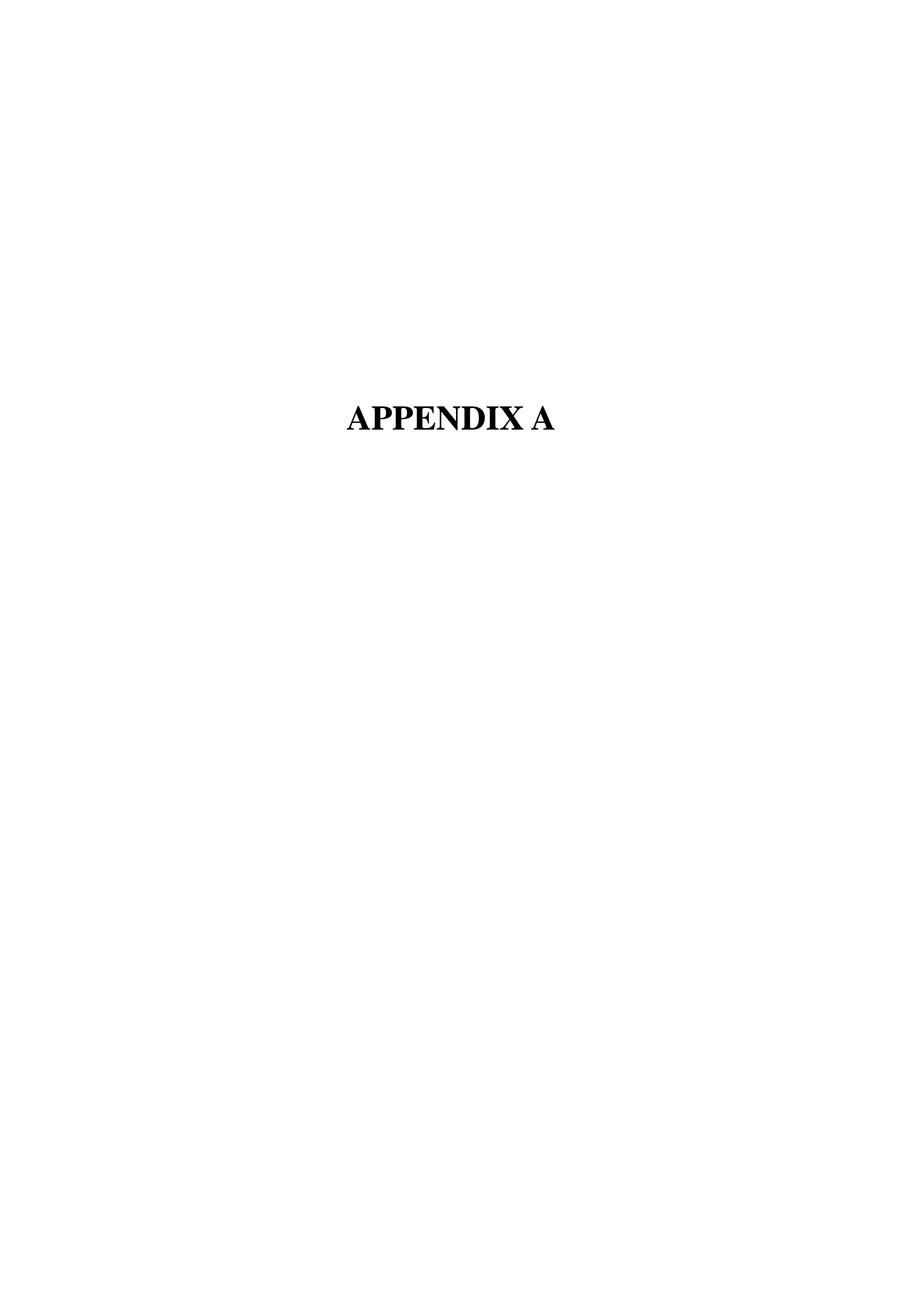

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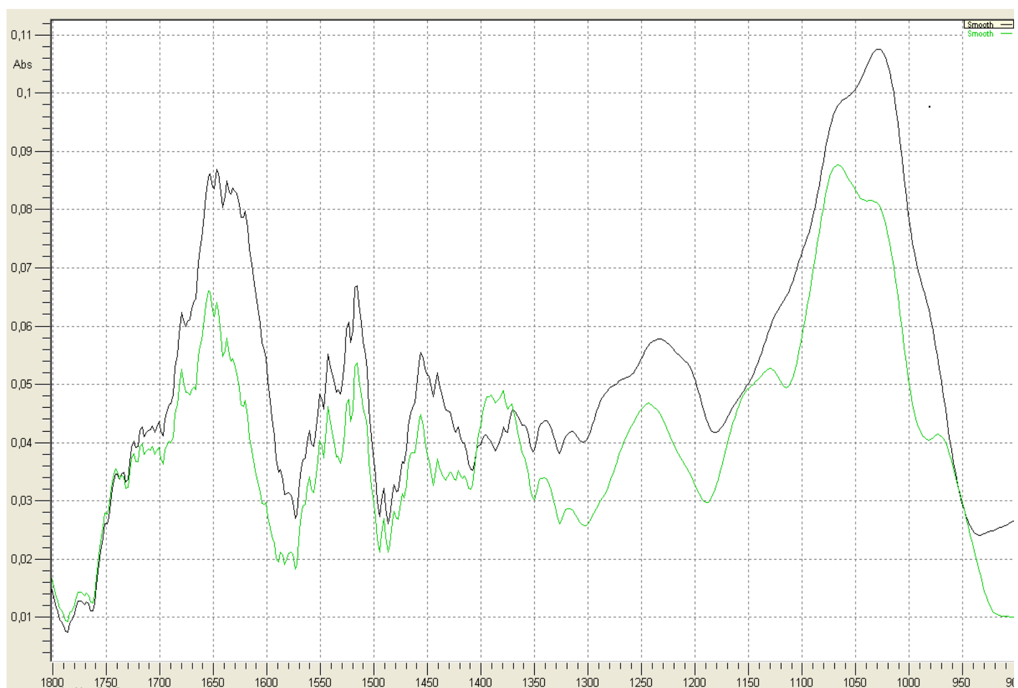


Figure A1. Absorption spectrum of white wines (Dafni: --, Vilana: ---) directly after vinification

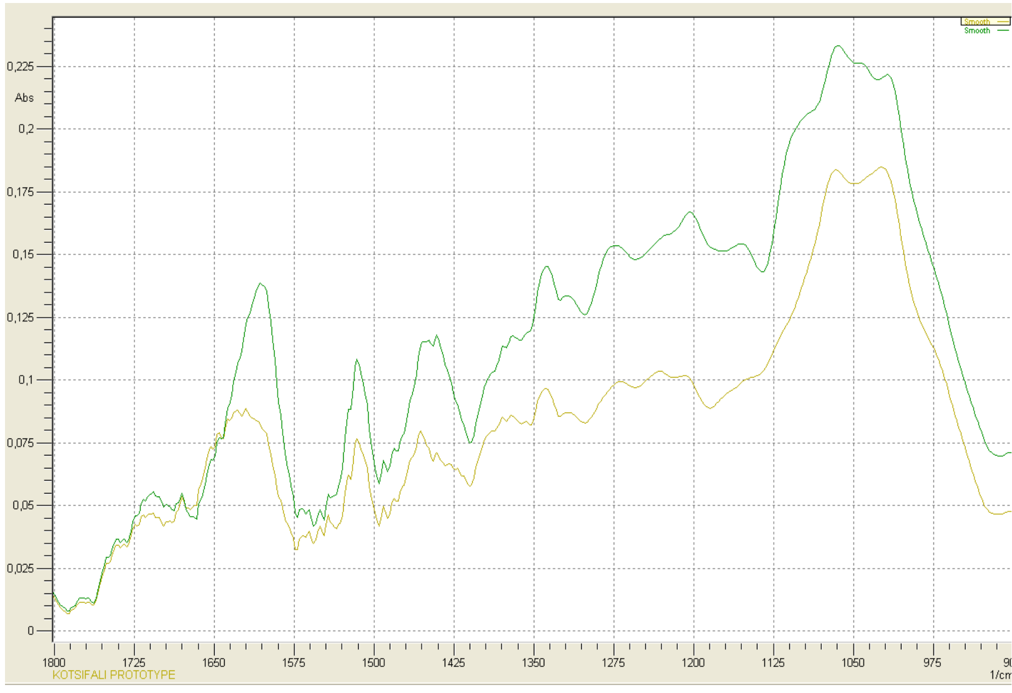


Figure A2: Absorption spectrum of red wines (Mandilari:---, Kotsifali:---) directly after vinification

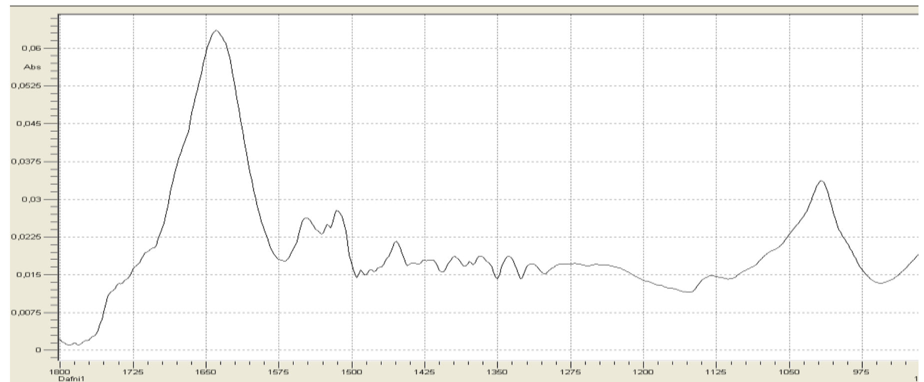


Figure A3: Absorption spectrum of Dafni, after 3 months ageing in stainless steel container.

