Study of phenolic characterization of walnut wood extracts for potential use in oenology

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ABSTRACT

The goal of this study is to identify the quantities of phenolic compounds that can be extracted from walnut wood extracts (*Juglans regia*), comparing them with those of oak wood extracts (*Quercus petarea*). These chips of the two different types of wood come in three different levels of toasting, light, medium and strong. The extracts were macerated in a known alcoholic solution for two different maceration times, respectively 15 and 30 days. All samples were replicated once to make this study statistically acceptable. After the two different maceration times, the samples were analyzed for 3 different compounds, total phenols, flavonoid phenols and non-flavonoid phenols, and for the color intensity of the solution. The results were statistically analyzed with two different methods, the first analyzes each sample by comparing it with the others at both maceration times, the second analyzes the samples for factors (type of wood, toasting and maceration time), studying their influence of individuals or interaction in pairs or all three. Walnut wood behaved differently from oak wood, having less influence on the content of phenolic compounds in the alcoholic solution, but resulted in a higher coloring intensity than oak. Toasting and maceration times also influence the content of phenolic compounds regardless of the type of wood.

KEYWORD: walnut, oak, wood, phenols, toasting level.

RESUMO

O objetivo deste estudo foi efetuar uma análise comparativa entre a composição fenólica de extratos de madeira de nogueira (*Juglans regia*) e de extratos de madeira de carvalho (*Quercus petraea*). Foram utilizados três níveis de tosta nas aparas utilizadas das duas madeiras: leve, média e forte. Os extratos produzidos das duas madeiras foram obtidos a partir de soluções modelo de vinho, tendo-se para tal utilizado 15 e 30 dias de extração. Os extratos foram produzidos em duplicado de modo a obter resultados com validade estatística. Após os 2 tempos de extração considerados, as amostras dos extratos obtidos foram analisadas, tendo por base os seguintes parâmetros fenólicos: compostos fenóis totais, flavonóides e não-flavonóides, e ainda a intensidade da cor. Os resultados obtidos foram analisados estatisticamente, utilizando-se como fatores de análise, a análise comparativa entre tempos de maceração, a espécie de madeira e o nível de tosta, quer forma isolada, quer em termos de interação entre os vários fatores. Os extratos de madeira de nogueira apresentaram resultados diferentes relativamente aos extratos de madeira carvalho, tendo sido quantificados menores teores em compostos fenólicos, embora com valores mais elevados de intensidade da cor. O nível de tosta e os tempos de maceração também influenciaram os teores em compostos fenólicos, independentemente da espécie de madeira.

PALAVRAS-CHAVE: nogueira, carvalho, madeira, fenóis, nível de tosta.

RESUMO ALARGADO

O objetivo deste estudo foi identificar as quantidades de compostos fenólicos extraíveis de extratos de madeira de nogueira (*Juglans regia*), comparando-as com as de extratos de madeira de carvalho (*Quercus petraea*). O uso da madeira na vinificação é muito difundido na enologia, tanto para os barris mais clássicos, quer para na utilização de materiais alternativos, como sejam, os chips, as aduelas etc. Além destes materiais, nos últimos anos, vários estudos têm também analisado a potencial utilização de outras espécies de madeiras alternativas à madeira de carvalho. Além das espécies alternativas de madeira mais estudadas, como seja, a cerejeira, o castanheiro e a acácia, no presente estudo optou-se por estudar a madeira de nogueira. Uma vez que não existem estudos anteriores sobre esta espécie de madeira, este trabalho pretende, pois, obter informação, sobre a potencial utilização futura desta espécie na enologia.

Assim, foi-se estudar extratos de madeira de nogueira obtidos a partir de aparas com diferentes níveis de tosta (ligeira, média e forte), tendo-se para tal utilizado soluções modelo de vinho como meio de maceração. Utilizaram-se 2 tempos de maceração, 15 e 30 dias. Os extratos foram produzidos em duplicado, tendo os resultados médios obtidos tratados estatisticamente. Os extratos foram analisados ao nível de 3 parâmetros fenólicos, compostos fenólicos totais, flavonóides e não flavonóides, e ainda ao nível da intensidade da cor dos extratos. Assim, pretendeu-se saber se a madeira de nogueira apresenta uma capacidade de libertar compostos fenólicos em meio alcoólico semelhante ou não à madeira de carvalho. Os resultados obtidos foram analisados estatisticamente, utilizando-se como fatores de análise, a análise comparativa entre tempos de maceração, a espécie de madeira e o nível de tosta, quer forma isolada, quer em termos de interação entre os fatores. Estudou-se também a interação entre os vários fatores considerados.

Os resultados obtidos permitiram concluir que os extratos de madeira de nogueira apresentaram valores em compostos fenólicos totais mais baixos, comparativamente aos extratos de madeira de carvalho, embora os valores de intensidade da cor tenham sido mais elevados. Verificou-se ainda que o nível de tosta e os tempos de maceração também influenciaram o conteúdo fenólico quantificado, independentemente da espécie de madeira estudada.

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1. INTRODUCTION

1.1. Wood in wine aging

Traditionally the great red wines are stored in oak barrels. Initially the use of these containers helped the transport of wine, only after it was discovered that the wood had a significant influence on the evolution of the wine, in color, aroma and clarity (Ribéreau-Gayon *et al.,* 2017). The wooden barrels have been used for the aging and storage of wine for centuries. It was seen that oak wood was the most suitable for this purpose, it offers good wine conservation and an improvement in its quality. The main wood compounds are flavonoids, non-flavonoids, and volatile aromatic compounds (Zhang *et al.,* 2015). This practice was abandoned in the second half of the last century because there were often microbial contaminations and hints of mold. Only in recent decades, with the new knowledge, has the use of these types of containers been re-evaluated (Ribéreau-Gayon *et al.,* 2017).

Over the years all the characteristics of wood and the aging of barrel wines have been studied. Different species of oak have been studied, coming from different geographical areas. The quality and characteristics of the wood mainly depend on the geographical origin, such as American, French or Eastern Europe, but also from the species and extraction practices (de Simón *et al*., 1999; Spilmann *et al*., in 2004; Prida and Puech, 2006).

In more recent years, the use of wooden chips has been studied to bring the characteristics of barrel aging to the wine, at least on an aromatic level (del Álamo Sanza *et al.,* 2004).

Wood adds to wine, or increases, polyphenols, coumarins, polysaccharides, volatile phenols (guaiacol, eugenol, ethyl phenols, vinyl phenols, etc.), *cis*- and *trans*-β-methyl-γ-octalactone, volatile phenol aldehyde, and furanic aldehydes (Flamini *et al.,* 2017). In addition, barrel aging helps the natural clarification of wine and color stabilization (del Álamo Sanza *et al.,* 2004).

The ellagitannin content is very important for the sensorial qualities of the wine, conferring astringency and favoring the pigmentation of the coloring substances, with a stabilizing effect (Zhang *et al.,* 2015). Furthermore, the phenolic substances, which pass into the wine from the wood, have an important antioxidant and anticarcinogenic effect, that is, they are positive for human health (Madrera *et al.,* 2010). The ellagitannins, which correspond to about 10% of the heartwood, are mainly represented by castalagin, vescalagin, grandinin and roburin A-E. The first two are those that are usually found in greater concentration (Jordão *et al.,* 2007). 50% of the total ellagitannins cannot be removed from the heartwood with the most common used solvents as water and alcohol, organic solvents (Viriot et *al.,* 1993). The concentration of ellagitannins depends on the geographical origin of the wood and if the wood is green, only seasoned or even toasted (Sanz *et al.,* 2011).

1.2. Oak wood

Oak is an angiosperm belonging to the Fagacea family, we find the most important species in Europe, mainly in France (*Q. robur* and *Q. petraea*), and in America (*Q. alba) (Figure 1)*. New species have been studied in Spain, Russia and Eastern, beyond quercus petraea (Zhang *et al.,* 2015). The pedunculate oak (*Quercus robur* or *Quercus peduncoIata*) dominates in Limousin, present in Burgundy and even in the south of France. It is rich in extractable polyphenols and relatively low in aromatic compounds. The sessile oak (*Quercus petraea* or *Quercus sessilis*) is present mainly in the forests of the Center and the Vosges, has slow annual growth and a fine grain. This type of oak has a good aromatic potential and a low quantity of removable ellagitannins. In the United States, the dominant species is the white oak of America (*Quercus alba*) which is poor in phenolic compounds and rich in aromatic substances, as methyloctalactone, this compound has a marked influence on the aromatic and chemical characteristics of the wine during its aging (Ribéreau-Gayon *et al.,* 2017).

But recently also *Quercus pyrenaica* has been subjected to many studies on wood chemical composition, and the effects on wine (Jordão *et al.,* 2007; Fernandez de Simon *et al.,* 2006). *Quercus pyrenaica* is one of the oak species common in the European continent. This is widespread mainly in the west of France and in the Iberian Peninsula (Spain and Portugal) (Figure 2). Its structural properties make its use in oenology possible (Fernández de Simón *et al.*, 2006; Castro-Vázquez *et al.,* 2013b).

This species has been the subject of various studies to identify its chemical composition and determine its content in ellagitannins, ellagic acids, low molecular weight compounds and aromatic ones, often in comparison with the classic oak species used and chestnut. Studies on this type of wood were mainly carried out with alternative products to barrels, such as chips or staves. (Cadahía *et al.,* 2001; De Coninck *et al.,* 2006; Jordão *et al.,* 2007; Fernández de Simón *et al*., 2009; Fernandez de Simón *et al*., 2010; Gallego *et al.,* 2012; Castro-Vázquez *et al.,* 2013b). Studies on the ellagitannin composition of Portuguese oak show how their concentration in this type of wood is higher than American oak, but with French oak they are very similar. The most present ellagitannins are vescalagin and castalagin, while roburin B, C and D, grandinin and ellagitannins are the least abundant (Cadahía *et al.,* 2001; Jordão *et al.,* 2007; Castro-Vázquez *et al.,* 2013b).

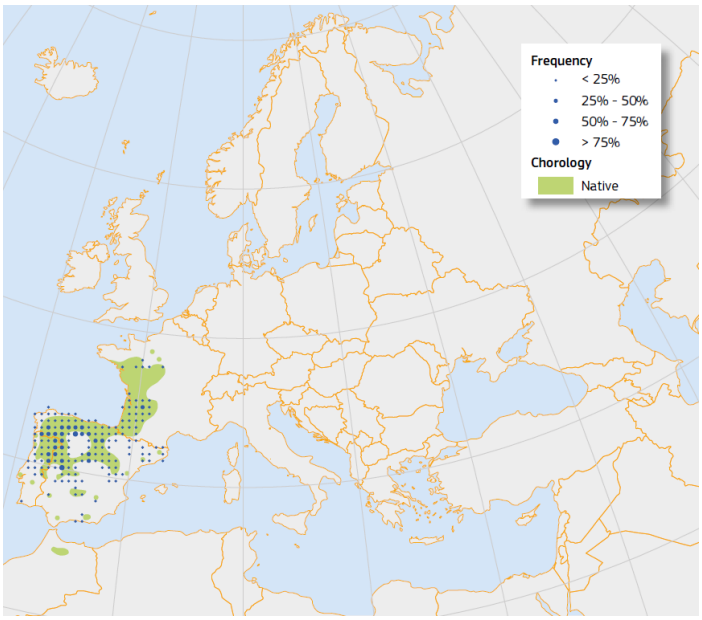


Figure 1. *Distribution of the Quercus pyrenaica in Europe (Nieto Quintano et al., 2016).*

According to Fernández de Simón and Cadahía (2004), the content of ellagitannins is higher in *Q. robur*, followed by *Q. petraea* and lastly *Q. alba*. Furthermore, the content of ellagitannins, volatile substances and low molecular weight compounds does not depend only on the species but also on the cooperage processing, on forestry factors and on the differences between the individual trees. The higher concentration of ellagitannins and other phenolic substances in European woods has also been demonstrated in a study by Prida and Puech in 2006 and by de Simón *et al*. in 1999.

Spilmann *et al*. in 2004 they showed how in wines aged in contact with French oak wood, the concentration of oak-lactones is higher than wines aged in oak of American origin. The same has been demonstrated for eugenol, while the opposite has been seen for ethylphenol and ethylguaiacol.

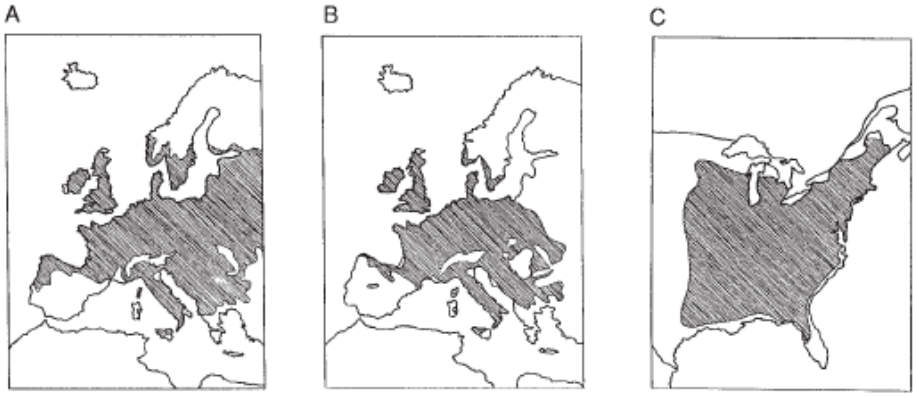


Figure 2. Distribution map of A) Quercus Sessil, B) Quercus Robur and C) Quercus Alba *(Jackson, 2008).*

1.2.1. Phenolic composition of oak

Being the type of wood, most used for the aging of wines, oak has been studied several times in its phenolic composition, to determine the role of factors such as geographical origin, the level of toasting, the cooperage processes, etc.

More than 8000 wood polyphenols known today in the plant kingdom, divided by classes according to their structure, derive from glucose metabolism (Zhang *et al.,* 2015).

Flavonoids are the most important and most present group of polyphenolic compounds. In addition, there is in fact another classification method, which divides flavonoids and non-flavonoids. Flavonoids are usually glycosidated with glucose, but also with galactose, rhamnose and xylose. The flavonoid group consists of compounds such as chalcones, dihydrocalcones, aurones, flavonols, dihydroflavonols, flavanones, flavones, isoflavonoids, biflavonoids and anthocyanidins. The group of non-flavonoids is made up of compounds such as hydroxybenzoic acids, hydroxycinamic acids, volatile phenols, stilbenes and miscellaneous compounds (lignins and coumarins). There is another very large group of phenols called tannins, classified into two classes, namely condensed and hydrolyzable ones. The latter are mainly gallotannins and ellagitannins (Zhang *et al.,* 2015).

Ellagitannins are very important compounds for the aging of wine because they can protect the wine from oxidation, directly absorbing the oxygen entering the barrel, causing easy hydroperoxidation of the wine's substances. This allows the ellagitannins to hinder a rapid evolution of the phenolic compounds of the wine and therefore the variation of the yellow-brick-colored coloring substances. They also have the peculiarity of reducing the astringency of young wines through polymerization reactions with wine polyphenols. Their content, as we know, changes according to the type of wood (Castro-Vázquez *et al.,* 2013a).