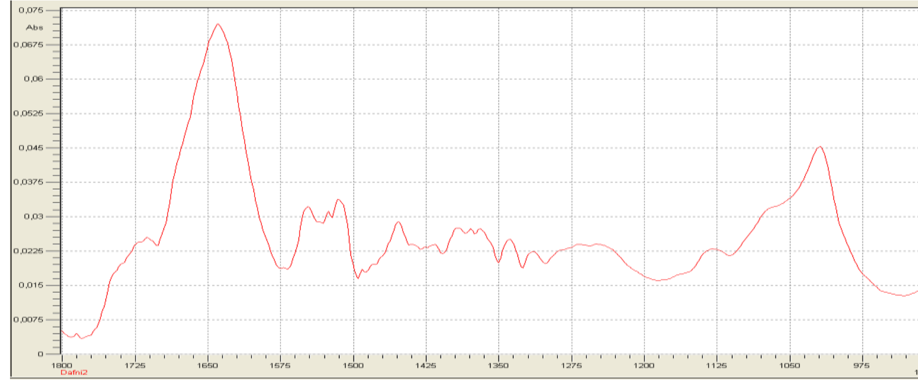
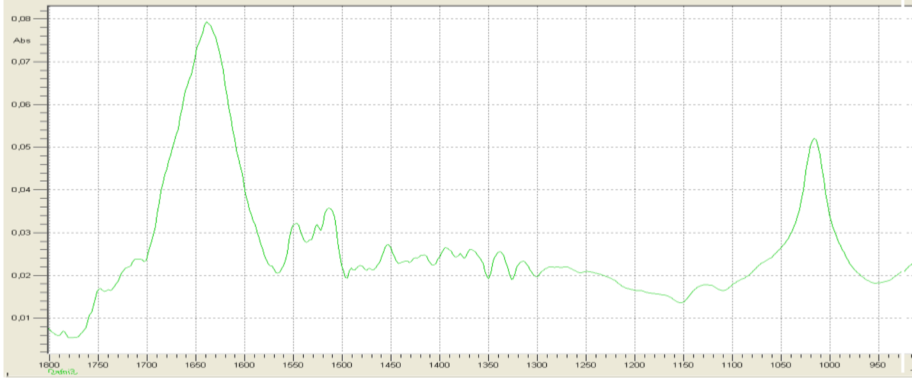


Figure A4 Absorption spectra of Dafni after 3 months of ageing in stain.steel with oenosticks.container



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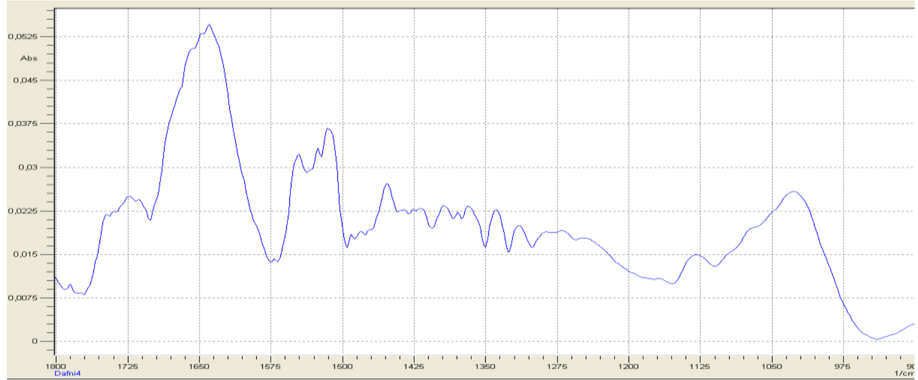

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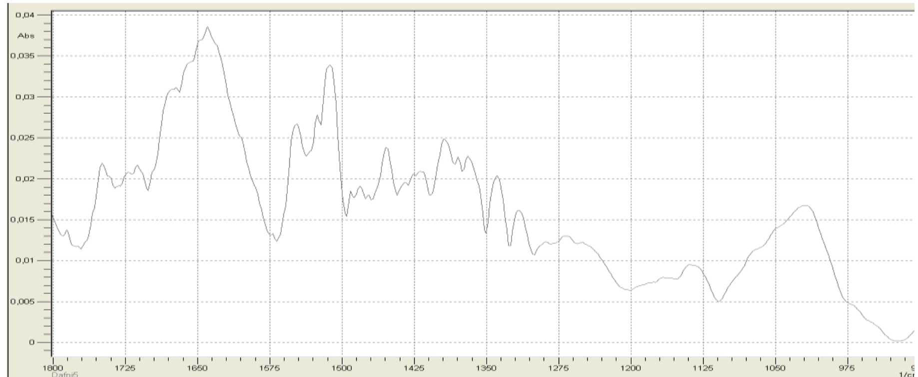


Figure A7: Absorption spectrum of Dafni after 3 months ageing in French oak barrel.

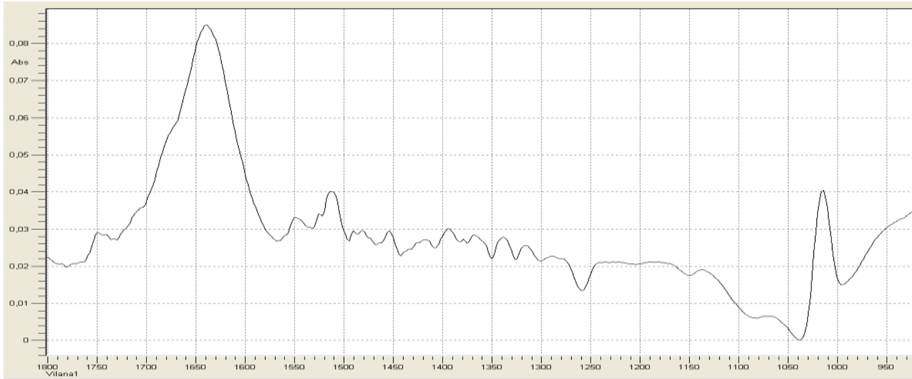


Figure A8: Absorption spectra of Vilana after 3 months of ageing in stainless steel container

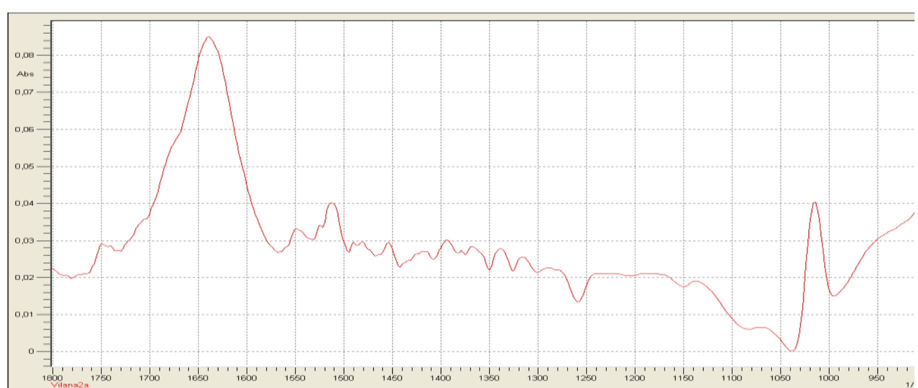


Figure A9: Absorption spectra of Vilana after 3 months of ageing in st. steel with oenosticks container

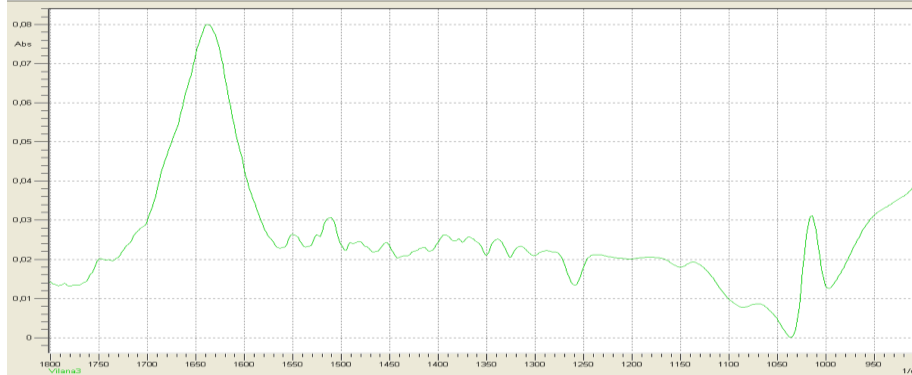
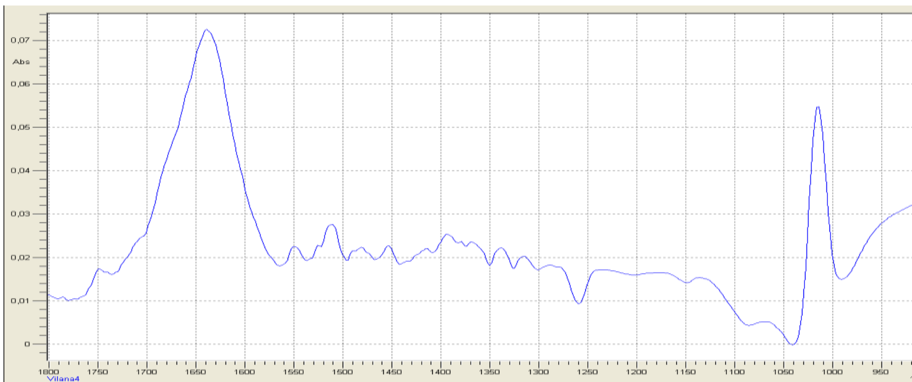


Figure A10: Absorption spectra of Vilana after 3 months of ageing in American oak barrel



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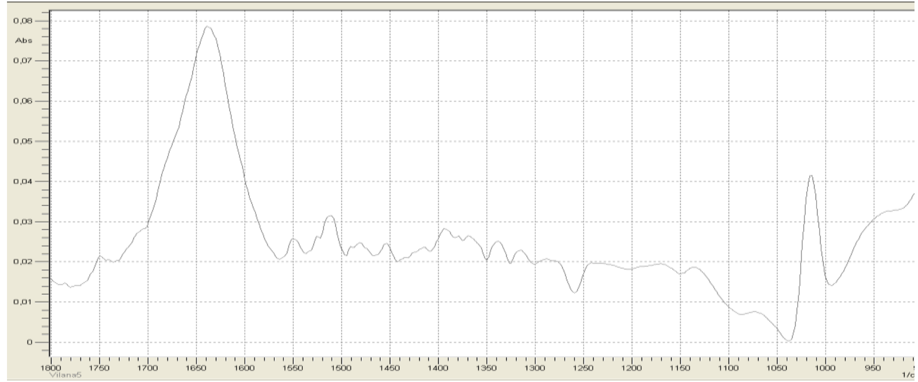

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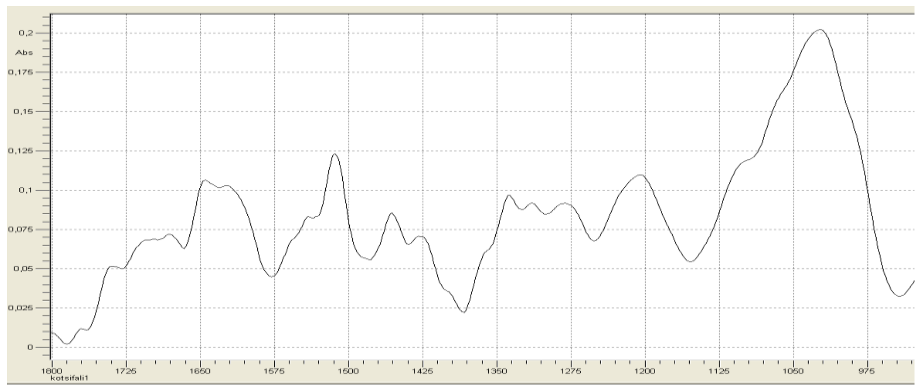

Figure A13: Absorption spectra of Kotsifali after 3 months of ageing in stainless steel container

Figure A14: Spectra of Kotsifali after 3 months of ageing in st.steel with oenosticks container

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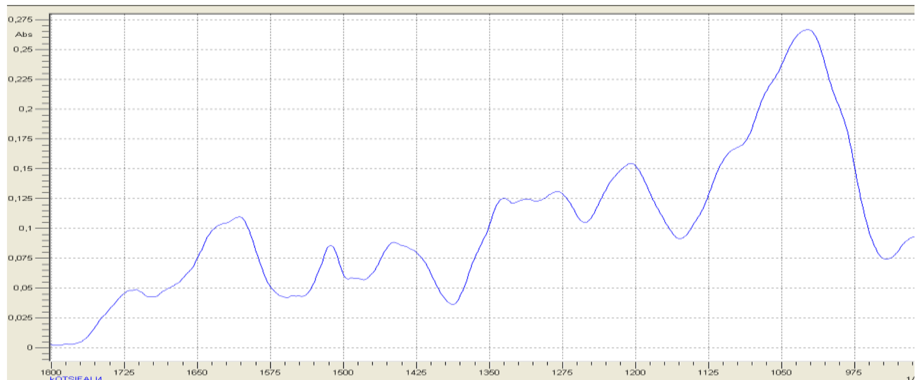