The ellagitannins derive from the secondary metabolism of the plant. In oak they are found in heartwood and sapwood. It has been seen that the ellagitannins present in the oak wood are the following: Castalagin, Vescalagin, Roburin E, Grandinin, Roburin A, Roburin B, Roburin C, Roburin D, Acutissimin A, Acutissimin B, Punicalagin and Pedunculagin (Castro-Vázquez *et al*., 2013a; de Simón *et al*.,1999; Zhang *et al.,* 2015).

1.3. Seasoning and toasting of the wood

The seasoning of the wood for the manufacture of barrels is an essential process for the loss of humidity which then allows easy processing (Chatonnet *et al.,* 1994). This process can be natural or artificial. The natural one usually lasts from 24 to 36 months. It takes place in the open air and the first 10 months are indicated as ripening, in which the physical-mechanical and aromatic characteristics are improved. During drying, the wood is colonized by a microflora. 80% from *Aureobasidium pullulans*, less than 20% from *Trichoderma harzianum* and *Trichodema homingii*, plus other organisms in minimal part (Ribéreau-Gayon *et al.*, 2017).

This microflora leads to transformations in wood, mainly there is a decrease in ellagitannins with a consequent decrease in astringency and in the color of the wood extracts (Chatonnet *et al.,* 1994; Doussot *et al.,* 2002). Drying also leads to changes on an aromatic level. It has been shown that an increase or decrease in compounds such as eugenol, syringe aldehyde and vanillic from degradation lignin, the two isomers of β-methyl-γ-octalacton, with an isomeric ratio in favor of the cis form, the most fragrant (Sefton *et al.,* 1993; Chatonnet, P. 1995; Ribéreau-Gayon *et al.*, 2017). Artificial drying, on the other hand, takes place inside a stove ventilated at 40-60°C. This practice allows a faster drying of the wood but with loss of quality, mainly due to the formation of cracks in the wood, with all the consequences of the case. After the refined level of essically the mature staves are assembled to form a barrique with the help of metal circles, and then toasting is carried out. (Ribéreau-Gayon *et al.*, 2017).

Toasting of the wood barrels for the conservation of the wine is commonly used to give sensorial notes to the aged wine, in fact, it modifies the polyphenolic content and reduces the astringency, especially castalagin and vescalagin, for example, are degraded into less astringent substances (Castro-Vázquez et al. 2013a). The chemical composition of the wood also depends on the toasting levels to which they are subjected. The toasting, depending on the level, leads to an important transformation of the substances contained in the wood, the phenolic compounds besides the ellagitannins, by pyrolysis and hydrothermolysis, thus changing the organoleptic sensations of aged wine in contact with wood. Even lignin and hemicellulose undergo alterations, with the formation of various substances, including several aromatic compounds (Doussot *et al.,* 2002; Jordão *et al.,* 2007).

According to Ribéreau-Gayon *et al.* (2017) the toasting operation has a strong impact on the sensorial evolution of the wine in contact with the wood during aging. The conditions in which this operation is carried out varies according to the cooperage and the human variant, being an operation done by hand and therefore without perfect control. There are several factors that affect toasting: type of fuel (wood, gas, electricity), heating mode (closed or open barrel), homogeneity of heating, duration and intensity and humidification.

Commonly there are three types of toasting:

-Light: usually 5 minutes at temperatures between 120° and 180°C. This causes a modification of the hemicellulose and lignins while maintaining the cellulosic structure;

-Medium: about 10 minutes at a surface temperature of 200°C. The wood wall components disappear by fusion;

-Strong: lasting more than 15 minutes at about 230°C. The surface of the wood is swollen with multiple cracks and the cellular structure disorganized.

The types of toasting described above are indicative for this process, each cooperage factory has its own personal toasting process.

1.3.1. Influence of toasting on phenolic compounds

With the toasting process, several authors showed that the level of total phenols increases by out 30%, as well as the content of ellagic acids but by 40%, instead the ellagitannins decrease dramatically, up to more than 90%. As already mentioned, the increase in ellagic acids is directly related to the decrease in ellagitannins, mainly due to hydrolysis reactions (Cadahía *et al.,* 2001; Doussot *et al.,* 2002; Jordão *et al.,* 2007)

Extracts of oak wood analyzed after toasting have shown how the degradation of ellagitannins occurs, especially starting from the medium toasting. It is also noted that ellagic acid is more resistant to temperature than gallic acid which is more degraded (Table 1) (Ribéreau-Gayon *et al.,* 2017).

Table 1*. Effect of toasting on phenolic compounds of oak wood (Ribéreau-Gayon et al. 2017).*

Phenolic compounds	Not toasted	Light	Medium	Strong
Ellagitannins (mg/L)	333	267	197	101
Gallic acid (mg/L)	20	103	9.8	2
Ellagic acid (mg/L)	21	18	13.8	13.7

The concentration of ellagic acid increases with toasting, while, on the contrary, the concentration of gallic acid decreases with increasing toasting. The increase in ellagic acid with toasting is correlated with the degradation of ellagitannins, same with gallotannins and gallic acid (Sanz *et al.,* 2010a).

As found by Chira and Teissedre in 2015, the light and medium toasting did not show significant differences, maintaining high concentrations of ellagitannins in the wood. These authors have also proven that gallic acid is the most sensitive compound to thermal degradation, in fact they have seen that its decrease has always been very rapid in all tests during toasting.

During toasting, lignin is depolymerized in relation with the intensity, with the production of hydroxybenzoic acids, hydroxycinnamic aldehydes and hydroxybenzoic aldehydes. Their final concentration depends on the level of toasting and the type of wood we are using, being related to the lignin content. With toasting these same aldehydes are degraded, with the production of other compounds such as volatile phenols (Sanz *et al.,* 2011).

1.4. Wood alternatives to barrels

Besides barrels, there are alternative systems for aging wines. These involve the use of staves, cubes, chips, granulates etc., derived from wood. Their use allows to obtain similar results to the barrel in faster times, if accompanied by the micro-oxygenation system, with a saving

mainly at an economic level (Fernandez de Simon *et al.,* 2010).

The effectiveness of the wood alternatives depends on the botanical species, the origin, the size of the fragments, the level of toasting, the quantity added to the wine and the contact time (Caldeira *et al.,* 2010).

It has also been seen that for the same contact times, the phenolic and aromatic components are extracted more with the use of chips than the barrel (Pérez-Coello *et al.,* 2000).

In recent years they have been used to bring refined aromas and scents to wines. It has also been seen that the effect of these chips is less than that of the classic barrels, although the use of the chips allows for faster aging, with a better degree of polymerization (del Álamo Sanza *et al.,* 2004). Chips have also been shown to cause changes in wine faster than in a barrel, therefore even in short contact. It has been seen that in a wine that has undergone a rapid maturation process in contact with wood chips, there was however an extraction of volatile compounds deriving from wood and an increase in the coloring intensity (Sánchez-Palomo *et al.,* 2017).

Wood chips have long been used in new wine-growing areas, such as South Africa, Australia and Chile. It is only since 2005 that the European Union regulates their use [Council Regulation (EC) No.2165/2005 of 20 December 2005] and the denomination of the wines produced in this way [Commission Regulation (EC) No. 1507/2006 dated 11 October 2006]. The use of oak chips is permitted and regulated by the European Union (Commission Regulation (EC) N 606/2009) and the international Vine and Wine organization (OIV, Resolution OENO 3/2005).

In Europe, countries with an ancient winemaking tradition have stronger resistance when it comes to the use of wooden chips to defend that tradition. Today every single EU state can apply the use of chips in its own way (Sánchez-Palomo *et al.,* 2017).

Their use is increasing, in fact it has increased in relation to a decrease in the use of classic barrels, especially in low and medium price wines, because the relationship between production costs and profit margins are in favor of the use of chips (Rudnitskaya *et al.,* 2017).

1.4.1. Wood alternatives in different winemaking stages

There are several moments in the evolution of wine when it is possible to make an addition of wooden chips, staves, cubes or other fragments. Numerous studies have been carried out at every stage, from alcoholic fermentation to malolactic fermentation up to storage and aging process, but knowledge of these practices is still limited (Pérez-Coello *et al.,* 2000; Kyraleou *et al.*,2015; Sánchez-Palomo *et al.,* 2017).

Pérez-Coello et al. (2000), studied the use of American and French oak wood chips during the alcoholic fermentation of wine. The results obtained by them showed how the wines fermented in contact with the wooden fragments showed higher fermentation yields and a high concentration of volatile substances produced during fermentation. Furthermore, these wines

had aromatic components deriving from wood, which contributed to increasing the complexity of the wine. Compounds such as lactones, eugenol, vanillin and furfural are extract from oak wood chips. However, the use of fragments allows fermentation in steel tanks to preserve the fruity aromas and avoid negative color changes for white wines.

Instead, a study carried out by Sánchez-Palomo et al. in 2017, it highlights the differences between an addition made in fermentation with post-fermentation, in a white wine. They showed how wines with added post-fermentation chips showed a higher concentration of lactones, furanic compounds and benzoic compounds. On the other hand, wines with chips added during fermentation resulted in a higher concentration of alcohols, ethyl acetate, hexyl acetate, isaomyl acetate and ethyl esters of fatty acids. Both wines, however, showed fruity, sweet and spicy aromas with greater intensity than the samples that had not had contact with the wooden fragments.

Also Kyraleou et al. (2015), compared the addition of wood fragments in wine during and after fermentation. They found that the addition in fermentation did not bring advantages in the extraction of ellagitannins and in the condensation reactions of the tannins and stabilization of the anthocyanins. They also detected that in wines fermented in contact with wood fragments there were higher concentrations of volatile compounds such as lactones and eugenol, but also branched ethyl esters and acetates. Where instead the chips were added after fermentation, they found higher concentrations of ethyl esters, guaiacol, methyl guaiacol and vanillin. However, they saw that the condensation and stabilization reactions take place faster in the fermented wine in the presence of fragments, although in less effective way, making it a wine ready to be drunk.

1.5. Alternative wood botanical species

In recent years, the growing demand for oak wood has led to an increase in the costs of the base material and a decrease in the availability of wood in the forests. This meant that alternative woods began to be used, such as chestnut (*Castanea sativa*), acacia (*Robinia pseudoacacia*), cherry (*Prunus avium*) and in minor extent also mulberry (*Morus alba* and *Morus nigra*) and ash (*Fraxinus excelsior*) (Tavares *et al.,* 2017). In the past, the use of chestnut wood in winemaking was widespread, thanks to its great availability and low cost (De Rosso *et al.,* 2009).

Each type of alternative wood has different phenolic characteristics, also compared with oak woods. In fact, acacia wood has a significant content of benzene aldehyde, that of chestnut a high content of polyphenols and an easy extraction of eugenol and vanillin, the cherry wood releases methoxyphenols, while that of mulberry has been shown to have a low content of volatile substances. For these reasons, wines aged in contact with alternative woods have

different sensorial characteristics that have been studied over time. (De Rosso *et al.,* 2009; Tavares *et al.,* 2017).

1.5.1. *Castanea sativa*

Another alternative type of wood is that of sweet chestnut (*Castanea Sativa* Mill.). This is a native species of Europe and today its distribution is mainly concentrated in Italy, France, Spain, Portugal, Switzerland, the Balkans and in the Mediterranean islands (Figure 3). The area covered by sweet chestnut trees occupies approximately 2.5 million hectares. In these countries several tons of sweet chestnut wood are produced, mostly used as firewood, but also to produce furniture, barriques, shingles, sleepers, etc. Its fruits are also highly sought after and are collected for food consumption (Conedera *et al.,* 2016; Viriot *et al.,* 1994). Chestnut wood has been used in the past to produce barrels for its low cost and its high availability. On a phenolic level there are many similarities with oak, for example, chestnut wood, it is very rich in low molecular weight polyphenols, but also in hydrolysable tannins and gallic acid, hence its use to produce oenological tannins. Furthermore, it has also been seen that the phenolic compounds of chestnut wood behave in a similar way to those of oak during seasoning and toasting. (Canas *et al.,* 2000; Caldeira *et al.,* 2006; Fernández de Simón *et al.,* 2009; Sanz *et al.,* 2010a).

Vescalagin, castalagin and ruburin E are the most common ellagitannins in chestnut wood. While minor amounts of roburin B, C and D, grandin and ellagitannins have been found. The quantities of these ellagitannins are very similar to those of European and American oak woods (Viriot *et al.,* 1994; Sanz *et al.,* 2010).

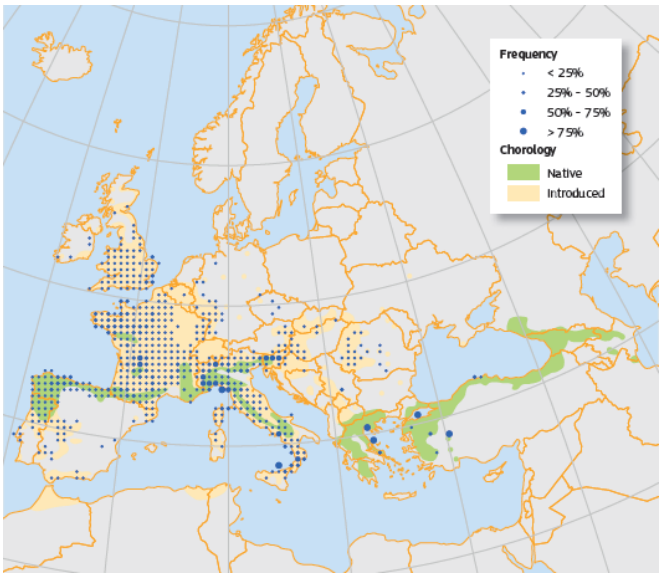


Figura 3. *Distribution of Castanea sativa in Europe (Conedera* et al., *2016)*

1.5.2. *Prunus avium*

Wild cherry is a mainly European species. It is a tree that grows very fast but that does not have a long life, usually 100-150 years. It is distributed in Europe (Figure 4), North Africa, West Asia and Anatolia. The cultivated subspecies are widely used by man for their fruits. However, the wild species, often used as an ornamental tree, has its real importance in its wood, very valuable being dense and solid, also used for musical instruments (Welk *et al.,* 2016). Wild cherry wood is also usually used for the construction of barrels for the aging of vinegars and red wines. It has also been seen that non-toasted seasoned wood gives the wine special characteristics but being very permeable it is recommended to use it for rapid aging with low contact time. (Cerezo *et al.,* 2008; De Rosso *et al.,* 2009). In general, cherry wood barrels have been shown to cause faster wine evolution than oak wood, both in terms of color and for the transition of phenolic compounds from the wood into the wine. It is therefore potential recommended, for short wine contact time. The use of larger barrels, up to 1000 liters of capacity, to exploit the characteristics of cherry wood, expanding the evolution of the wine over time (Chinnici *et al.,* 2011; Chinnici *et al.,* 2015).

Compared to the types of wood most used in winemaking, such as oak, which mainly possess gallic, ellagic and ellagitannin acids, it has been seen that cherry wood has high concentrations of flavonoids and procyanidins. Toasting, on the other hand, radically transforms cherry wood, reducing the quantitative differences between procyanidins and hydrolysable tannins and increasing the content of ellagitannins (Sanz *et al.,* 2010b; Chinnici *et al.,* 2015). Cherry wood is not yet fully known, some components are still unknown and there is no perfect knowledge of the effect on wine. For these reasons, several studies will still be needed to characterize this type of wood well.