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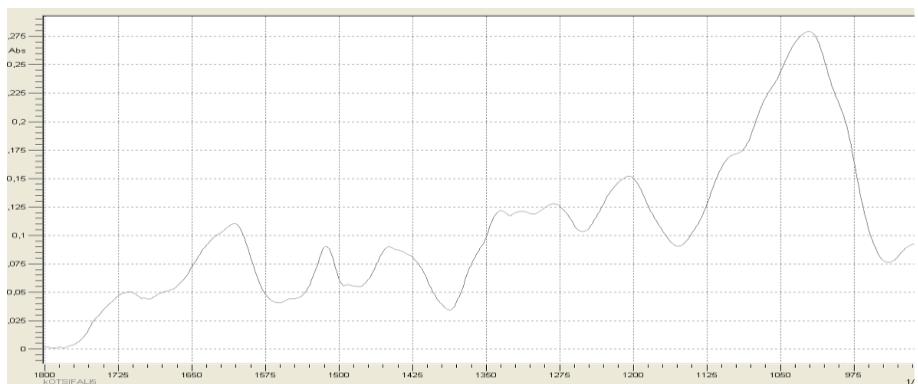


Figure A17: Absorption spectra of Kotsifali after 3 months of ageing in French oak barrel

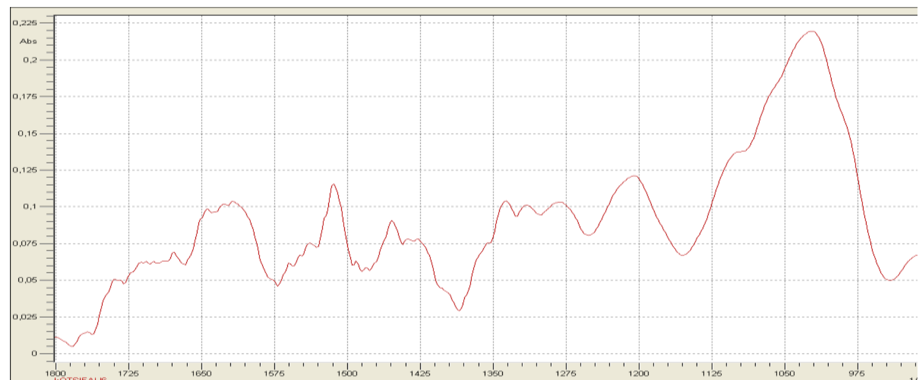
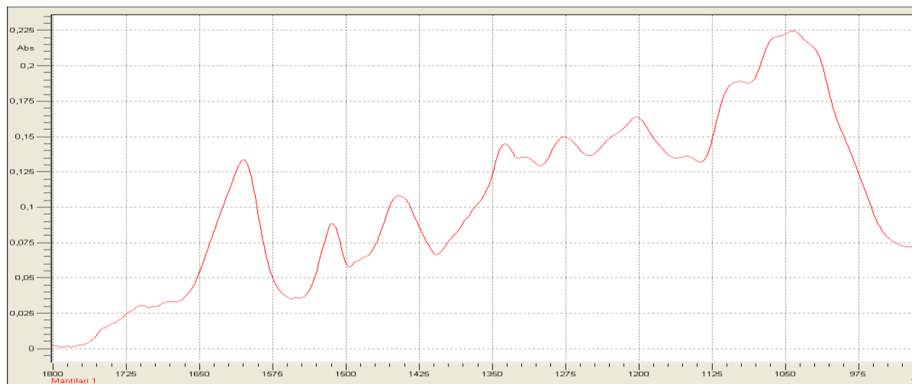


Figure A18: Absorption spectra of Kotsifali after 3 months of ageing in Chestnut barrel



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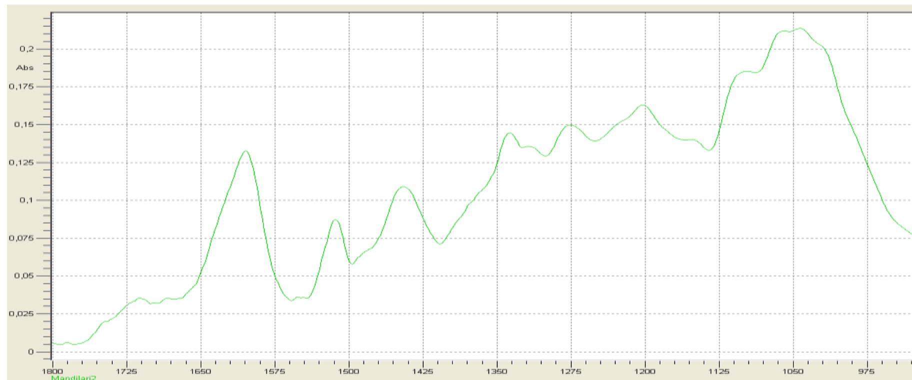


Figure A20: spectra of Mandilari after 3 months of ageing in stainless steel with oenosticks container

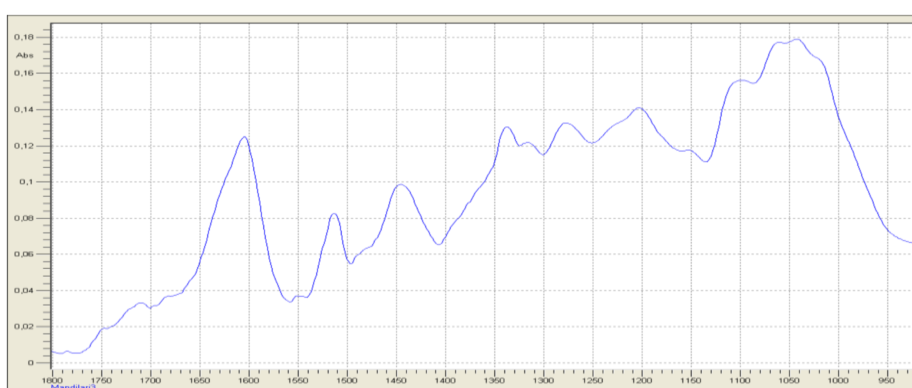


Figure A21: Absorption spectra of Mandilari after 3 months of ageing in American oak barrel

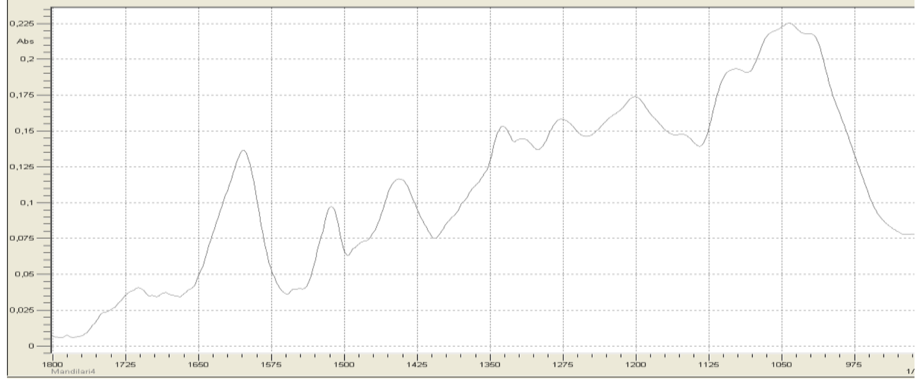
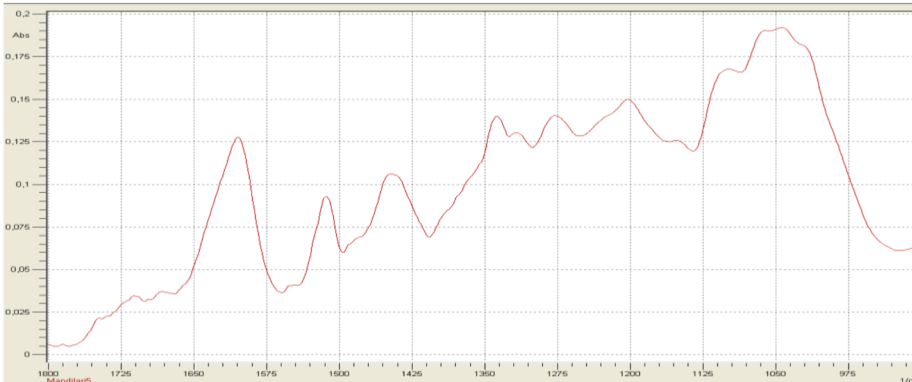


Figure A22: Absorption spectra of Mandilari after 3 months of ageing in acacia barrel



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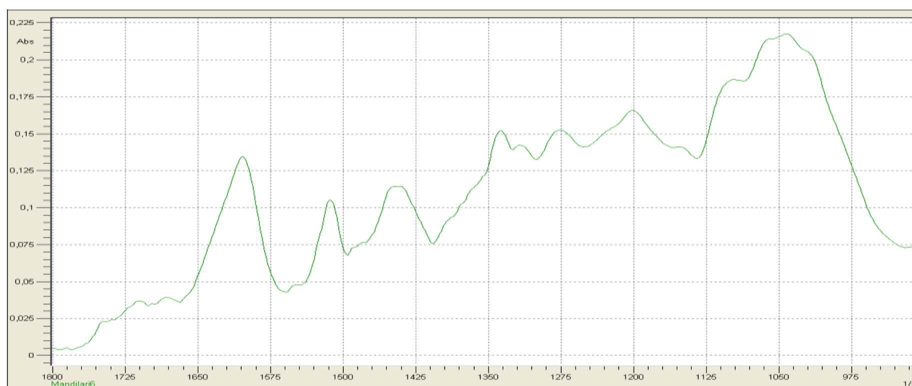

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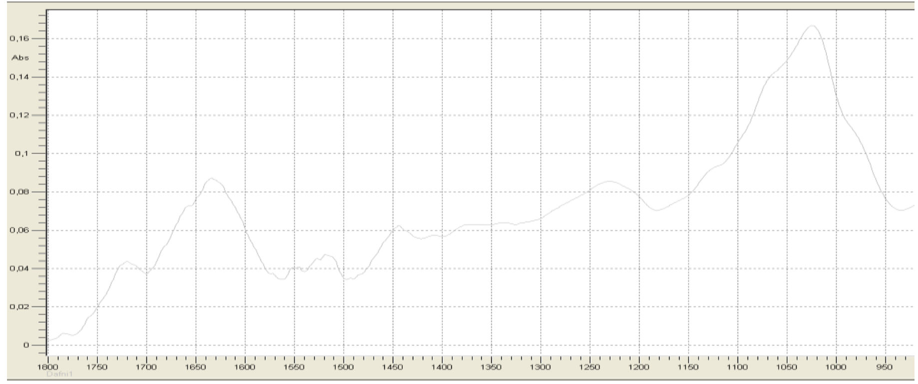

Figure A25: Absorption spectra of Dafni after 6 months of ageing in stainless steel container