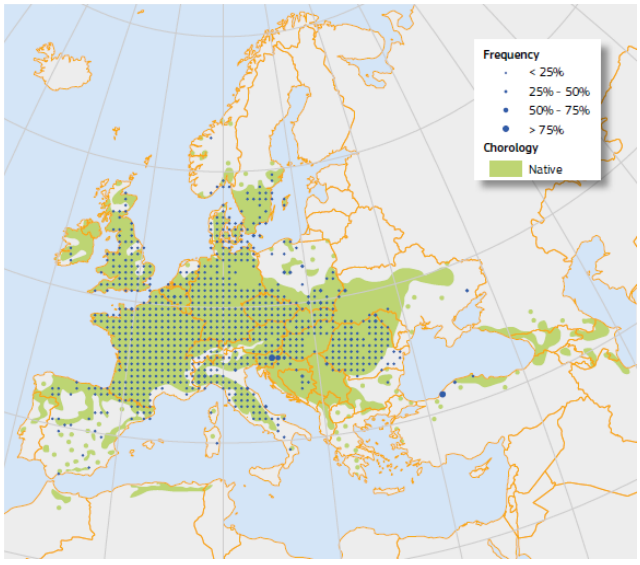


Figura 4. *Distribution of Prunus avium in Europe (Welk* et al., *2016).*

1.5.3. *Robinia pseudoacacia*

The *Robinia pseudoacacia* is known by the name of black locust, it is native to North America, but has been massively transported to Europe in the 17h century. In Europe it is distributed extensively (Figure 5), from the far west in Portugal to the Caucasus region, and from Northern Europe to Sicily. Its wood is very resistant and durable, widely used in the carpentry industry to build furniture, fences, building, boats, railways, but also as firewood (Sitzia et *al.,* 2016). In addition to these types of use, *Robinia pseudoacacia* wood has been evaluated, through numerous studies and tests, as an alternative wood to oak, for the aging of wines. On a phenolic level, acacia wood contains polyphenolic compounds of different structure and different families. Acacia heartwood has high flavonoid content, and as is usual with this species, these compounds are different from those found in other wood species. Furthermore, it has been seen that the heartwood has no ellagitannins and has a low concentration of condensed tannins, unlike the oak and cherry rich in ellagitannins and procyanidins (Sanz *et al.,* 2012; Tavares *et al.,* 2017). In acacia dihydrorobetin and robinetin are the most present flavonoid compounds, and together with fustin and butin, can be used as markers for those wines that have been aged in contact with this wood, facilitating its recognition (Fernandez de Simon *et al.,* 2009; Sanz *et al.,* 2010; Sanz *et al.,* 2012; Fernández de Simón *et al.,* 2014).

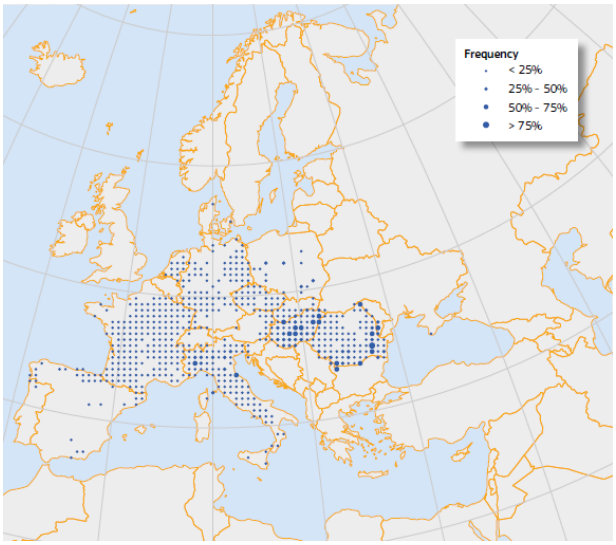


Figure 5. *Distribution of Robinia pseudoacacia in Europe (Sitzia et al., 2016).*

1.6. The walnut as an alternative wood species for potential oenological use

The walnut tree (*Juglans regia* and *Juglans nigra*) is one of the most important woody plants. It is mainly used for its fruits, but also for production of oil and wood. The walnut tree requires specific climatic situations, being sensitive to frost when young. It prefers temperate climates with reduced temperature changes during spring and with milder winters which favor its germination. Usually, it is found as a single tree and woods of the same species are rarely

found. (Bottema, 1980; Peng, S., & Jay-Allemand, 1991; de Rigo *et al.,* 2016; Tomás, 2000; Polleggioni *et al.,* 2017).

The nut fruit is a very important food worldwide (Figure 6), having a high nutritional intake. Walnut is rich in oils of fatty acids, proteins, vitamins and minerals. It is also rich in polyphenols considered important for human health due to their peculiar antioxidant, antiallergenic and anti-inflammatory properties (de Rigo *et al.,* 2016; Núñez-Sánchez *et al.,* 2016; Fukuda *et al.,* 2003).

Figure 6. *Fruit and walnut tree (de Rigo et al., 2016).*

The wood of the walnut tree is highly appreciated for its qualities and for its ease of processing (Figure 7). Even the standard wood has high prices, in fact the most valuable pieces reach very high figures compared to other woods used in carpentry (de Rigo *et al.,* 2016).

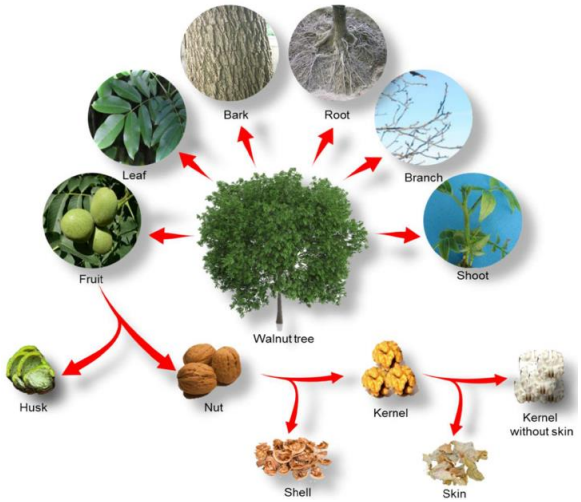


Figure 7. *Different parts of the wood and fruit of the walnut tree (Jahanban-Esfahlan* et al., *2019a).*

1.6.1 History of walnut tree

Walnut is a plant grown since ancient times, especially in China, Eurasia and eastern Europe. The few study carried out on the origin of this species made it difficult to determine the place where it was born and developed initially. In recent times, however, molecular markers have been developed capable of highlighting, at the molecular level, genetic traces belonging to the *Juglans* species. This allowed to find a population of wild walnut in Asia and fossils containing pollen in Europe. From here they then started to connect the various to create an evolutionary map of the species (Polleggioni *et al.,* 2017).

The walnut tree is native to ancient Persia, it was then transported by man, simultaneously to other species such as *Platanus* and *Castanea*, in the southeast of Europe and in the south of Turkey (Bottema, 1980).

On the other hand, during the last ice age, the walnut completely disappeared from these areas. It only managed to survive in warmer areas near the Caspian Sea and the Black Sea. The walnut then spread to the rest of Europe, mainly in the south of Spain and then to Portugal, France, Germany, Belgium, central Italy, the southern Alps and the Balkans (Figure 8). Further on the walnut tree was brought to the Americas by the Spanish conquerors. The plant then settled in the hot areas of Chile and California. Furthermore, walnut can be found in Eastern Europe, North Africa and Eastern Asia, mainly in China. (Tomás, 2000; Polleggioni *et al.,* 2017).

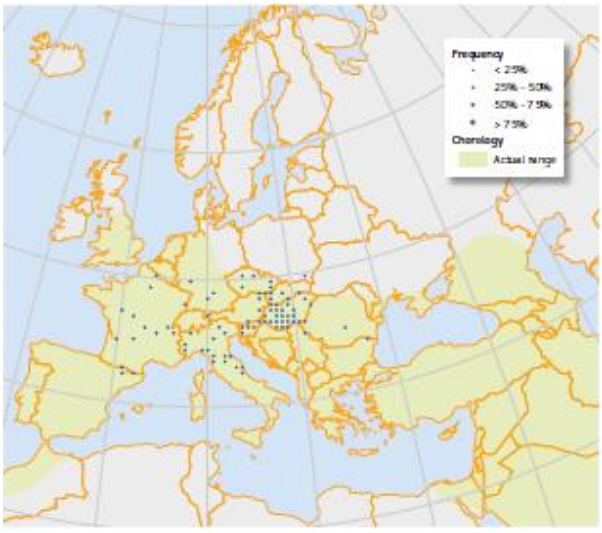


Figure 8. *Distribution of Juglans regia in Europe (de Rigo et al. 2016).*

1.6.2. Phenolic composition of walnut

The phenolic compounds of walnut, both on the type and on the extraction practices, have been the subject of numerous studies because they are very important for human health. This is because, especially polyphenols, they are highly antioxidant compounds. In fact, the

demand for antioxidant phenolic substances for pharmaceutical, nutraceuticals, cosmetic or food purposes is increasing. These compounds have been researched and studied in many parts of the walnut tree, from seed to wood and fruit. *Juglans* species have been shown to possess gallic acid, ellagitannins, naphthoquinones, flavonoids and various phenolic acids (Jahanban-Esfahlan *et al.,* 2019a and b; Fernández-Agulló *et al*., 2020).

It has been seen that several polyphenols are contained in the sapwood and heartwood *Juglans nigra* species: α-hydrojuglone-4-glucoside, myricetine, myricitrine, sakuranetine, sakuranine and neosakuranine (Gupta *et al.,* 1972). Naphthoquinones, such as Juglone and its derivatives, and flavonoids such as sakuranetine and myricetine have also been found in the bark, pericarp and nut fruit of *Juglans regia* and *nigra* species (Binder *et al.,* 1989).

It has also been seen that the most present compound in walnut wood is Juglone (Figure 9), an allelopathic compound that inhibits the growth of other plants and that causes blackening, blistering and peeling. It also has an antibiotic, antifungal, anti-tumor and tranquilizing effect (Hashemi & Latibari, 2011).

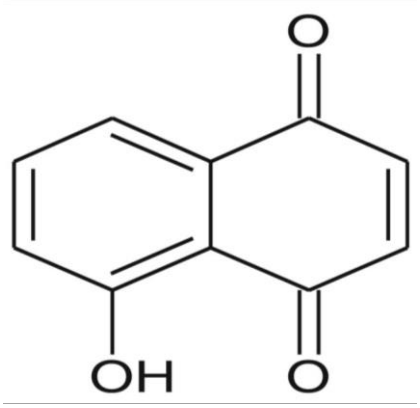


Figure 9. *Chemical composition of the Juglone (Bukhari et al., 2017).*

As for the *Juglans regia* species, it has been seen, by Colaric *et al.* in 2005, that in the skin of the nut kernel the most abundant phenolic compounds are juglone, syringic and ellagic acid, in higher concentrations than the kernel. Instead in the leaves, containing high concentrations of phenolic compounds (Nour *et al.,* 2016), the most present compound, in fresh leaves, is the juglone, but they are also considered an important source of flavonoids (Qa'dan *et al.,* 2005; Abuajah *et al.,* 2015).

While in the shoot, according to Claudot *et al.* 1997, the latest analyzes carried out have highlighted the presence of compounds such as flavanol myricitrin, hydrojuglone -d-glucopyranoside as a glucoside derived from juglone. Always in the shoots of Juglans regia species have been found: phenolic acids as p-coumaric, gallic, vanillic, syringic, ellagic and chlorogenic acid; flavonoids as quercetin, catechin, and myricetin; quinones as juglone and 1,4-naphthoquinone (Solar, *et al.,* 2006; Cheniany, *et al.,* 2013).

A study conducted by Fukuda *et al.* in 2003, he identified 14 polyphenolic compounds of walnut, analyzing seed extracts. These compounds are as follows: Glansrin A, Glansrin B, Glansrin C, 2,3-HHDP-d-glucopyranose, Isostrictinin, Pedunculagin, Casuarictin, Strictinin, Tellimagrandin I, Tellimagrandin II, 1,2-di-O-galloyl-4,6-HHDP-b-d-glucopyranose, Rugosin C, Casuarinin and Stenophyllanin A.

The compounds contained in the different parts of the plant are put together in Table 2.

Table 2. *The compounds contained in the different parts of the plant of juglans regia (Claudot* et al. *1997; Fukuda* et al. *in 2003; Qa'dan* et al., *2005; Solar,* et al., *2006; Cheniany,* et al., *2013; Abuajah* et al., *2015; Nour et al., 2016).*

	Parts of the plant			
	Skin of nut kernel	**Leaves**	**Shoots**	**Seed**
Compounds	Juglone	Juglone	Myricitrin	Glansrin A
	Syringic acid		Hydrojuglone -d-glucopyranoside	Glansrin B
	Ellagic acid		p-coumaric acid	Glansrin C
			Syringic acid	2,3-HHDP-d-glucopyranose
			Ellagic acid	Isostrictinin
			Gallic acid	Pedunculagin
			Vanillic acid	Casuarictin
			Chlorogenic acid	Strictinin
			Quercetin	Tellimagrandin I
			Catechin	Tellimagrandin II
			Myricetin	1,2-di-O-galloyl-4,6-HHDP-b-d-glucopyranose
			Juglone	Rugosin C
			1,4-naphthoquinone	Casuarinin
				Stenophyllanin A

2. AIM OF THE STUDY

The use of wood in the practice of aging wine has been commonly practiced for decades. The barrels have accompanied our wines for all this time and only in recent times that the way of conceiving wood in wine is changing. In fact, in recent years the use of chips or wood extracts has developed, which allow faster aging if accompanied by good micro-oxygenation, to extract more quickly those compounds characteristic of aging in contact with the wood we are used to, to give in a shorter time that refined character and those sensorial and chemical characteristics of wine. The wine industry, always expanding, has used these alternative methods to keep up with the times and recover that waste material from barrel construction.

The wood that made up these materials, however, used in oenology, was mainly oak, until some time ago. In fact, in recent years we have been looking for alternative wood species, studying their characteristics, their practicality and the goodness of their pairing with wine. Woods such as acacia, cherry, chestnut have been studied lately. However, other wood species could be evaluated as a potential source of bioactive compounds with a positive impact on wine composition and sensory properties.

Thus, the aim of this study is to characterize walnut wood at a phenolic level, for its possible future use in enology. The diffusion of this type of wood in the oenological field is not well known, So, a first step to study the potential use of walnut wood in enology it will be analyze their composition, especially in term of global phenolic composition and compare with the oak wood. Consequently, this work can be considered with pioneering.

During this study, chips from walnut and oak were put in maceration during two different periods (15 and 30 maceration days) in a model solution, which was then the basis of the analyzes to detect the extracted phenolic substances and make a comparison between the two wood species.

3. MATERIAL AND METHODS

3.1. Wood and extraction solution

The wood chips used for the experiment derive from two different types of wood. The first type, which we can also define as "control", belongs to oak wood of the genus *Quercus petraea*, manufactured by AEB Bioquímica Portuguesa S.A. The second type of wood is walnut, belonging to the *Juglans Regia* species, manufactured by AEB Bioquímica Portuguesa S.A. Both types of chips have three levels of toasting, light, medium and strong. Oak wood was toasted by an industrial process, while walnut wood was toasted at a laboratory scale process. Light toasting took place at 180°C for 5 minutes. The medium toasting took place at 160°C for 20 minutes. And last the strong toasting took place at 260°C for 27 minutes. These wooden chips will be left to macerate inside a model alcoholic solution for 15 days and 30 days. You will have a sample for each type of wood and each toasting level, with a replica of each. The samples will be a total of 24, 12 for each maceration time. The wood chips concentration used for both species was 2.5 g/500mL of model wine solution. The model wine solution was prepared using ethyl alcohol and distilled water. The pH was then adjusted to 3.2 using L (+) tartaric acid. The sample containers (Figure 10) were then closed and stirred every 5 days and storaged in the dark conditions at 20 ºC.

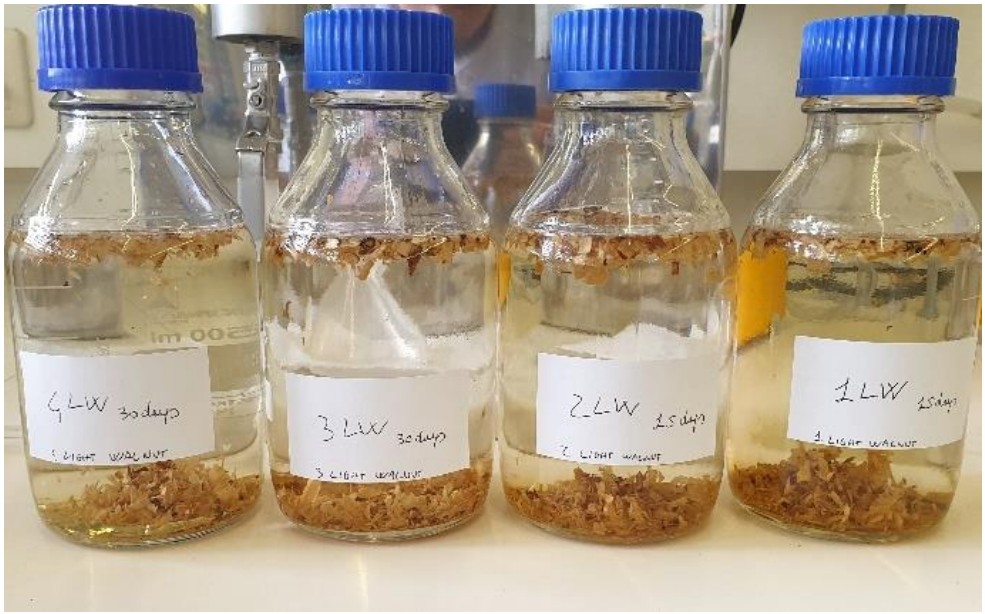


Figure 10. *Examples of ready and closed samples.*

3.2. Chemical analyses

The analyzes were carried out in the oenology laboratory inside the Ferreira Lapa building at the Superior Institute of Agronomy of the University of Lisbon.

At the end of the maceration time, the liquid part of the samples was separated from the wooden chips and then proceeded with the various analyzes. The mathematical expressions comes from a standard curve obtained in the laboratory with water solutions of gallic acid.

3.2.1. Total phenols

The concentration of total phenols is determined following the method described by Ribéreau-Gayon (2017). In a 100 ml volumetric flask the sample was diluted with distilled water to 1:100. The absorbance at 280 nm wavelength was then read through the spectrophotometer.
To obtain the value of total phenols, the index of total phenols must first be calculated, using the following equation:

$$Total\ phenol\ index\ = ABS \times 100\ (au)$$

Finally, to have the value of the concentration of total phenols expressed in mg/L of gallic acid, we use this equation:

$$Total\ phenols\ = \frac{(ABS\ \times 100) + 0.0344}{0.038}$$

3.2.2. Flavonoids and non-flavonoids phenols

Flavonoids and non-flavonoids phenols were quantified following the Kramling and Singleton method (1969). 10mL of sample are placed inside a centrifuge cuvette together with 10ml of HCL (1: 4) and 5mL of formaldehyde concentrated at 8mg/mL. The container with the mixture was then inertized with nitrogen, sealed and placed to rest in a dark room for 24 hours. After this period, the samples were centrifuged at 3500 rpm for 10 minutes. Then a 1:10 dilution was made with distilled water, 5mL of sample in 45mL of distilled water. The absorbance at 280 nm of the diluted sample spectrophotometer was then read.
The results obtained from the analysis were multiplied by the dilution of 10.

$$Absorbance\ units = ABS \times 10\ (au)$$

The concentration of non-flavonoids expressed in mg/L of gallic acid through the following equation:

$$Non\text{-}flavonoids = \frac{(ABS\ \times 10) + 0.0344}{0.038}$$

Finally, the concentration of flavonoids is obtained by subtracting the concentration of the total phenols mentioned above and the concentration of total non-flavonoids obtained, through the following equation:

$$Flavonoids = [Total\ phenols] - [non\text{-}flavonoids]$$

3.2.3. Color analysis

The color analysis was carried out following the indications of the OIV method MA-AS2-07B: R2009 (Type IV method). The optical reading at 420nm took place directly on the centrifuged

samples, in a 10mm cuvette. The analysis was carried out as if the sample were of a white wine and not of a red wine, being the model solution more like the characteristics of a white wine, that is, at 420nm (yellow) and not at 520 and 620nm (blue and red). The results are expressed with the absorbance value at 420nm.

3.2.4. Statistical analysis

To study the statistical significance of the data obtained from the analyzes, these were analyzed through an analysis of variance (ANOVA), through two different approaches to data organization. The first compares the results of all the samples analyzed with each other, without studying the effects of individual factors. While the second approach is based on ANOVA with three fixed variables with interactions, to understand both the main effects of the individual variables (type of wood, level of toasting and maceration time), the effects of the interactions between the variables in pairs or taken all three together. Post hoc tests were carried out on the significance highlighted by the analysis of variances using the "Bonferroni" marginal means comparison method. The statistical analyzes were carried out using the statistical software "R Studio" (R version 4.0.0). The statistical significance taken into consideration was $p < 0.05$.

4. RESULTS AND DISCUSSION

4.1. Global results for the color and phenolic composition analysis on the oak and walnut extracts.

The approach used in the following statistical analyzes allowed us to compare the individual samples with each other, at several days of maceration, without considering the interactions between factors.

In the following figures, the letters placed above the columns indicate whether there are significant differences. This significance considers both maceration times.

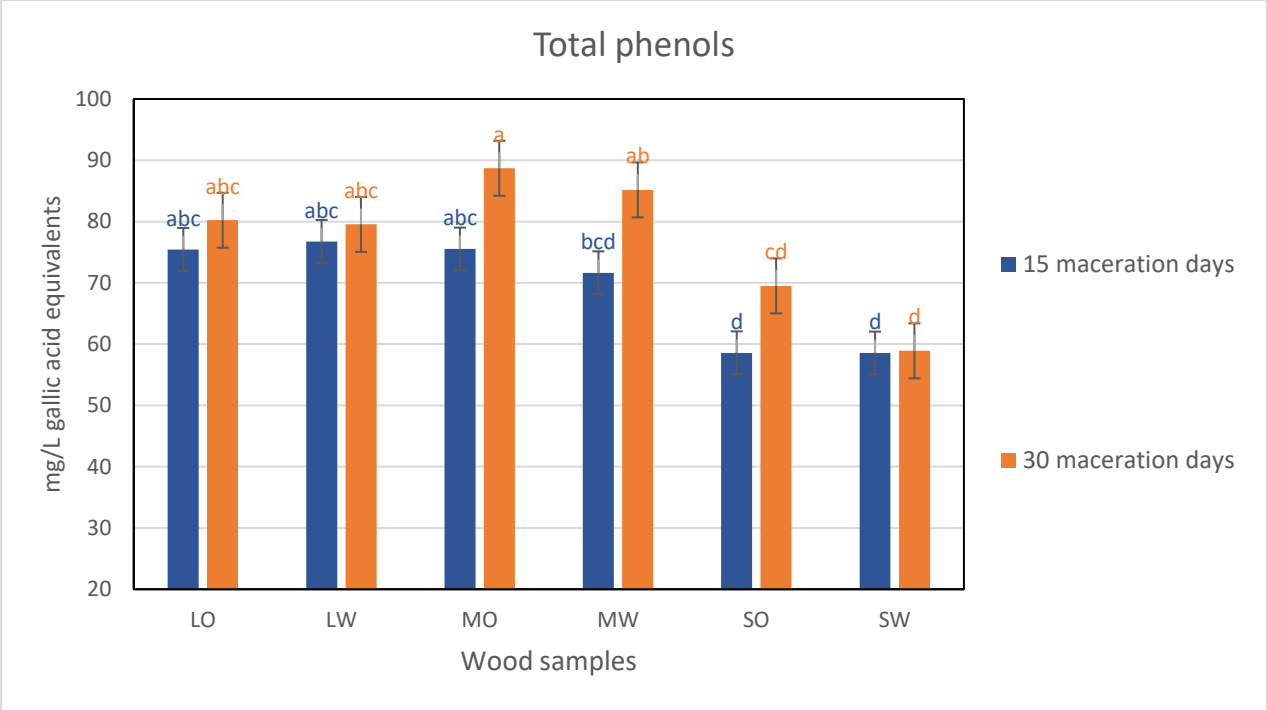


Figure 11. *The total phenols from the different wood chip extracts species obtained at two different maceration time (15 and 30 maceration days). The results with same letters are not significantly different (p<0.05). Legend: (LO) Light toasted oak wood; (LW) Light toasted walnut wood; (MO) Medium toasted oak wood; (MW) Medium toasted walnut wood; (SO) Strong toasted oak wood; (SW) Strong toasted walnut wood.*

In Figure 11 the samples were statistically compared for their total phenol content.

As can be seen in the Figure 11, the highest total phenol values are found in the sample containing medium toasted oak wood extract with 30 days of maceration, indicating how the combination of these three factors led to a greater extraction of total phenols in solution. In this sample the content of total phenols extracted in solution was 88.7 mg/L gallic acid equivalents. Immediately below these values we find the analogous but walnut sample, with 85.2 mg/L gallic acid equivalents. This tells us that the difference between the two woods leads to a lower total phenol content in walnut, but the difference is not significant.

The values that we can consider average, or intermediate to almost all, except for the lowest ones, are those that contain walnut wood and those with oak wood with light toasting, at both

maceration times, also there is the oak wood sample with medium toasting at 15 days of maceration, the values are between 80.2 and 75.4 mg/L gallic acid equivalents.

One step below is the value of the walnut wood sample with average toasting at 15 days of maceration of 71.6 mg/L gallic acid equivalents.

Between the latter sample and those with the lowest values, there is an intermediate, or the oak wood sample with strong toasting at 30 days of maceration, which contains 69.5 mg/l gallic acid equivalents.

The lowest significantly values, are found in the samples of strong toasted walnut, in both maceration times, and in those of strong toasted oak wood with 15 days of maceration, between 58.9 and 58.5 mg/L gallic acid. In addition, it was not totally clear the real impact of the maceration time on total phenolic content from the different wood extracts, except for wood extracts obtained from the two chip species with medium toasting.

The values of total phenols extracted from the samples containing the walnut wood are in line with those found by Jahanban-Esfahlanet et al. (2019a) in a study in which walnut wood extracts were analyzed.

In another work by Jahanban-Esfahlan et al. (2019b) the analysis of total phenols was carried out on the husk of the nut fruit, giving different results, with higher concentrations than the previous work and compared to the results obtained in this work.

Instead, in a work by Queirós et al. (2020), analyzes carried out on the nut fruit shell, the results of the total phenols shown are much higher than in the two works mentioned previously. This tells us that, depending on the part of the walnut tree analyzed, the results are different and therefore there may be discordances also in the literature.

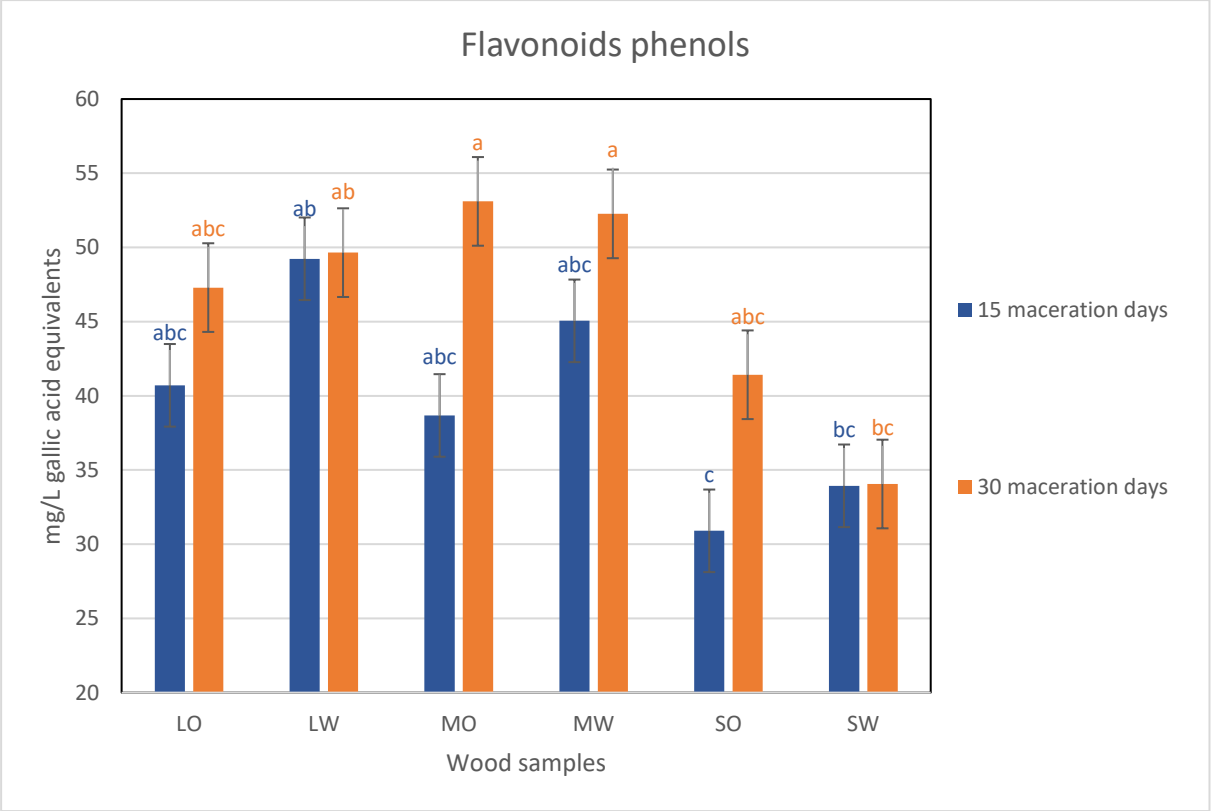


***Figure 12**. Evolution of the flavonoid phenols of the different two-step maceration samples. The results with same letters are not significantly different (p<0.05). Legend: LO) Light toasted oak wood; LW) Light toasted walnut wood; MO) Medium toasted oak wood; MW) Medium toasted walnut wood; SO) Strong toasted oak wood; SW) Strong toasted walnut wood.*

Figure 12 shows the statistical results of the values of the flavonoids content in the analyzed samples.