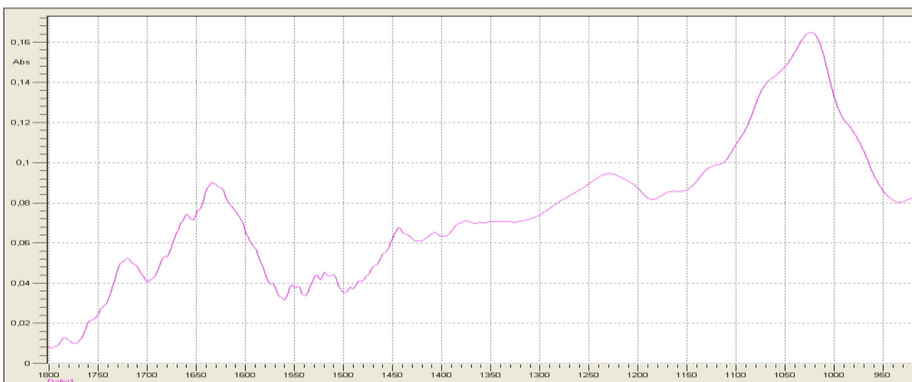

Figure A26: Spectra of Dafni after 6 months of ageing in stain. steel with oenosticks container

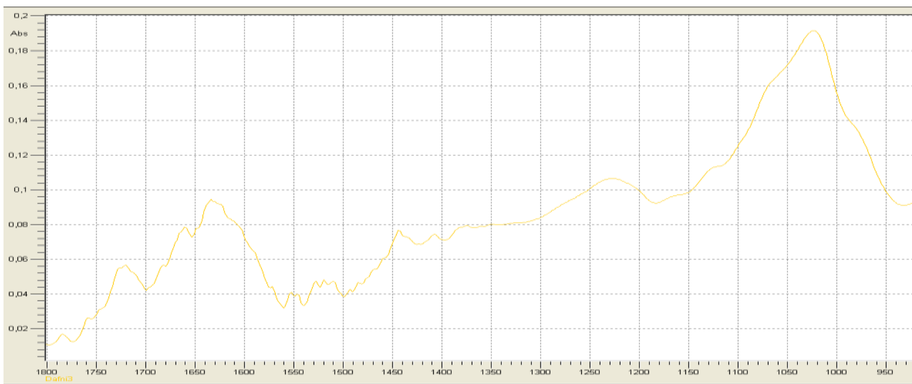

Figure A27: Absorption spectra of Dafni after 6 months of ageing in American oak barrel

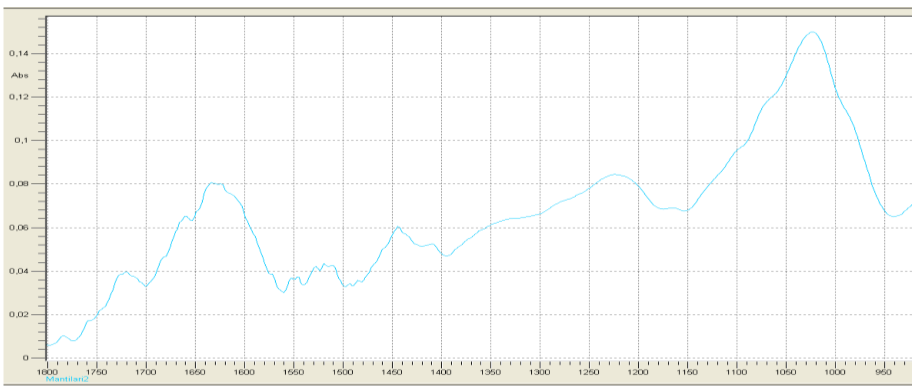

Figure A28: Absorption spectra of Dafni after 6 months of ageing in acacia barrel

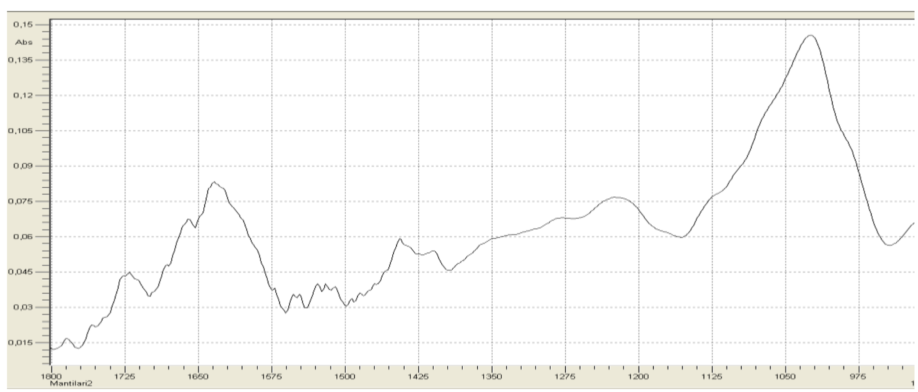

Figure A29: Absorption spectra of Dafni after 6 months of ageing in French oak barrel

Figure A30: Absorption spectra of Vilana after 6 months of ageing in stainless steel container

Figure A31: Spectra of Vilana after 6 months of ageing in st. steel with oenosticks container

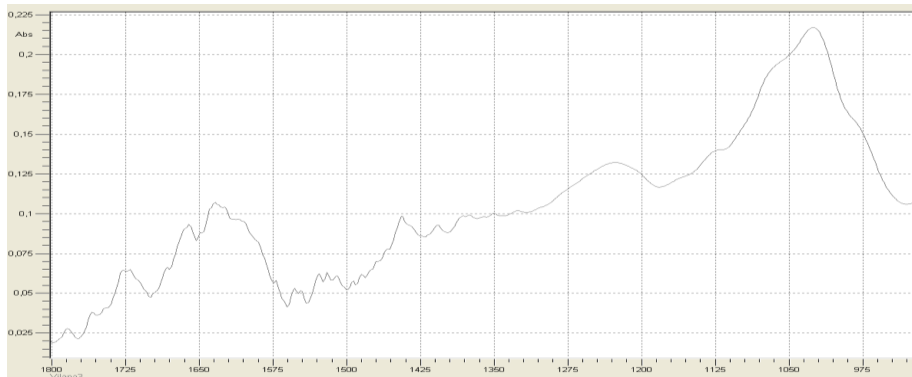

Figure A32: Spectra of Vilana after 6 months of ageing in American oak barrel

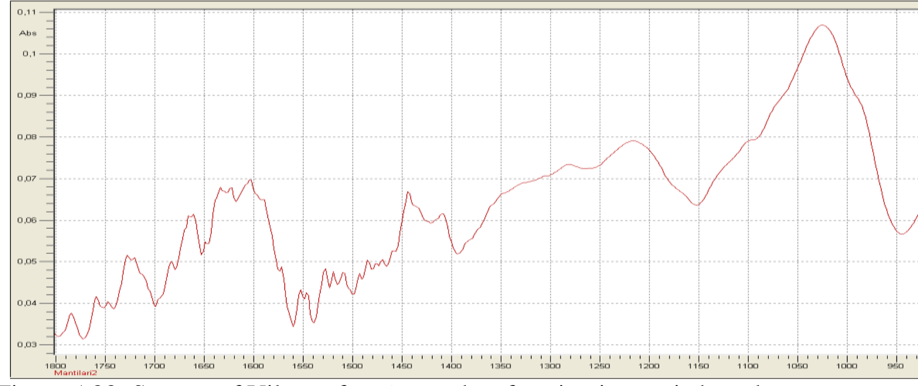

Figure A33: Spectra of Vilana after 6 months of ageing in acacia barrel

Figure A34: Spectra of Vilana after 6 months of ageing in French oak barrel

Figure A35: Absorption spectra of Kotsifali after 6 months of ageing in stainless steel container

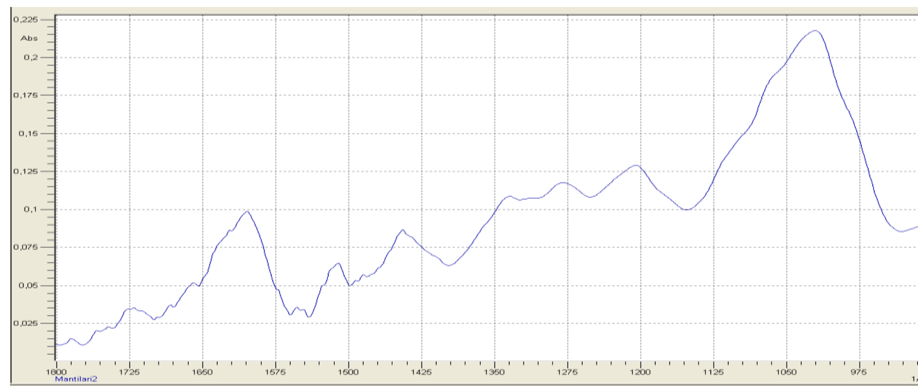

Figure A36: spectra of Kotsifali after 6 months of ageing in st steel with oenosticks container

Figure A37: Absorption spectra of Kotsifali after 6 months of ageing in American oak barrel

Figure A38: Absorption spectra of Kotsifali after 6 months of ageing acacia barrel

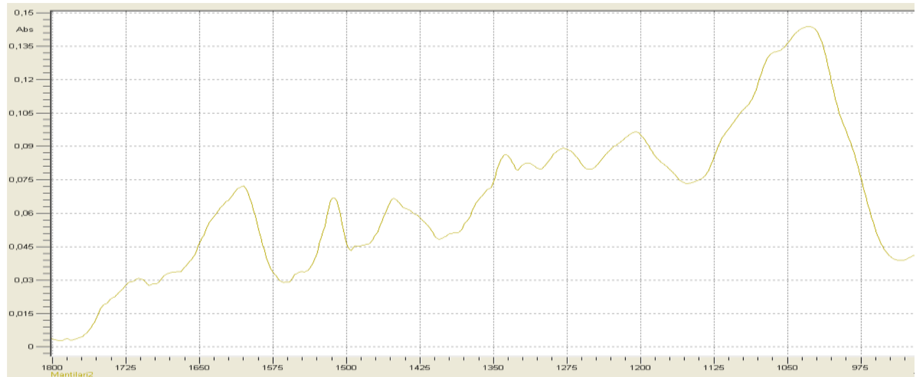

Figure A39: Absorption spectra of Kotsifali after 6 months of ageing French oak barrel

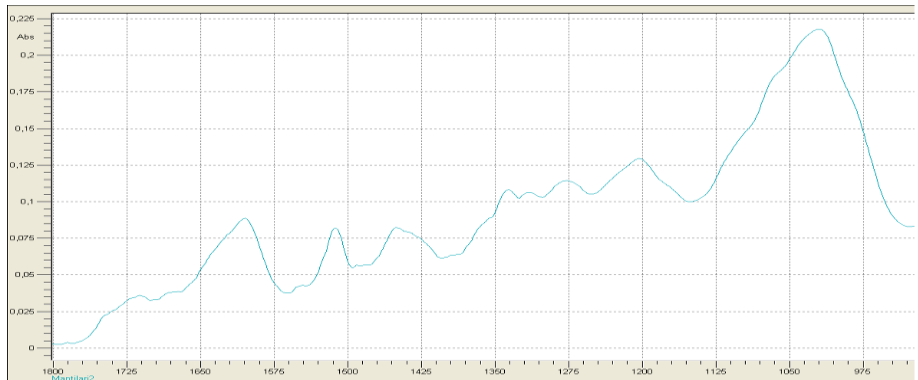

Figure A40: Absorption spectra of Kotsifali after 6 months of ageing in Chestnut barrel