The highest values were recorded in the walnut and oak wood samples, both with medium toasting and at 30 days of maceration, with contents respectively of 52.3 and 53.1 mg/L gallic acid equivalents.

On the other hand, the walnut wood samples with light toasting at both maceration times, have values slightly lower than the first ones, that is 49.2 mg/L gallic acid at 15 days of maceration and 49.6 mg/l gallic acid equivalents at 30 days of maceration

The intermediate values ranging from 47.3 (LO 30 days) to 38.7 (MO 15 days) mg/L gallic acid equivalents. The lowest flavonoid phenols content was obtained for the walnut samples with strong toasting (both maceration time, 33.9 and 34.1 mg/L) and oak samples with strong toasting obtained after 15 maceration days (30.9 mg/L).

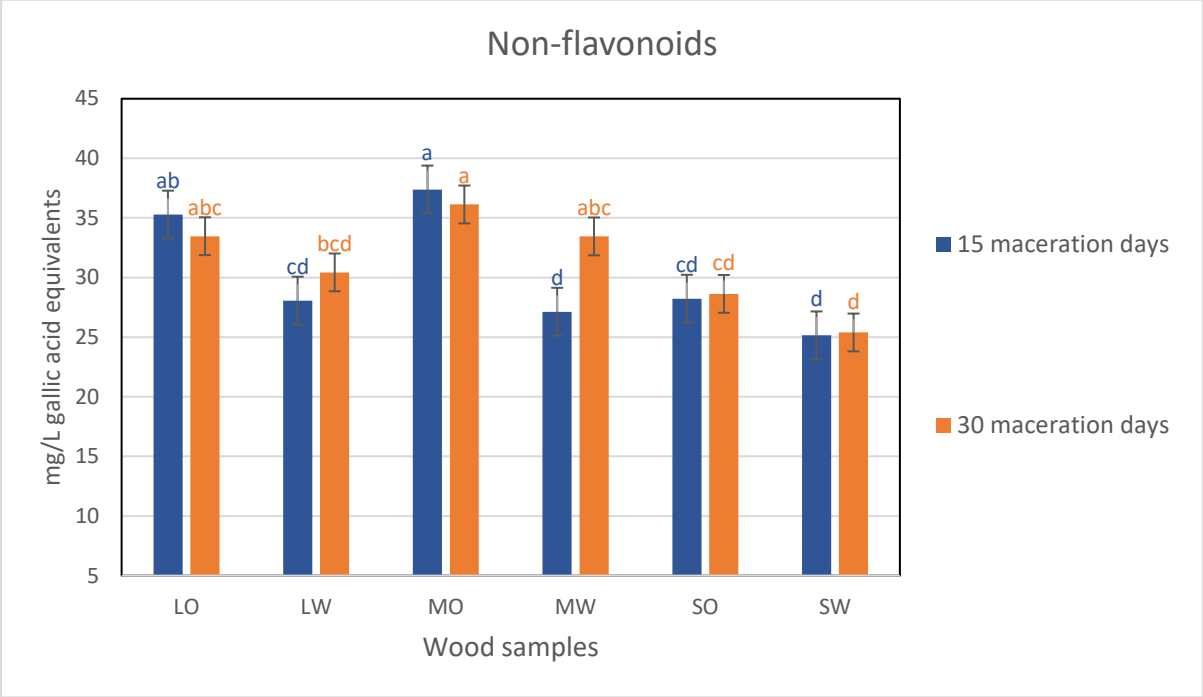


Figure 13. *Evolution of the non-flavonoids of the different two-step maceration samples. The results with same letters are not significantly different (p<0.05). Legend: LO) Light toasted oak wood; LW) Light toasted walnut wood; MO) Medium toasted oak wood; MW) Medium toasted walnut wood; SO) Strong toasted oak wood; SW) Strong toasted walnut wood.*

Regarding the extracted content of non-flavonoids, Figure 13 shows us the results of the statistical analyzes on the values obtained through the analysis of the samples. The highest values belong to the samples of oak wood with medium toasting, in both maceration times (37.4 and 36.1 mg/L gallic acid equivalents, respectively after 15 and 30 maceration days). Just below these values we find the sample at 15 days of maceration, always in oak wood but with light toasting, with an average value of 35.3 mg/L gallic acid of non-flavonoid phenols. Even lower values, but always close to the highest, were given by the samples with 30 days of maceration with lightly toasted oak wood extract and medium toasted walnut wood, respectively of 33.5 and 33.5 mg/L gallic acid. The sample of light toasted walnut extract and 30 days of maceration, with the value of 30.4 mg/L of non-flavonoid phenols, is intermediate to most of the samples.

Instead, the samples that gave results closer to the lower ones found during the analyzes, are those of light toasted walnut extract obtained after 15 days of maceration, with 28.1 mg/L gallic acid, and those of oak with strong toasting at both maceration times, with 28.6 mg/L for 30 days and 28.2 mg/l for the other. The lowest values found for non-flavonoid phenols were given from extract samples from walnut wood with medium toasting and obtained after 15 days of maceration (27.1 mg/L gallic acid) and with strong toasting at both times of maceration (25.4 mg/L and 25.2 mg/L, respectively for 30 and 15 maceration days).

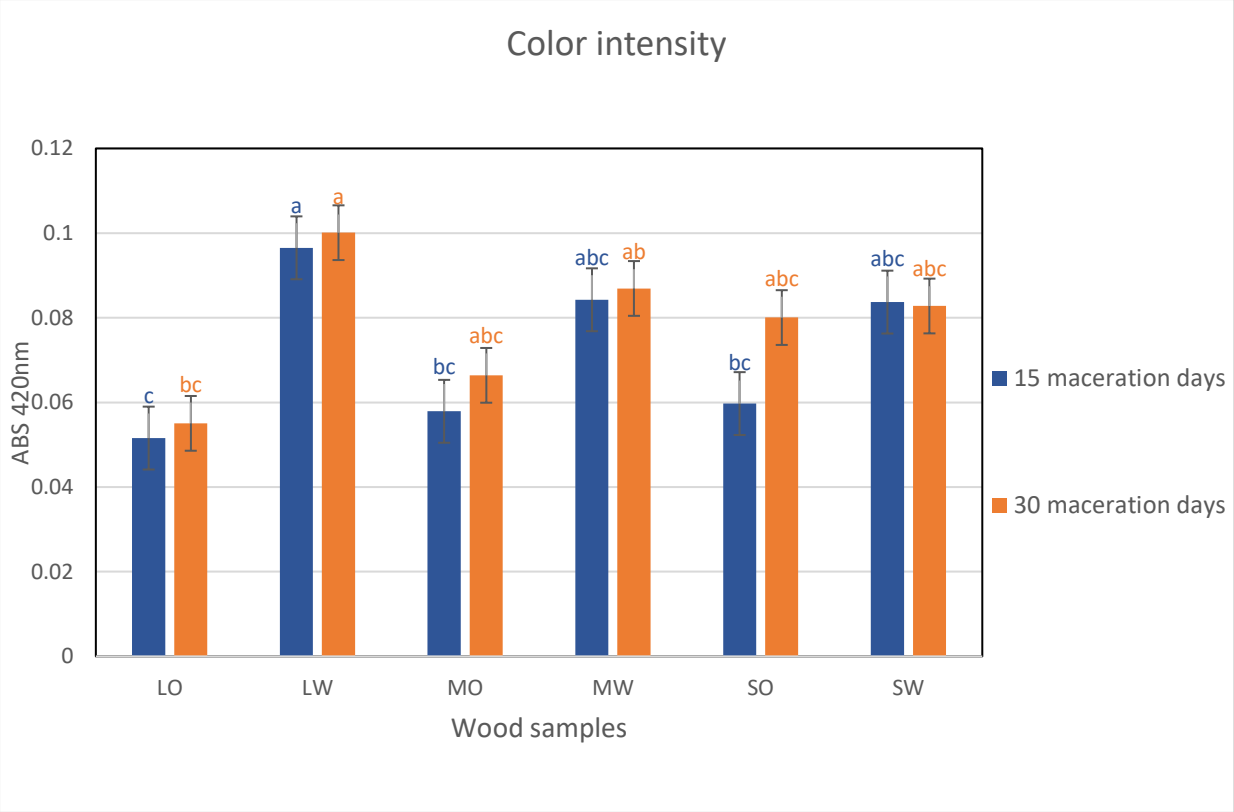


Figure 14. *Color intensity at 420 nm from different wood chip extracts obtained at different maceration days. The results with same letters are not significantly different ($p<0.05$). Legend: LO) Light toasted oak wood; LW) Light toasted walnut wood; MO) Medium toasted oak wood; MW) Medium toasted walnut wood; SO) Strong toasted oak wood; SW) Strong toasted walnut wood.*

The color analysis carried out with the study of absorbance at 420nm, treating the samples as if they were white wines, allowed us to obtain very interesting results.

As can be seen from Figure 14, the highest values are those of light toasting walnut extract samples with both maceration times, 0.10 abs untis for the one with 30 days of maceration and 0.096 abs units for the one with 15 maceration days.

This difference is very relevant if compared with the samples that have the same level of oak toasting, in fact, as indicated by the letters above the columns, these samples have the lowest values recorded in the analyzes, with 0.0551 for the 30-day one of maceration and 0.0516 for the one with 15 days of maceration.

This trend is also confirmed by the other samples, in fact those of walnut with medium and strong toasts, have values closer to the high ones, or in any case intermediate ones.

Samples of oak wood with similar toasts, except the one with strong toasting at 30 days of maceration, have tendencies that are lower.

The results visible in Figure 14 indicate how the influence on color by walnut wood is much stronger and more important than that of oak wood. The samples, in fact, had more intense colors visible even with the naked eye.

4.2. Toasting level, wood specie and maceration time effects on the total phenols content of the wood extracts

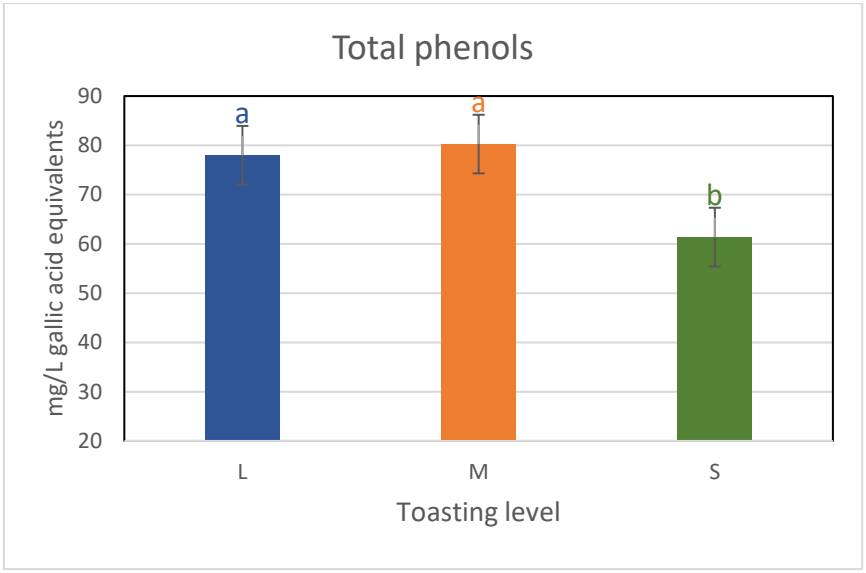
From here on, the results derived from a different statistical approach will be reported. In this method, the three different factors are analyzed (type of wood, level of toasting and days of maceration), taken individually or studied the interaction in pairs and in trio. The significance, indicated by letters placed above the columns of the following figures, indicate the differences found by the statistical analysis between the variances of each sample with that of any other, by comparing a single factor or the interactions between them. The single factor analyzes study the differences between the variances of the results only and only due to that factor, not considering the influence of the other factors. While for the interaction analyzes only the interaction between the two factors is considered, without considering the third factor.

This paragraph shows the results of the statistical analysis for the content of total extracted phenols. In Figure 15 a) the statistical analysis of total phenols shows us how there has been a significant decrease in the woods that had undergone the strong toasting process. This result was expected because, as is well known, the toasting process usually leads to a decrease in the total phenols of the wood (Ribéreau-Gayon *et al.,* 2017). In this case it occurred in both types of wood used in this study, demonstrating how toasting affects the characteristics of the wood regardless of the type of wood used, this is also seen in the statistical analyzes of the other compounds and the study of the interactions. Another significant difference highlighted by statistical analyzes regarding total phenols, we find it in the type of wood. From the analyzes conducted, it has been shown (Figure 15 b)) how the botanical origin of wood influences the content of total phenols within the model solution. In this case, it has been seen that oak wood has released in solution a significantly higher quantity of total phenols than walnut. This result suggests that the impact of walnut on a wine put in contact with it, at least for total phenols, could be less than in oak. Another factor studied was that of maceration time.

The impact of maceration times on the extraction of total phenols from wood chips is evident. In figure 15 c) we see, in fact, how the longer maceration time, i.e. that of 30 days, led to a significantly higher total phenol content, compared to that of 15 days, within the model solution in which it is maceration and then extraction took place. It is therefore evident that the longer maceration has allowed a greater extraction of total phenols in solution.

Once the individual factors have been analyzed, having seen what the differences are made on the number of total phenols extracted and where they are significant, it is right to analyze the interaction between the different factors, in order to analyze which influence they work simultaneously.

a)



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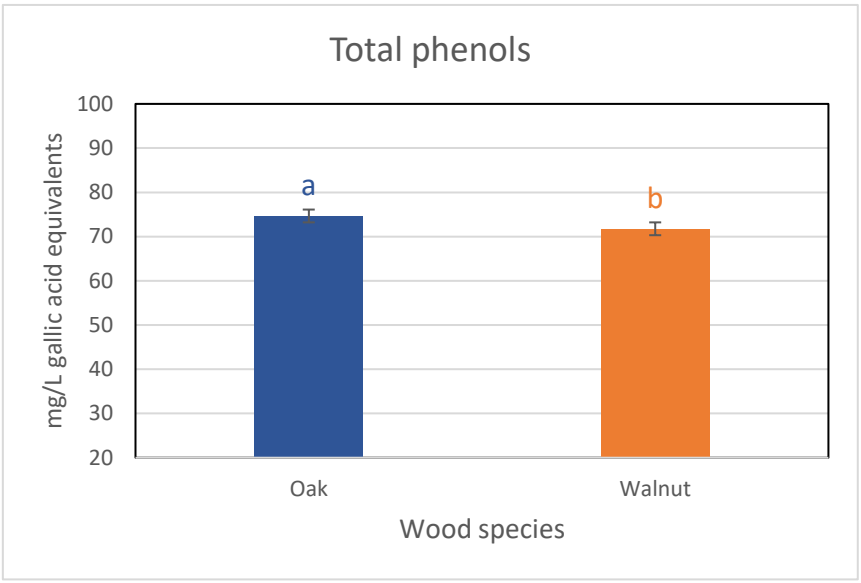

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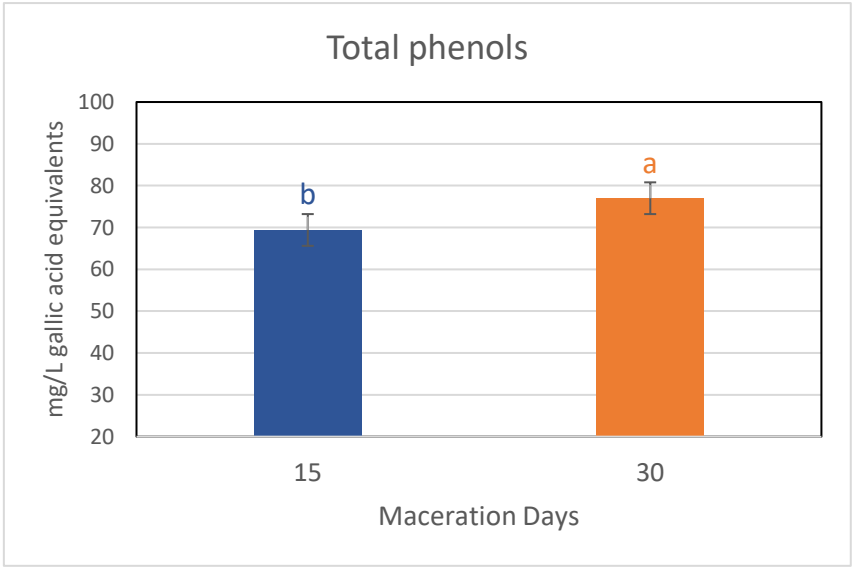


Figure 15. *a) Impact of the level of toasting on the concentration of total phenols extracted; b) Impact of wood species on the concentration of total phenols extracted; c) Impact of maceration time on the concentration of total phenols extracted. Results with the same letter are not significantly different (p<0.05). Legend: L) Light toast; M) Medium toast; S) Strong toast.*

Moving on to the study of the interactions of the different factors, we see that, again for the total phenol content, there are no significant differences in the statistical analysis of the interaction between the type of wood and the level of toasting, as shown in the figure 16 a). The absence of interaction between the two factors tells us that the two types of wood react very similarly to the different levels of toasting.

It is evident how the extractability from the wood, regardless of the walnut wood, is linked to the level of toasting, in fact it is clear how the medium toasting leads to greater extractability while with the toasting it decreases widely. This can also be seen in Figure 15 b).

In Figure 16 b) we see that there are no significant results for the interaction between the maceration time and the type of wood.

This means that the content of total phenols, inside the sample solution, changes regardless of the relationship between the two factors studied.

As we saw earlier in the statistical analysis of the individual factors, type of wood and days of maceration, there were significant differences within them, however these differences are not linked to each other as demonstrated in the interaction analysis.

During the statistical analysis of the interaction between the toasting level and the days of maceration, several significant differences were found, highlighted through Bonferroni's post hoc.

As can be seen in Figure 16 c), the highest values of total phenols extracted were found in the samples consisting of the medium toasting chips put in maceration for 30 days.

The value immediately below this is found in the sample with light toasting, always with 30 days of maceration. In third place for total phenol content there are those samples that have undergone 15 days of maceration with light and medium toasting levels.

The lowest values were found in the strong toasting samples in both maceration periods. These results indicate that there is a strong relationship between toasting and maceration days in the samples studied. In fact, it is noted that there is a strong influence of strong toasting on the total phenol content, being that which leads to lower values, while the days of maceration have a great influence in light and medium toasted, with the lower values having the times of shorter maceration.

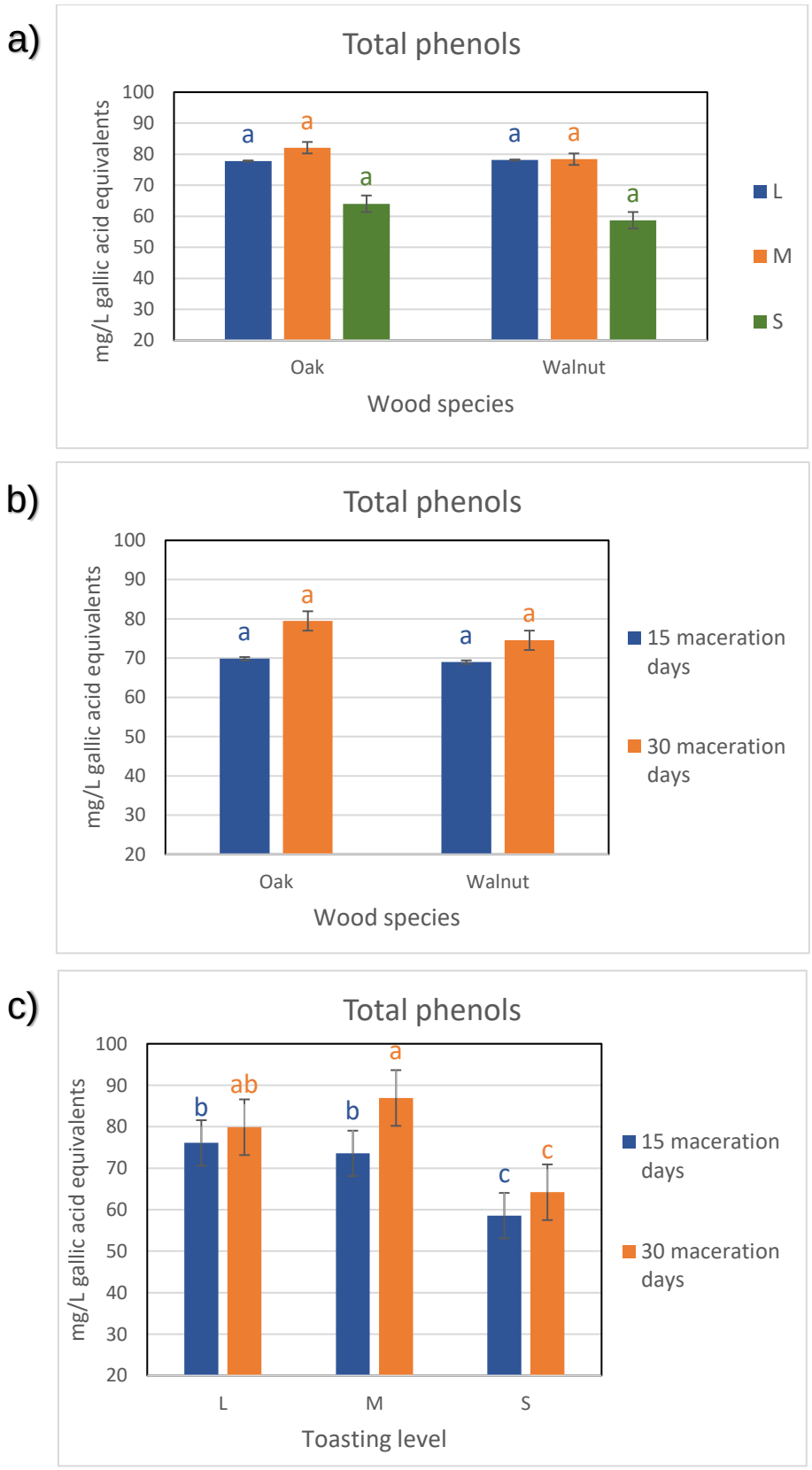


Figure 16. *a) Impact of the interaction between toasting level and wood species on the concentration of total phenols extracted; b) Impact of the interaction between maceration time and wood species on the concentration of total phenols extracted; c) Impact of the interaction between toasting level and maceration time on the concentration of total phenols extracted. Results with the same letter are not significantly different (p<0.05). Legend: L) Light toast; M) Medium toast; S) Strong toast.*

The statistical analysis carried out also included the study of the three-factor interaction, i.e. days of maceration, type of wood and level of toasting.

Observing the Figure 17 there are no significant differences, therefore no type of interaction was found between the three factors for the concentration of total extracted phenols.

We can therefore deduce that the three factors significantly influence this content individually or in pairs, as we have already seen, while in relation to each other it does not.

In fact, it is clearly seen, in Figure 17, that the trends of the columns are very similar to each other, so we can say that the samples behave similarly to each other when the interaction of all their factors is studied.

The non-significance of this type of three-factor interaction demonstrates how the two types of wood under the same conditions (toasting and maceration time) have no difference on the extraction of total phenols, thus behaving in a very similar way.

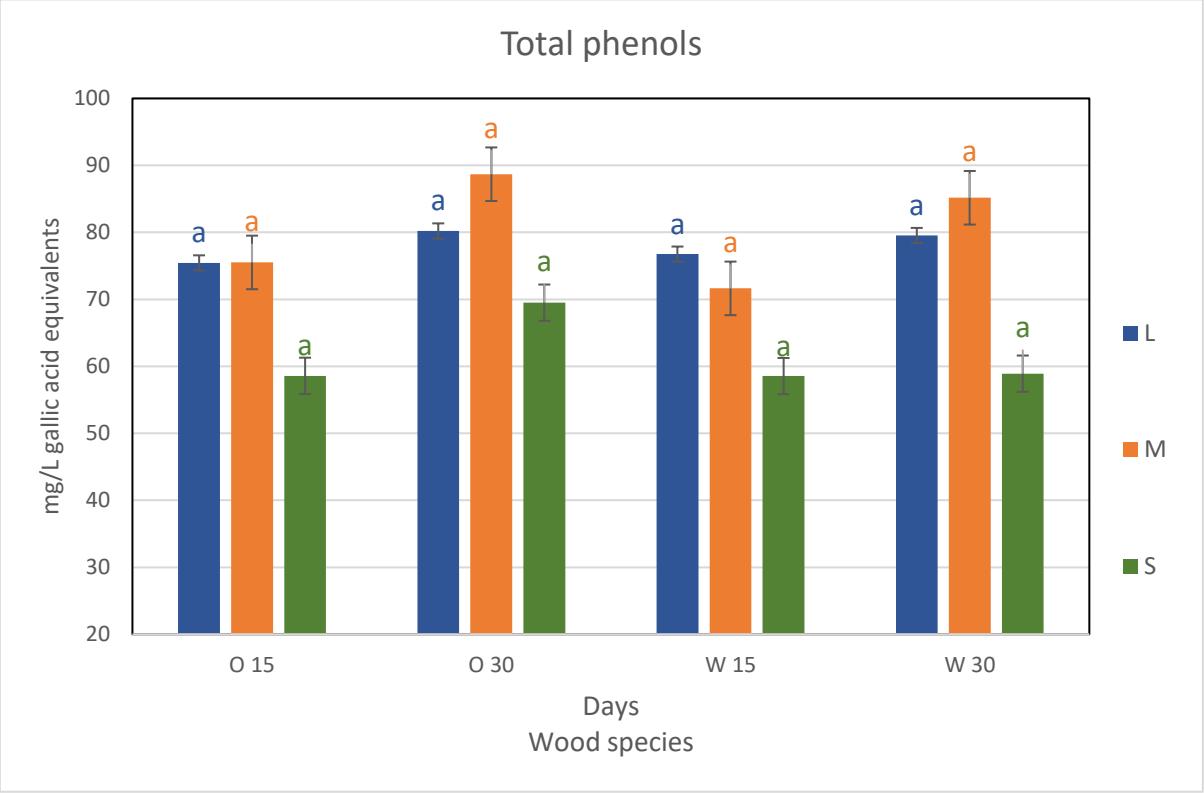


Figure 17. *Impact of the interaction between toasting level, maceration time and wood species on the concentration of total phenols extracted. The statistical analysis considers the interaction at three factors between the toasting level, the maceration time and the wood species. Results with the same letter are not significantly different ($p<0.05$). Legend: L) Light toast; M) Medium toast; S) Strong toast; O) Oak wood; W) Walnut wood.*

4.3. Toasting level, wood specie and maceration time effects on the flavonoid phenols content of the wood extracts

As shown in Figure 18 a), the flavonoid phenols content extracted within the analyzed samples shows significant differences depending on the level of toasting.

In fact, it can be seen how strong toasting causes a significant decrease in the flavonoid extract content, while it remains the same in light and medium toasting.

These differences between the toasts are independent of the other factors, i.e. the type of wood and the days of maceration.

This makes us understand how the toasting process of the wood leads to a decrease of the flavonoids inside it and therefore a lower extraction in alcoholic solution.

Instead, as regards another variable of the analyzes, namely the type of wood, no significant differences were found between oak and walnut.

As shown in Figure 18 b), the flavonoid phenols content extracted between the two woods is almost the same. The samples containing the walnut wood, therefore, released in the alcoholic solution a quantity of flavonoids is like those with the oak wood.

The wood species therefore did not directly affect the final content in flavonoids, as shown by statistical analysis results.

The maceration time, however, had a significant influence on the extraction of flavonoid phenols.

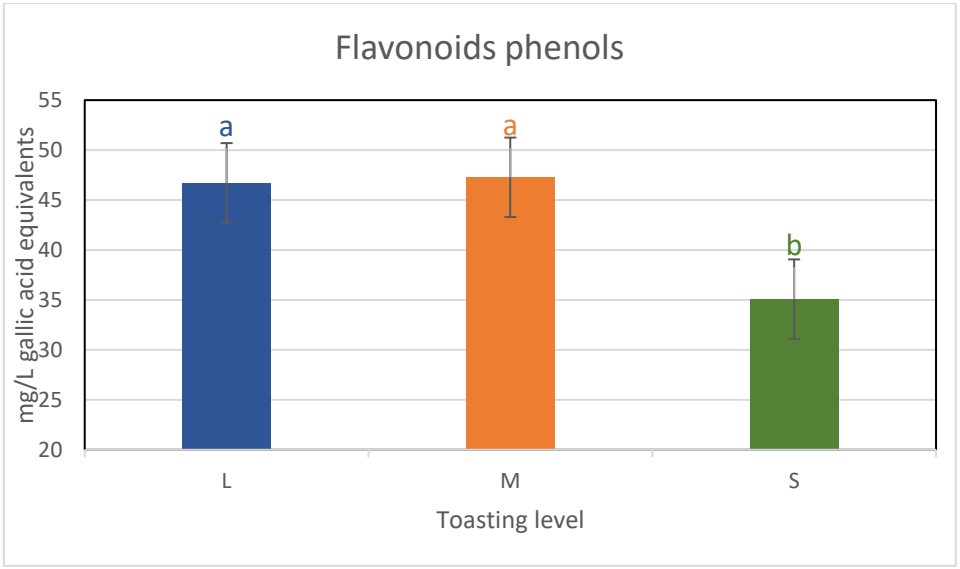
In fact, as it is visible in Figure 18 c), 30 days of maceration meant that the final content of flavonoids, within the model solution analyzed, was significantly higher than the samples with 15 days of maceration.

Even in this case the longer maceration time led to a higher extraction.