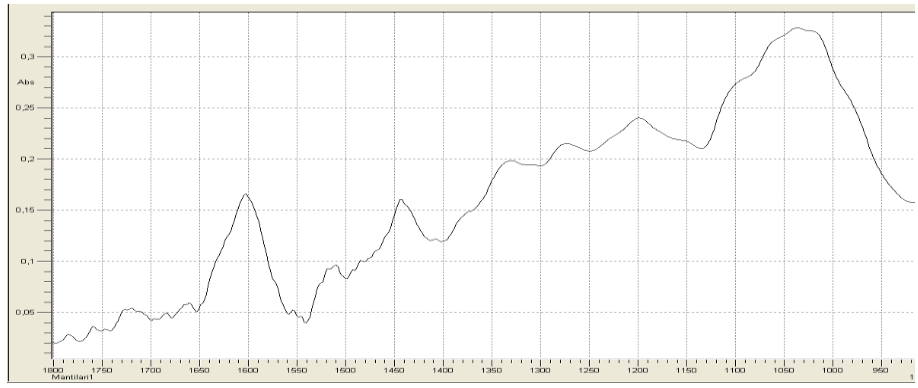

Figure A41: Absorption spectra of Mandilari after 6 months of ageing in stainless steel container

Figure A42: Absorption spectra of Mandilari after 6 months of ageing in st. steel with oenosticks container

Figure A43: Absorption spectra of Mandilari after 6 months of ageing in American oak barrel

Figure A44: Absorption spectra of Mandilari after 6 months of ageing in acacia barrel

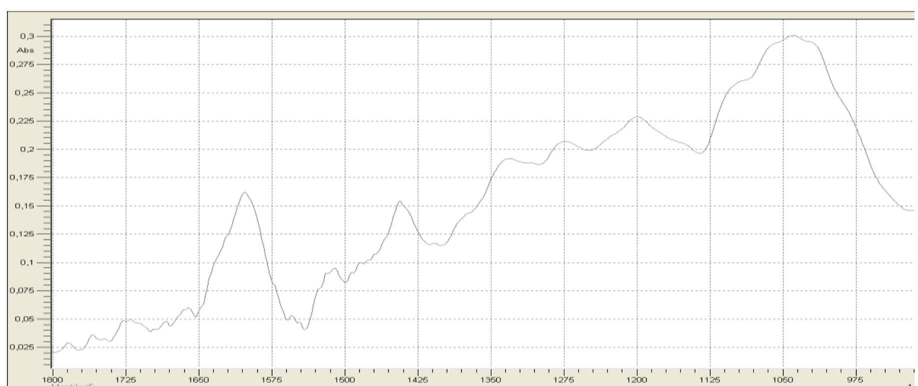
Figure A45: Absorption spectra of Mandilari after 6 months of ageing in French oak barrel

Figure A46: Absorption spectra of Mandilari after 9 months of ageing in Chestnut barrel

Figure A47: Absorption spectra of Dafni after 9 months of ageing in stainless steel container

Figure A48: Spectra of Dafni after 9 months of ageing in st. steel with oenosticks container

Figure A49: Absorption spectra of Dafni after 9 months of ageing in American oak barrel

Figure A50: Absorption spectra of Dafni after 9 months of ageing in French oak barrel

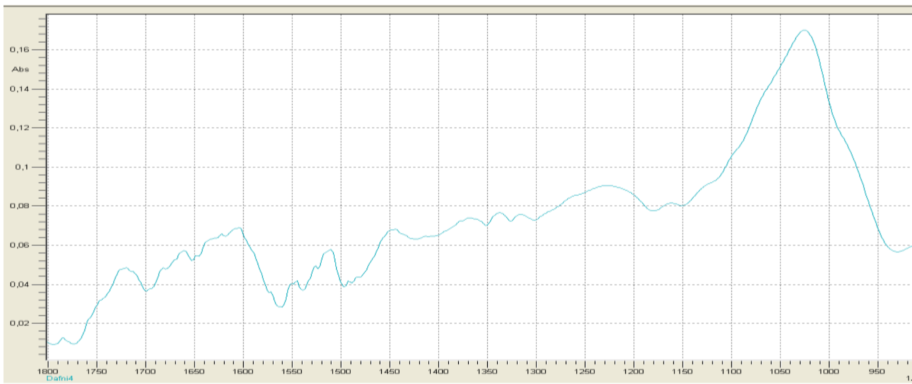

Figure A51: Absorption spectra of Dafni after 9 months of ageing in acacia barrel

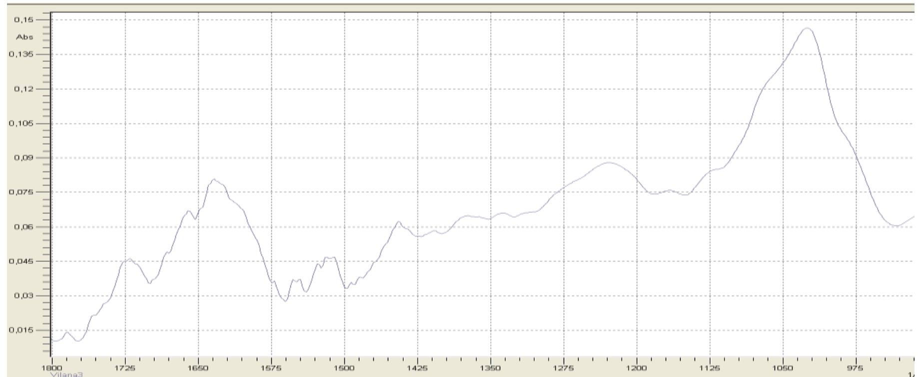

Figure A52: Absorption spectra of Vilana after 9 months of ageing in stainless steel container

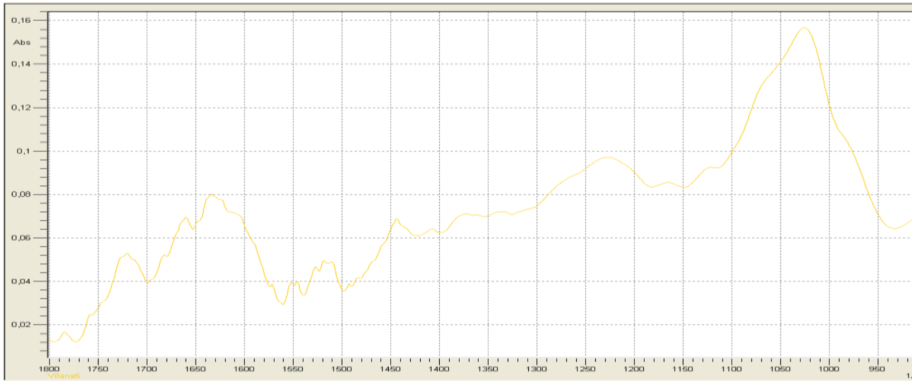

Figure A53: spectra of Vilana after 9 months of ageing in st. steel with oenosticks container

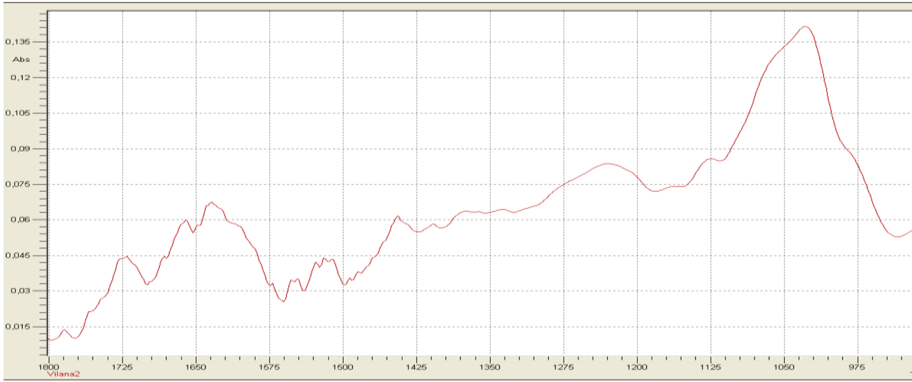

Figure A54: Absorption spectra of Vilana after 9 months of ageing in American oak barrel

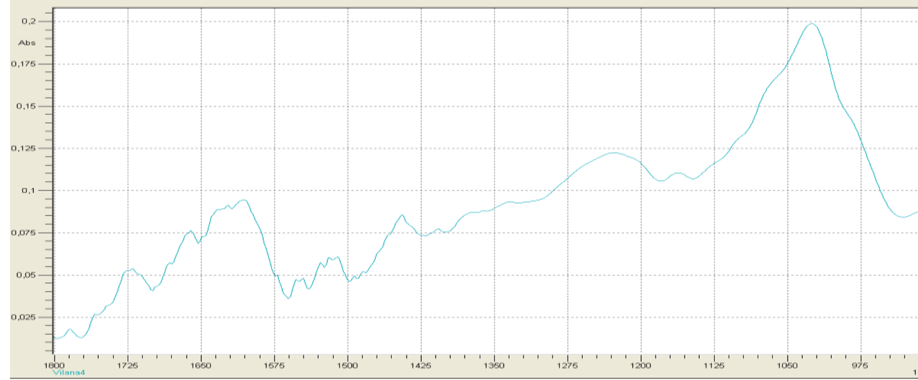

Figure A55: Absorption spectra of Vilana after 9 months of ageing in acacia barrel

Figure A56: Absorption spectra of Vilana after 9 months of ageing in French oak barrel

Figure A57: Absorption spectra of Kotsifali after 9 months of ageing in stainless steel container

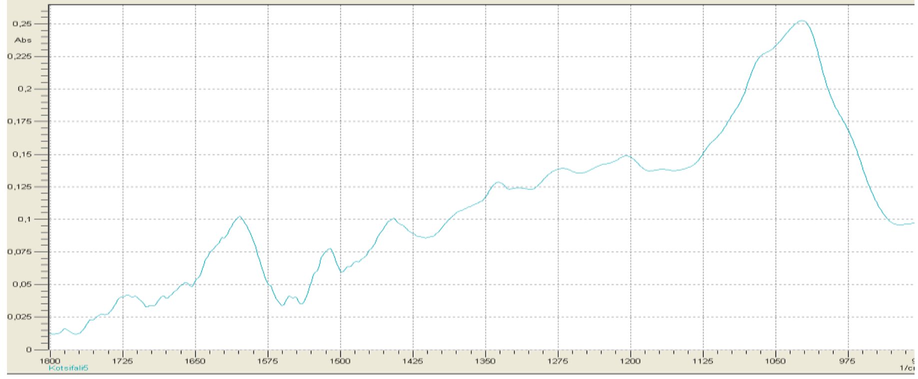

Figure A58: Spectra of Kotsifali after 9 months of ageing in st. steel with oenosticks container

Figure A59: Absorption spectra of Kotsifali after 9 months of ageing in American oak barrel

Figure A60: Absorption spectra of Kotsifali after 9 months of ageing in acacia barrel

Figure A61: Absorption spectra of Kotsifali after 9 months of ageing in French oak barrel

Figure A62: Absorption spectra of Kotsifali after 9 months of ageing in Chestnut barrel