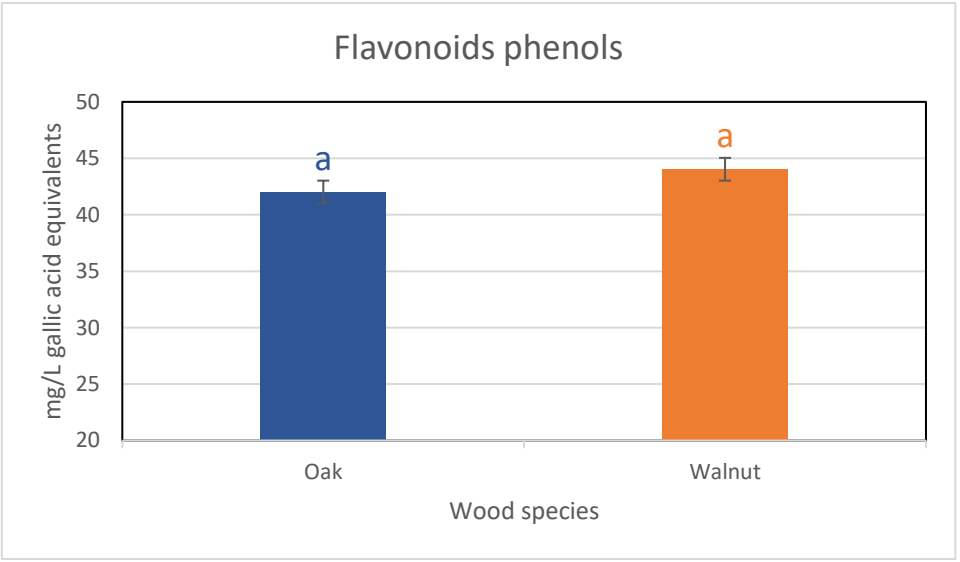
This effect is independent of the type of wood and the degree of toasting, only the main effect of the maceration time has been studied.

Statistical analysis of the interactions between the factors will also be carried out for the content of flavonoids extracted, to define their influence.

a)



b)



c)

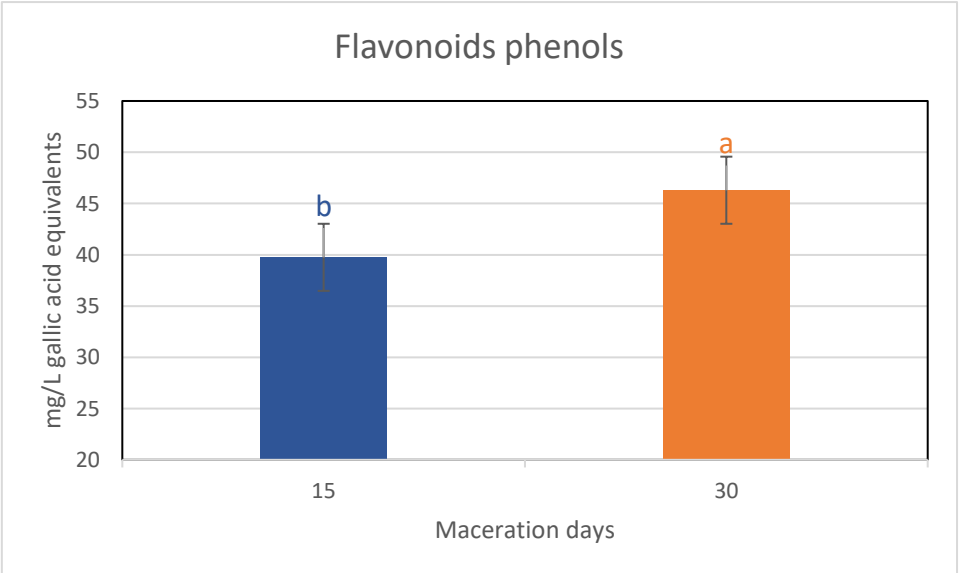
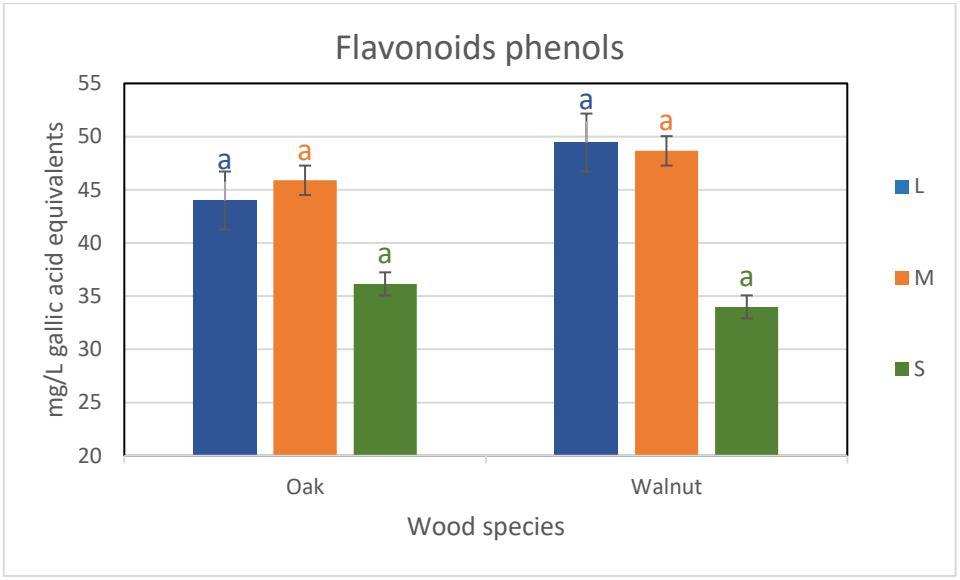


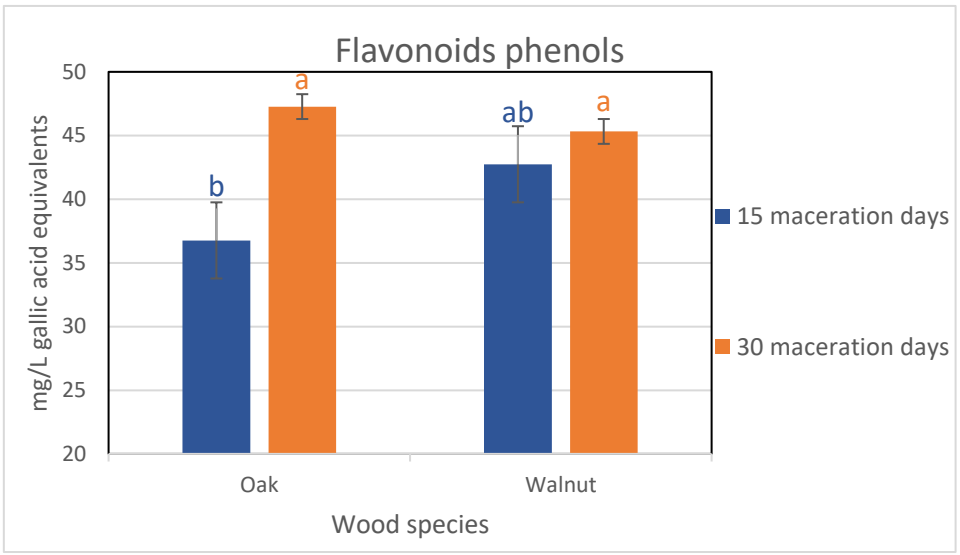
Figure 18. *a) Impact of the level of toasting on the concentration of flavonoid phenols extracted; b) Impact of wood species on the concentration of flavonoid phenols extracted; c) Impact of maceration time on the concentration of flavonoid phenols extracted. Results with the same letter are not significantly different ($p<0.05$). Legend: L) Light toast; M) Medium toast; S) Strong toast.*

The statistical analysis of the two-factor interaction did not give significant results when the toasting level factor and the type of wood factor were taken into consideration.
In any case, since there is no significance in these differences, we can say in Figure 19a) that the type of wood and the level of toasting do not interact in the final content of flavonoid phenols.
As regards, however, the interaction between the days of maceration and the type of wood, significantly differences were found during the statistical analysis, then analyzed through Bonferroni's post hoc.
As we can deduct from the results showed in Figure 19 b), both woods with 30-day maceration have a significantly higher flavonoid content.
Instead, the oak wood samples at 15 days of maceration, have the lowest value of flavonoid phenols extracted.
The intermediate value belongs to those samples that have walnut wood and a maceration lasting 15 days.
These results show us how the two woods interact in the same way at 30 days of maceration, presenting higher levels of flavonoid phenols, while at 15 days of maceration the influence of wood is greater, in fact oak wood has a significantly higher concentration compared to that of walnut.
It can therefore be said that after 15 days of maceration there are differences between the two woods, differences that flatten after 30 days of maceration, resulting practically equal.
The interaction between the level of toasting and the days of maceration did not lead to significant differences (Figure 19 c)). This tells us that the two factors do not work together with the modification of the flavonoid content.
In Figure 19 c), however, we can see how the columns of the samples with 30 days of maceration, are however slightly higher than those of 15 days, for all levels of toasting. This somewhat reflects what we have seen on the main effect of maceration time in Figure 18 c), but does not assure us that these differences are due to the joint action of the two factors.

a)



b)



c)

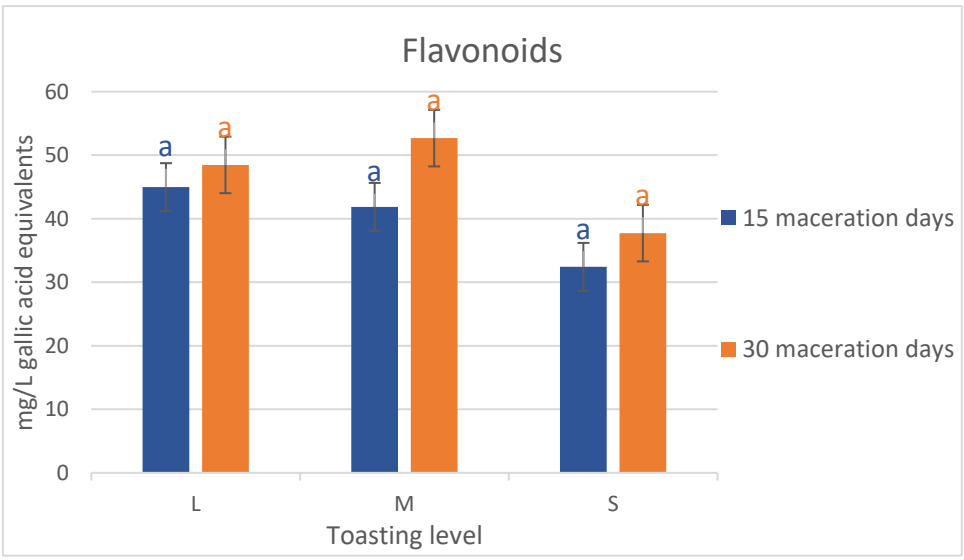


Figure 19. *a) Impact of the interaction between toasting level and wood species on the concentration of flavonoid phenols extracted; b) Impact of the interaction between maceration time and wood species on the concentration of flavonoid phenols extracted; c) Impact of the interaction between toasting level and maceration time on the concentration of flavonoids flavonoid phenols extracted. Results with the same letter are not significantly different (p<0.05). Legend: L) Light toast; M) Medium toast; S) Strong toast.*

The analysis of the three-factor interaction (toasting level, maceration time and wood species), as regards the flavonoid content, did not give any significant results.

As can be understood from the Figure 20, there are no significant differences between the samples due to the interaction between the type of wood, the toasting level and the maceration time. In fact, the trend of the columns is very similar to each other.

In this case we can say that the joint action of the three factors does not significantly affect the content of flavonoid phenols in the individual samples, therefore they behave similarly to each other if studied from this point of view.

However, it can be noted that there are differences between samples, simply by observing the heights of the columns, but these differences are due to the action of the individual factors or the interaction between two of them, as we have seen before.

Even in this case, therefore, it can be deduced that the two types of wood under the same conditions do not significantly influence the final content of flavonoid phenols, thus behaving in a similar way.

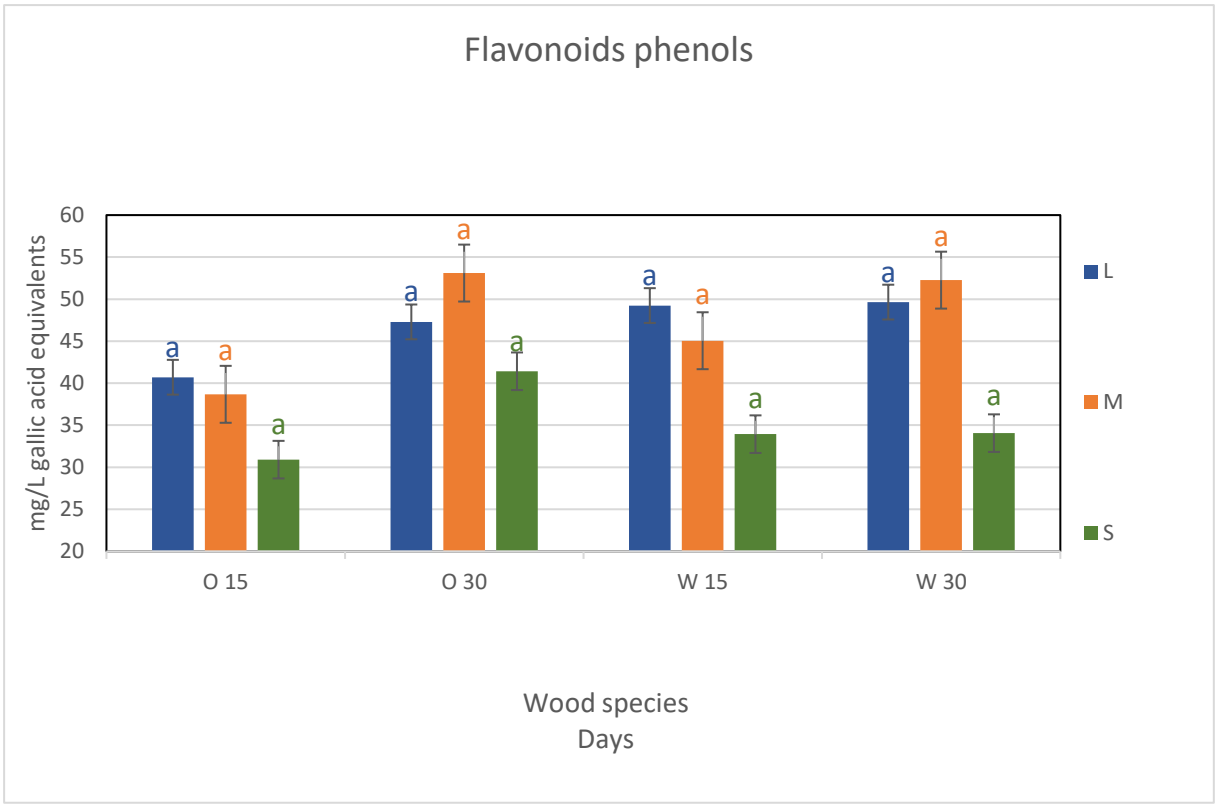


Figure 20. *Impact of the interaction between toasting level, maceration time and wood species on the concentration of flavonoid phenols extracted. The statistical analysis considers the interaction at three factors between the toasting level, the maceration time and the wood species. Results with the same letter are not significantly different ($p<0.05$). Legend: L) Light toast; M) Medium toast; S) Strong toast; O) Oak wood; W) Walnut wood; 15) 15 days of maceration; 30) 30 days of maceration.*

4.4. Toasting level, wood specie and maceration time effects on the non-flavonoid phenols content of the wood extracts

Also for the non-flavonoid phenols content, as for the total phenols and flavonoids, the statistical analysis showed significant differences due to the different level of toasting but only for strong toasting.