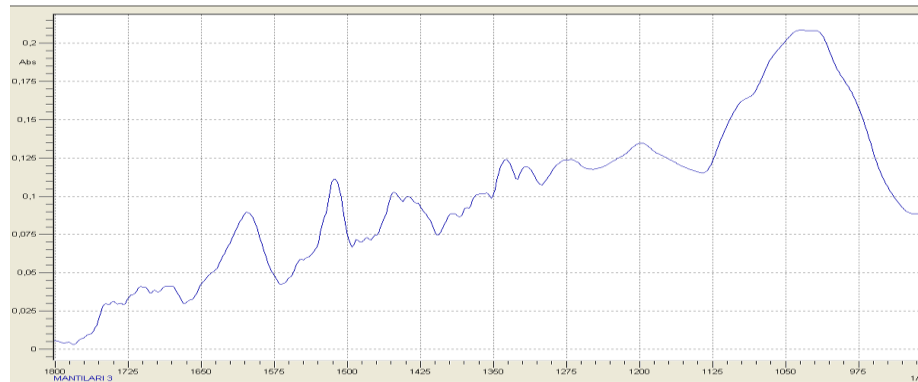

Figure A63: Absorption spectra of Mandilari after 9 months of ageing in stainless steel container

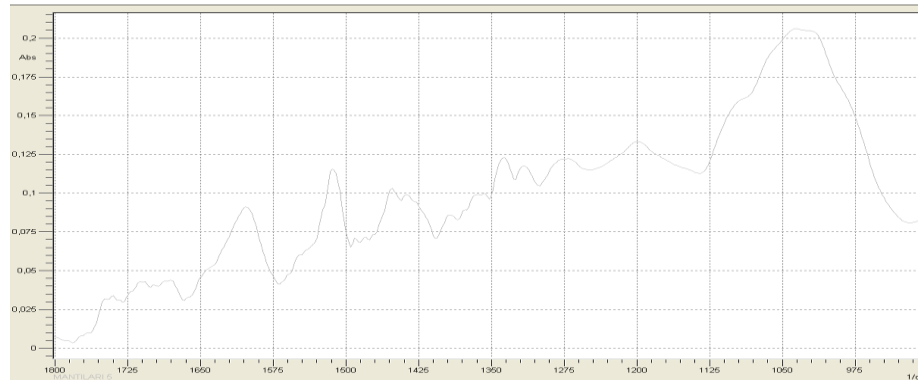

Figure A64: Spectra of Mandilari after 9 months of ageing in st. steel with oenosticks container

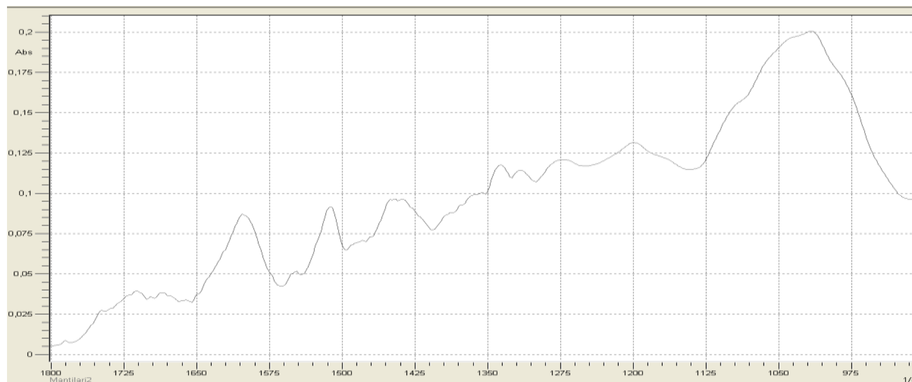

Figure A65: Absorption spectra of Mandilari after 9 months of ageing in American oak barrel

Figure A66: Absorption spectra of Mandilari after 9 months of ageing in acacia barrel

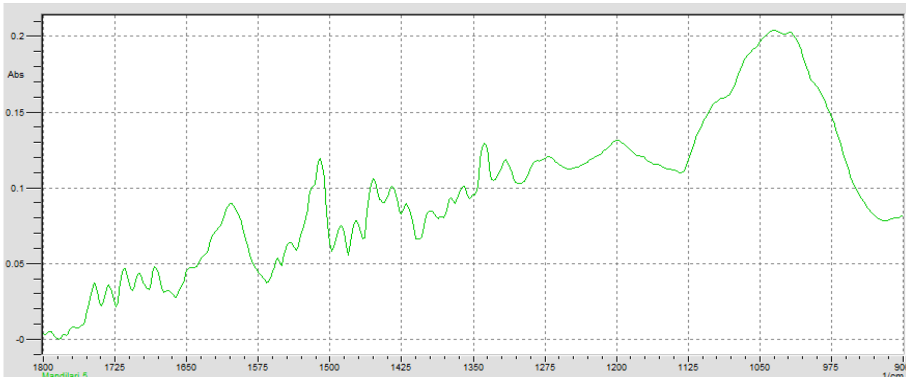

Figure A67: Absorption spectra of Mandilari after 9 months of ageing in French oak barrel

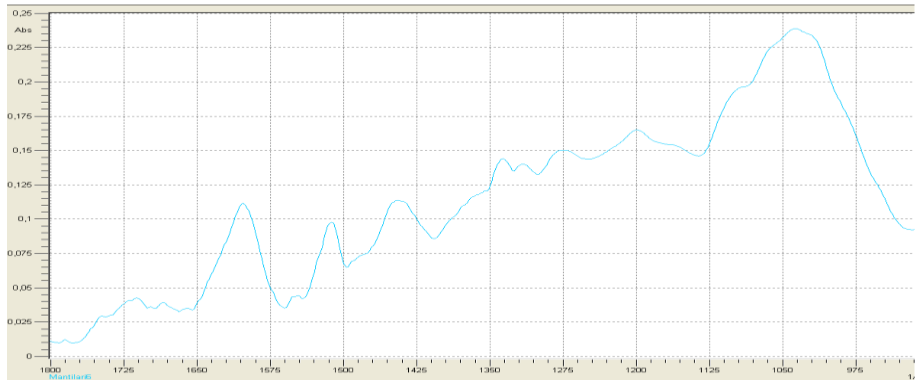

Figure A68: Absorption spectra of Mandilari after 9 months of ageing in Chestnut barrel

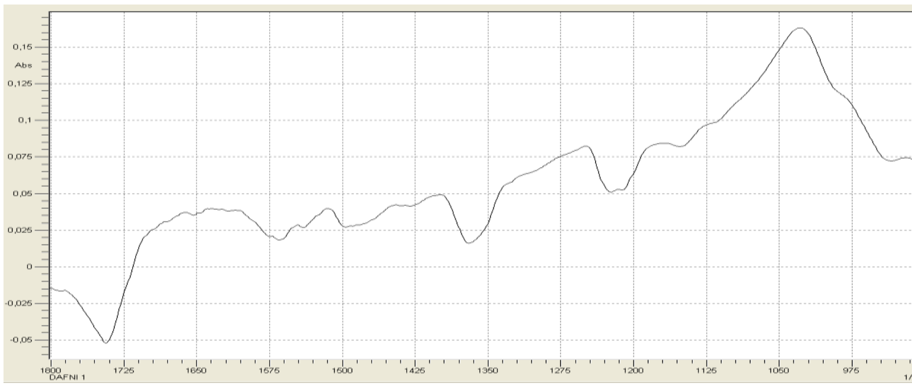

Figure A69: Absorption spectra of Dafni after 12 months of ageing in stainless steel container

Figure A70: Spectra of Dafni after 12 months of ageing in st. steel with oenosticks container

Figure A71: Absorption spectra of Dafni after 12 months of ageing in American oak barrel

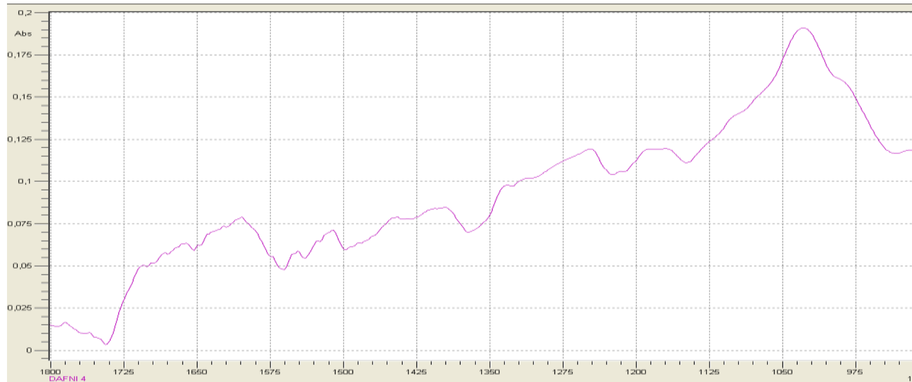

Figure A72: Absorption spectra of Dafni after 12 months of ageing in acacia barrel

Figure A73: Absorption spectra of Dafni after 12 months of ageing in French oak barrel

Figure A74: Absorption spectra of Vilana after 12 months of ageing in stainless steel container

Figure A75: Spectra of Vilana after 12 months of ageing in st. steel with oenosticks container

Figure A76: Absorption spectra of Vilana after 12 months of ageing in American oak barrel

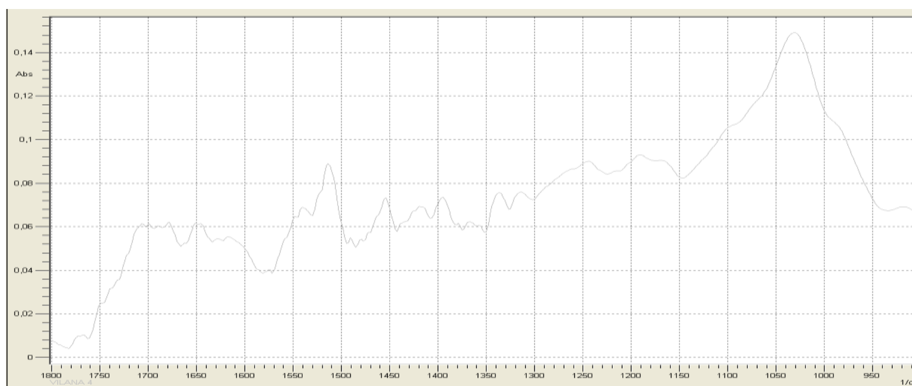

Figure A77: Absorption spectra of Vilana after 12 months of ageing in acacia barrel

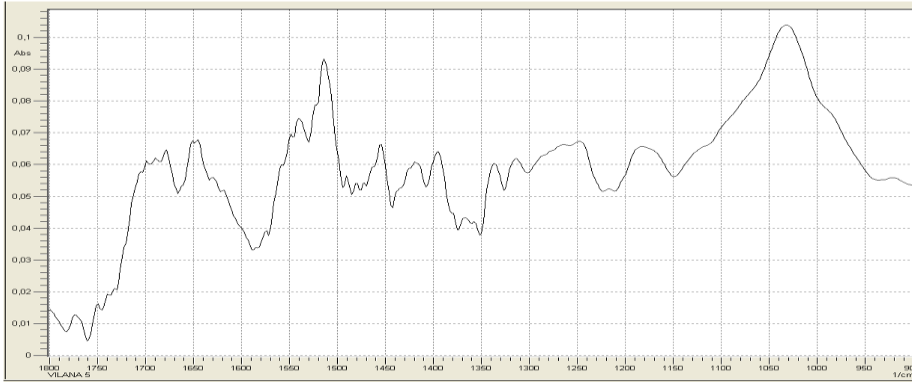

Figure A78: Absorption spectra of Vilana after 12 months of ageing in French oak barrel

Figure A79: Absorption spectra of Kotsifali after 12 months of ageing in stainless steel container

Figure A80: Spectra of Kotsifali after 12 months of ageing in st. steel with oenosticks container

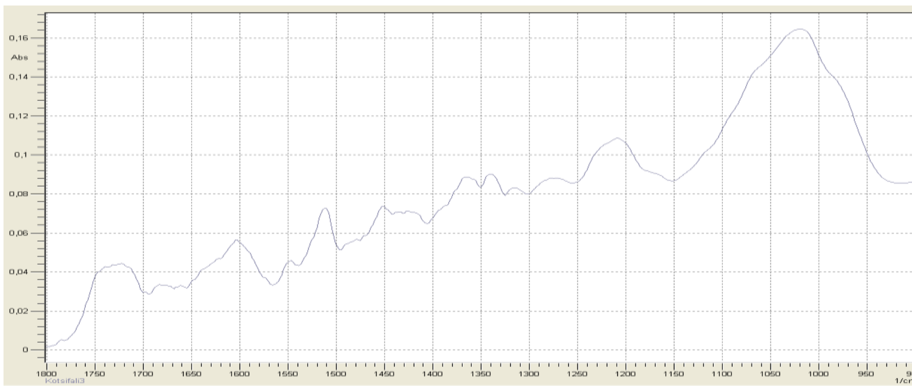

Figure A81: Absorption spectra of Kotsifali after 12 months of ageing in American oak barrel

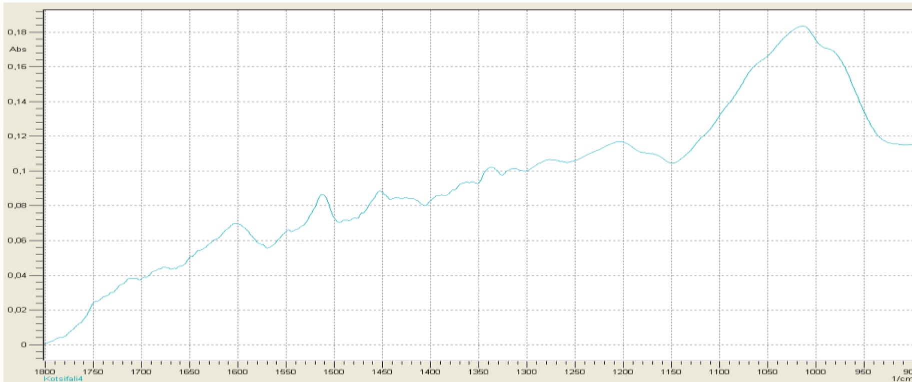

Figure A82: Absorption spectra of Kotsifali after 12 months of ageing in acacia barrel

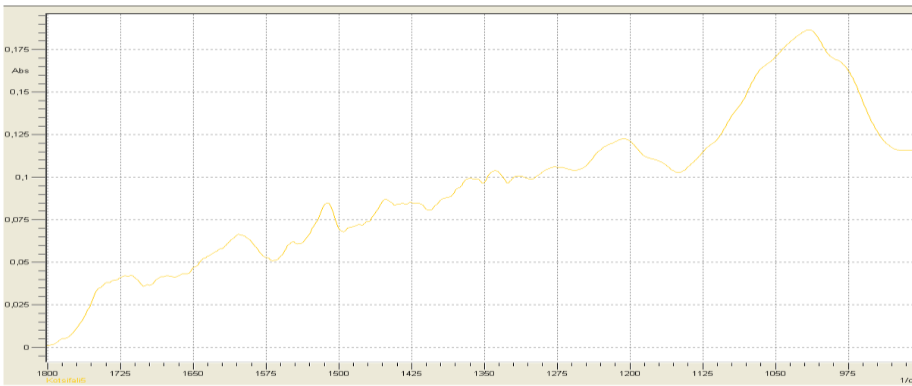

Figure A83: Absorption spectra of Kotsifali after 12 months of ageing in French oak barrel

Figure A84: Absorption spectra of Kotsifali after 12 months of ageing in Chestnut barrel

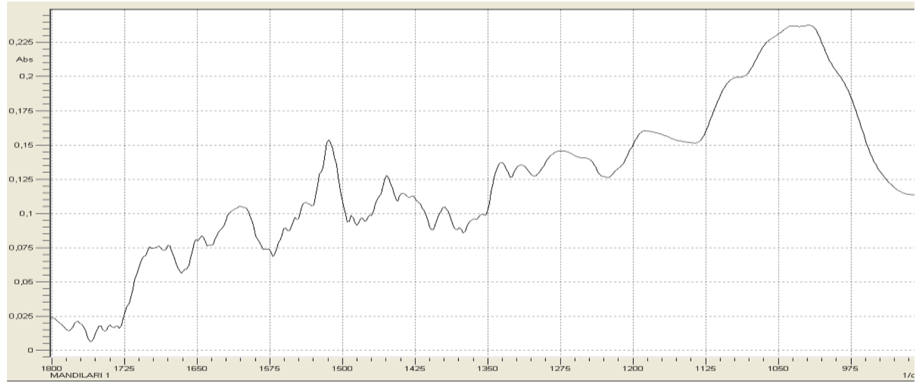

Figure A85: Absorption spectra of Mandilari after 12 months of ageing in stainless steel container

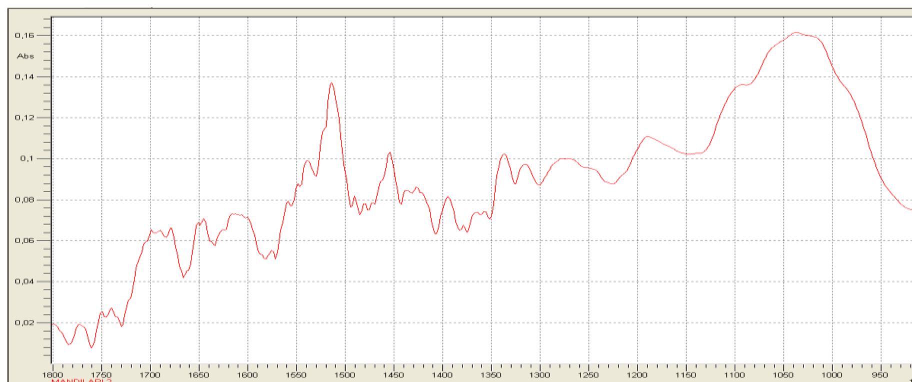

Figure A86: Spectra of Mandilari after 12 months of ageing in st. steel with oenosticks container

Figure A87: Absorption spectra of Mandilari after 12 months of ageing in American oak barrel

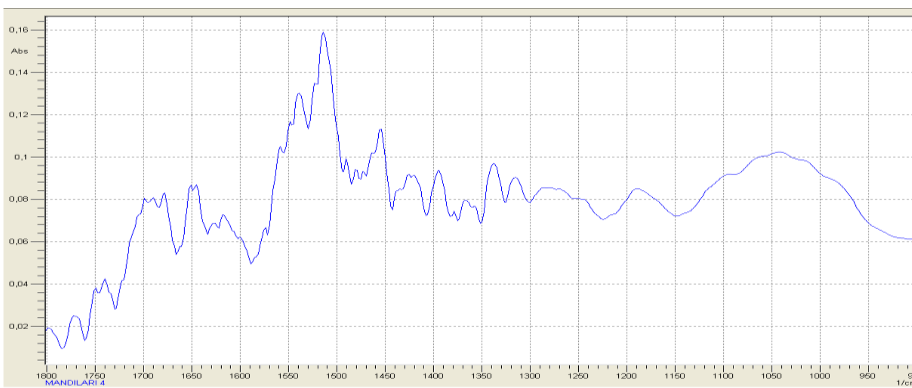

Figure A88: Absorption spectra of Mandilari after 12 months of ageing in acacia barrel

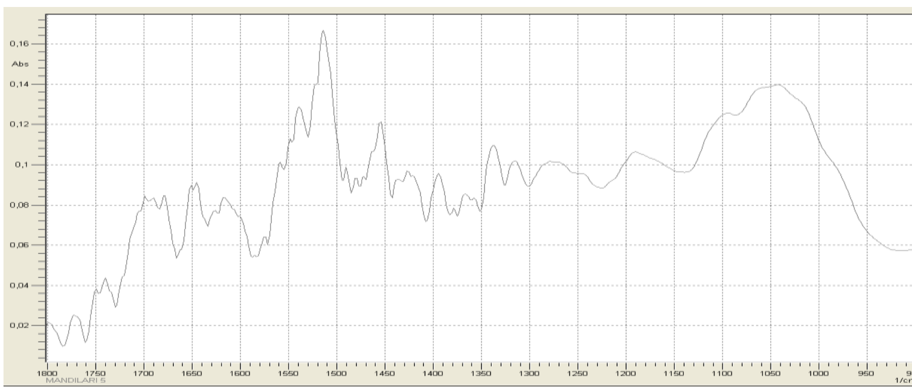

Figure A89: Absorption spectra of Mandilari after 12 months of ageing in French oak barrel

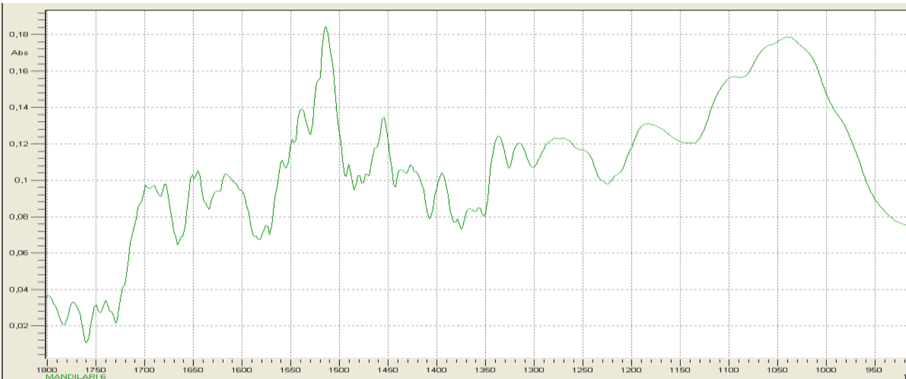

Figure A90: Absorption spectra of Mandilari after 12 months of ageing in Chestnut barrel

APPENDIX B

B1. Results of one-way analysis of variance (ANOVA) for total phenolic content and antioxidant activity of Vilana, Dafni, Kotsifali wines

Table B1: Results of one-way ANOVA (significance level p = 0.05). Comparison of the phenolic content of wines within each container during ageing.

	1st vinification	2nd vinification
	VILANA	
SS	$F_{4.10} = 19.017$, $p < 0.05$	$F_{4.10} = 45.925$, $p < 0.05$
SO	$F_{4.10} = 2.049$, $p = 0.171$	$F_{4.10} = 86.078$, $p < 0.05$
AO	$F_{4.10} = 201.420$, $p < 0.05$	$F_{4.10} = 112.918$, $p < 0.05$
Ac	$F_{4.10} = 136.477$, $p < 0.05$	$F_{4.10} = 177.504$, $p < 0.05$
FO	$F_{4.10} = 62.645$, $p < 0.05$	$F_{4.10} = 237.388$, $p < 0.05$
	DAFNI	
SS	$F_{4.9} = 6.208$, $p < 0.05$	$F_{4.11} = 19.702$, $p < 0.05$
SO	$F_{4.9} = 12.428$, $p = 0.05$	$F_{4.10} = 41.883$, $p < 0.05$
AO	$F_{4.9} = 144.401$, $p < 0.05$	$F_{4.11} = 191.712$, $p < 0.05$
Ac	$F_{4.9} = 19.183$, $p < 0.05$	$F_{4.11} = 7.217$, $p < 0.05$
FO	$F_{4.9} = 52.185$, $p < 0.05$	$F_{4.10} = 14.539$, $p = 0.05$
	KOTSIFALI	
SS	$F_{4.9} = 23.819$, $p < 0.05$	$F_{4.10} = 8.5513$, $p < 0.05$
SO	$F_{4.9} = 21.991$, $p < 0.05$	$F_{4.10} = 0.925$, $p = 0.484$
AO	$F_{4.9} = 73.215$, $p < 0.05$	$F_{4.10} = 156.875$, $p < 0.05$
Ac	$F_{4.9} = 332.257$, $p < 0.05$	$F_{4.10} = 104.543$, $p < 0.05$
FO	$F_{4.9} = 70.405$, $p < 0.05$	$F_{4.10} = 32.105$, $p < 0.05$
Ch	$F_{4.9} = 205.256$, $p < 0.05$	$F_{4.10} = 130.902$, $p < 0.05$
	MANDILARI	
SS	$F_{4.9} = 14.905$, $p < 0.05$	$F_{4.10} = 14.142$, $p < 0.05$
SO	$F_{4.9} = 40.757$, $p < 0.05$	$F_{4.10} = 5.421$ $p < 0.05$
AO	$F_{4.9} = 27.072$, $p < 0.05$	$F_{4.10} = 10.576$, $p = 0.05$
Ac	$F_{4.9} = 36.994$, $p < 0.05$	$F_{4.11} = 54.685$, $p < 0.05$
FO	$F_{4.9} = 42.707$, $p < 0.05$	$F_{4.11} = 11.604$, $p < 0.05$
Ch	$F_{4.9} = 83.698$, $p < 0.05$	$F_{4.11} = 43.477$, $p < 0.05$

Table B2: Results of one-way ANOVA (significance level p = 0.05). Comparison of the phenolic content of wines between the different containers at 3, 6, 9 and 12 months of ageing.

	1st vinification	2nd vinification
	VILANA	
3 MONTHS	$F_{4.10} = 1.290$, $p = 0.386$	$F_{4.10} = 5.959$, $p < 0.05$
6 MONTHS	$F_{4.10} = 5.858$, $p < 0.05$	$F_{4.10} = 84.258$, $p < 0.05$
9 MONTHS	$F_{4.10} = 4.669$, $p < 0.05$	$F_{4.10} = 185.681$, $p < 0.05$
12 MONTHS	$F_{4.10} = 334.148$, $p < 0.05$	$F_{4.10} = 306.798$, $p < 0.05$
	DAFNI	
3 MONTHS	$F_{4.10} = 4.693$, $p = 0.060$	$F_{4.10} = 4458$, $p < 0.05$
6 MONTHS	$F_{4.10} = 15.463$, $p < 0.05$	$F_{4.10} = 56.165$, $p < 0.05$
9 MONTHS	$F_{4.10} = 18.822$, $p < 0.05$	$F_{4.10} = 70.709$, $p < 0.05$
12 MONTHS	$F_{4.10} = 335.414$, $p < 0.05$	$F_{4.10} = 146.558$, $p < 0.05$
	KOTSIFALI	
3 MONTHS	$F_{5.6} = 16.238$, $p < 0.05$	$F_{5.12} = 0.377$, $p = 0.855$
6 MONTHS	$F_{5.12} = 23.819$, $p < 0.05$	$F_{5.12} = 13.449$, $p < 0.05$
9 MONTHS	$F_{5.12} = 119.253$, $p < 0.05$	$F_{5.12} = 58.664$, $p < 0.05$
12 MONTHS	$F_{5.12} = 50.531$, $p < 0.05$	$F_{5.13} = 15.245$, $p < 0.05$
	MANDILARI	
3 MONTHS	$F_{5.6} = 16.238$, $p = 0.05$	$F_{5.12} = 5.560$, $p < 0.05$
6 MONTHS	$F_{5.12} = 85.983$, $p < 0.05$	$F_{5.12} = 1.529$, $p = 0.258$
9 MONTHS	$F_{5.13} = 51.558$, $p < 0.05$	$F_{5.12} = 7.303$, $p < 0.05$
12 MONTHS	$F_{5.12} = 85.166$, $p < 0.05$	$F_{5.16} = 7.377$, $p < 0.05$

Table B3: Results of one-way ANOVA (significance level p = 0.05). Comparison of the antioxidant activity of wines within each container during ageing.

	1st vinification	2nd vinification
	VILANA	
SS	$F_{4.10} = 190.423$, $p < 0.05$	$F_{4\cdot10} = 122.743$, $p < 0.05$
SO	$F_{4\cdot10} = 334.871$, $p < 0.05$	$F_{4\cdot10} = 1923.48$, $p < 0.05$
AO	$F_{4\cdot10} = 351.337$, $p < 0.05$	$F_{4\cdot10} = 77.536$, $p < 0.05$
Ac	$F_{4\cdot10} = 542.639$, $p < 0.05$	$F_{4\cdot9} = 508.239$, $p < 0.05$
FO	$F_{4\cdot10} = 390.377$, $p < 0.05$	$F_{4\cdot10} = 404.573$, $p < 0.05$
	DAFNI	
SS	$F_{4\cdot10} = 44.783$, $p < 0.05$	$F_{4\cdot9} = 804.629$, $p < 0.05$
SO	$F_{4\cdot10} = 53.320$, $p < 0.05$	$F_{4\cdot9} = 105.848$, $p < 0.05$
AO	$F_{4\cdot10} = 143.617$, $p < 0.05$	$F_{4\cdot9} = 111.229$, $p < 0.05$
Ac	$F_{4\cdot10} = 628.101$, $p < 0.05$	$F_{4\cdot9} = 716.438$, $p < 0.05$
FO	$F_{4\cdot10} = 283.617$, $p < 0.05$	$F_{4\cdot9} = 496.682$, $p < 0.05$
	KOTSIFALI	
SS	$F_{4\cdot10} = 18.188$, $p < 0.05$	$F_{4\cdot9} = 3.083$, $p = 0.074$
SO	$F_{4.9} = 32.501$, $p < 0.05$	$F_{4\cdot9} = 4.742$, $p < 0.05$
AO	$F_{4\cdot10} = 41.948$, $p < 0.05$	$F_{4\cdot9} = 3.029$, $p < 0.05$
Ac	$F_{4\cdot10} = 27.696$, $p < 0.05$	$F_{4\cdot9} = 89.519$, $p < 0.05$
FO	$F_{4\cdot10} = 32.790$, $p < 0.05$	$F_{4\cdot9} = 27.986$, $p < 0.05$
Ch	$F_{4.9} = 20.399$, $p < 0.05$	$F_{4\cdot10} = 82.654$, $p < 0.05$
	MANDILARI	
SS	$F_{4\cdot10} = 118.945$, $p < 0.05$	$F_{4\cdot9} = 198.585$, $p < 0.05$
SO	$F_{4\cdot10} = 84.787$, $p < 0.05$	$F_{4\cdot9} = 254.269$, $p < 0.05$
AO	$F_{4\cdot10} = 40.729$, $p < 0.05$	$F_{4\cdot9} = 123.124$, $p < 0.05$
Ac	$F_{4\cdot10} = 82.623$, $p < 0.05$	$F_{4\cdot9} = 632.671$, $p < 0.05$
FO	$F_{4\cdot10} = 116.187$, $p < 0.05$	$F_{4\cdot9} = 166.924$, $p < 0.05$
Ch	$F_{4\cdot10} = 174.649$, $p < 0.05$	$F_{4\cdot9} = 168.986$, $p < 0.05$

Table B4: Results of one-way ANOVA (significance level p = 0.05). Comparison of the antioxidant activity of wines within each container at 3, 6, 9 and 12 months of ageing.

	1st vinification	2nd vinification
	VILANA	
3 MONTHS	$F_{4,10}$= 5.567, $p < 0.05$	$F_{4.10}$= 6.457, $p = 0.033$
6 MONTHS	$F_{4,10}$= 49.565, $p < 0.05$	$F_{4.10}$ = 160.142, $p < 0.001$
9 MONTHS	$F_{4,10}$= 735.75 $p < 0.05$	$F_{4.11}$ = 464.166, $p < 0.001$
12 MONTHS	$F_{4,10}$= 1566.03, $p < 0.05$	$F_{4.11}$ = 205.935, $p < 0.001$
	DAFNI	
3 MONTHS	$F_{4,10}$= 2.541, $p = 0.106$	$F_{4.10}$ = 2.602, $p = 0.161$
6 MONTHS	$F_{4,10}$ = 35.068, $p < 0.05$	$F_{4.10}$ = 80.473, $p < 0.001$
9 MONTHS	$F_{4,10}$= 509.365, $p < 0.05$	$F_{4.10}$ = 2529.179, $p < 0.001$
12 MONTHS	$F_{4,10}$ = 2837.747, $p < 0.05$	$F_{4.10}$ = 450.410, $p < 0.001$
	KOTSIFALI	
3 MONTHS	$F_{5,12}$= 6.929, $p < 0.05$	$F_{5.6}$ = 5.097, $p = 0.036$
6 MONTHS	$F_{5.12}$ = 40.027, $p < 0.05$	$F_{5.12}$ = 15.930, $p < 0.001$
9 MONTHS	$F_{5,12}$= 867.796, $p < 0.05$	$F_{5.12}$ = 56.832, $p < 0.001$
12 MONTHS	$F_{5.12}$= 379.119 $p < 0.05$	$F_{5.13}$ = 102.191, $p < 0.001$
	MANDILARI	
3 MONTHS	$F_{5\cdot 12}$ = 13.148, $p < 0.05$	$F_{5.6}$ = 2422, $p = 0.156$
6 MONTHS	$F_{5.12}$ = 231.47, $p < 0.05$	$F_{5.12}$ = 376.761, $p < 0.001$
9 MONTHS	$F_{5.12}$ = 458.824, $p < 0.05$	$F_{5.12}$ = 23.996, $p < 0.001$
12 MONTHS	$F_{5.12}$ = 292.965, $p < 0.05$	$F_{5.12}$ = 18.044, $p < 0.001$

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EVOLUTION OF THE TOTAL PHENOLIC AND THE TOTAL TANNIN CONTENT, AND THE ORGANOLEPTIC PROPERTIES OF WINES OF DAFNI VARIETY, DURING TWELVE MONTH STORAGE IN ACACIA AND FRENCH OAK BARRELS

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Summary

Ageing of white wines in wooden barrels is not a common practise as white wines' organoleptic properties can be strongly affected by the barrels' aromas. Hence, if ageing in wooden barrels is preferred, it is suggested to last only for a period of a few months. Apart from oak barrels, acacia barrels have been recently used for wine ageing, showing promising results. Twelve month ageing of wines of the white Cretan variety Dafni in Acacia and French oak barrels was studied. Wines from the region of Peza in Heraklion, Crete were used for the purposes of the experiments. Wine samples were taken at the begging and after six and twelve months of ageing and their total phenolic and total tannin content were determined. At the same time, organoleptic properties evaluation was performed by a qualified panel of wine experts, monitoring their aroma and astringency evolution. In comparison to French oak, wines in Acacia barrels were richer in both phenolic and tannic substances during ageing. However, in contrast to French oak, their organoleptic profile revealed stronger floral and fruit aromas instead of wood aromas, a quite interesting result as far as the use of Acacia barrels in white wine ageing, is concerned.

Key words

Astringency, sensory quality evaluation, floral bouquet.