This shows us how toasting has a major influence on the phenolic content of wood and probably their extractability.

In this case, as shown in Figure 21 a), it is the samples that have the wood with strong toasting to have the lowest non-flavonoid phenols content extracted, while the medium and light toasting have concentrations of these compounds extracted.

Strong toasting has in fact a great impact on the degradation of the phenolic compounds of the wood, and even in the case of non-flavonoid phenols, this decrease is evident by the results obtained.

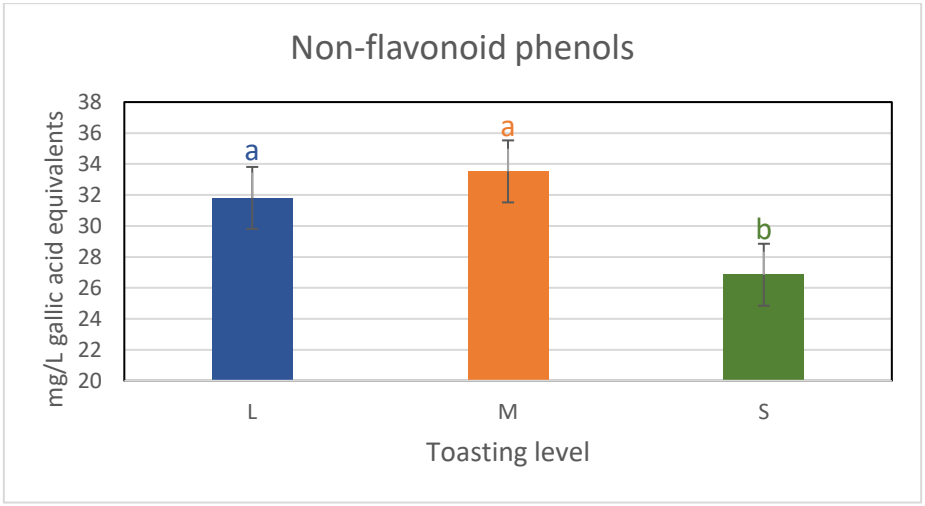
About the impact of the wood species, the results in Figure 21 b) shows that the type of wood has a significant effect on the non-flavonoid phenols content. Walnut wood, which is the main object of this study, shows that it has a lower quantity of non-flavonoid phenols than oak wood, regardless of the level of toasting and the maceration time.

In fact, the average non-flavonoid phenols content of walnut wood is 28.3 mg/L of gallic acid equivalents, significantly lower than that of oak wood of 33.2 mg/L of gallic acid equivalents.

Figure 21 c) shows us how the days of maceration do not significantly affect on the non-flavonoid phenols content extracted from the two wood species studied.
In fact, the content of these compounds does not change after 30 days of maceration, if not slightly. It can therefore be said that there is no further additional extraction of non-flavonoid phenols after 15 days of maceration.

Even in this case we proceeded with the statistical analysis of the interaction between the various factors. In this way it will be possible to see if the factors influence the content of non-flavonoid phenols taken at the same time.

a)



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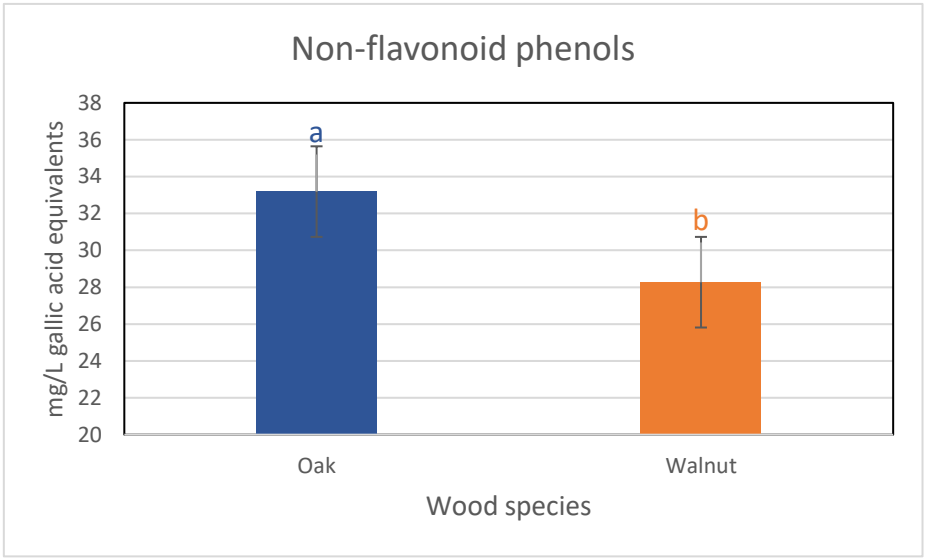

c)

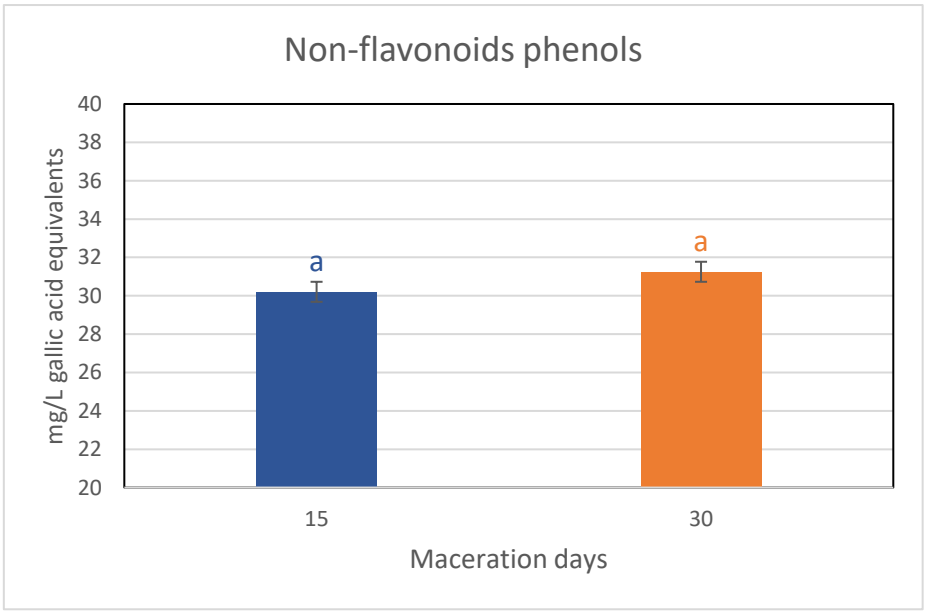

Figure 21. *a) Impact of the level of toasting on the concentration of non-flavonoid phenols extracted; b) Impact of wood species on the concentration of non-flavonoid phenols extracted; c) Impact of maceration time on the concentration of non-flavonoid phenols extracted. Results with the same letter are not significantly different ($p<0.05$). Legend: L) Light toast; M) Medium toast; S) Strong toast.*

The statistical analysis of the interaction between the type of wood and the level of toasting did not lead to significant differences.

As you can see in Figure 22 a), the differences in the non-flavonoid phenols content are due to the individual factors, in fact the columns have similar gaits for all variables. We have already seen the influence of individual factors in Figures 21.

Walnut wood and oak wood therefore behave very similarly in all toasting levels.

Instead, the interaction between the maceration time and the type of wood, analyzed statistically, reported significant differences in the non-flavonoid phenols content.

As can be seen in Figure 22 b) significantly higher results were found in the oak wood samples at both soaking times, this is mainly due to the strong impact of the wood type as we have seen above.

In the middle are the content of non-flavonoid phenols extracted from walnut wood samples with 30 days of maceration, demonstrating that the walnut wood needs more time for the extraction of this phenolic group.

In the lowest step we have the walnut wood samples at 15 days of maceration, this also proves the above.

Finally, it can be deduced that the walnut wood needs longer maceration times also compared to the oak wood, in fact the results approach only in the right columns of Figure 22 b), in which the difference is less than in the left columns.

The statistical analysis of the interaction between the toasting level and the maceration time did not produce any significant difference, as seen in the Figure 22 c)

This lack of interaction tells us that there is no relationship between the two factors, factors that taken individually have different effects on the non-flavonoid phenols content.

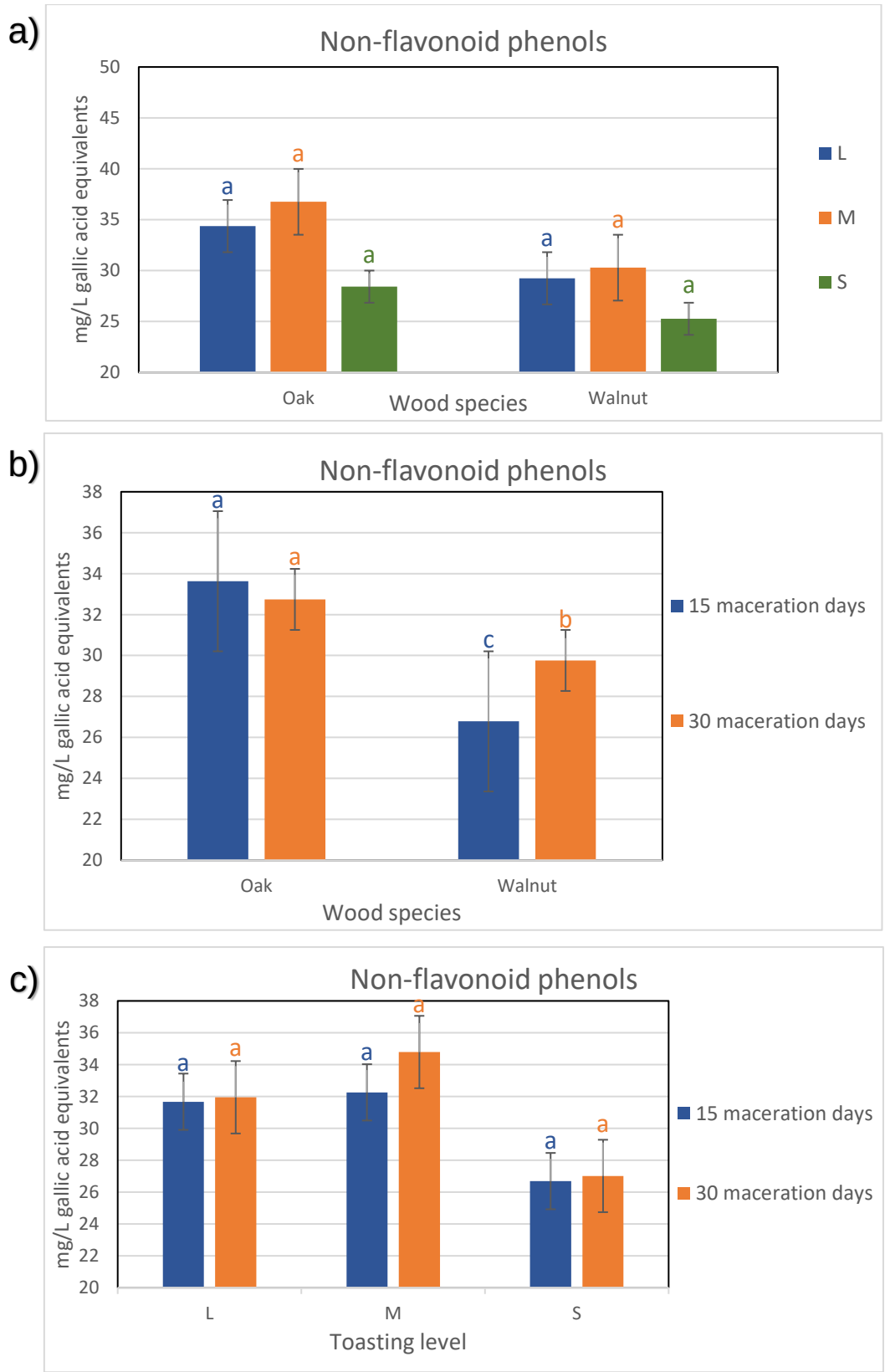

Figure 22.. *a) Impact of the interaction between toasting level and wood species on the concentration of non-flavonoid phenols extracted; b) Impact of the interaction between maceration time and wood species on the concentration of non-flavonoid phenols extracted; c) Impact of the interaction between toasting level and maceration time on the concentration of non-flavonoid phenols extracted ($p<0.05$). Results with the same letter are not significantly different. Legend: L) Light toast; M) Medium toast.*

In the statistical analysis of the three-factor interaction, showed in Figure 23, interesting results were obtained. The strong significance led to many differences between the samples analyzed. The highest concentrations of non-flavonoid phenols were identified in the medium toasted oak samples at both maceration times.

The lower ones were found in the walnut wood samples, with strong toasting of both maceration times, and the samples, always in walnut wood, with medium toasting and 15 days of maceration.

Instead, it was seen that the intermediate values are those of the samples of oak wood, with light toasting and 15 days of maceration.

Below the samples with lightly toasted oak wood at 30 days of maceration, and those with medium toasted walnut, always with 30 days of maceration.

Going further down, the walnut wood samples with light toasting and 30 days of maceration were identified i.e. intermediate to all samples except those with the highest values.

Those that are intermediate to the lowest values and to those mentioned above, are those made of oak wood, with strong toasting in both maceration times.

These results are interesting because they show us what the effects on the non-flavonoid phenols content of the three factors are related to each other.

Even in this statistical analysis, as we can see in Figure 23, there is a substantial difference between the groups of samples. It is evident that the oak samples have a constant content of non-flavonoid phenols in both maceration times, in fact they remain almost with the same significant letters, except for the values marked with "abc". In comparison, walnut does not have this constancy in its values. It is in fact evident that the shorter maceration time does not extract, from the wooden chips, an amount comparable to that of the samples with oak wood, you immediately notice that the columns are much lower, and this is highlighted by the letters of the significance that indicate the lowest levels of non-flavonoid phenols extracted.

The non-flavonoids phenols of walnut wood need more maceration time, as mentioned above, to approach the high values of oak wood. In fact, the samples with 30 days of maceration and light and medium toasts are those with the highest values and close to the oak wood.

In any case, the concentration of non-flavonoid phenols, extracted in model alcoholic solution, is higher in the samples containing oak wood chips, even if, with due maceration time, the samples with walnut wood chips approach these values.

Unlike the other three-factor statistical analyzes previously shown, in this case significant differences were found. These differences indicate how the two different types of wood, under the same conditions, have a different influence on the content of non-flavonoid phenols extracted in solution. It will certainly be useful in the future to analyze these non-flavonoids and

characterize them, to recognize the compounds that are released by walnut wood and quantify them, to understand if they are compatible with the demands of the wine world.

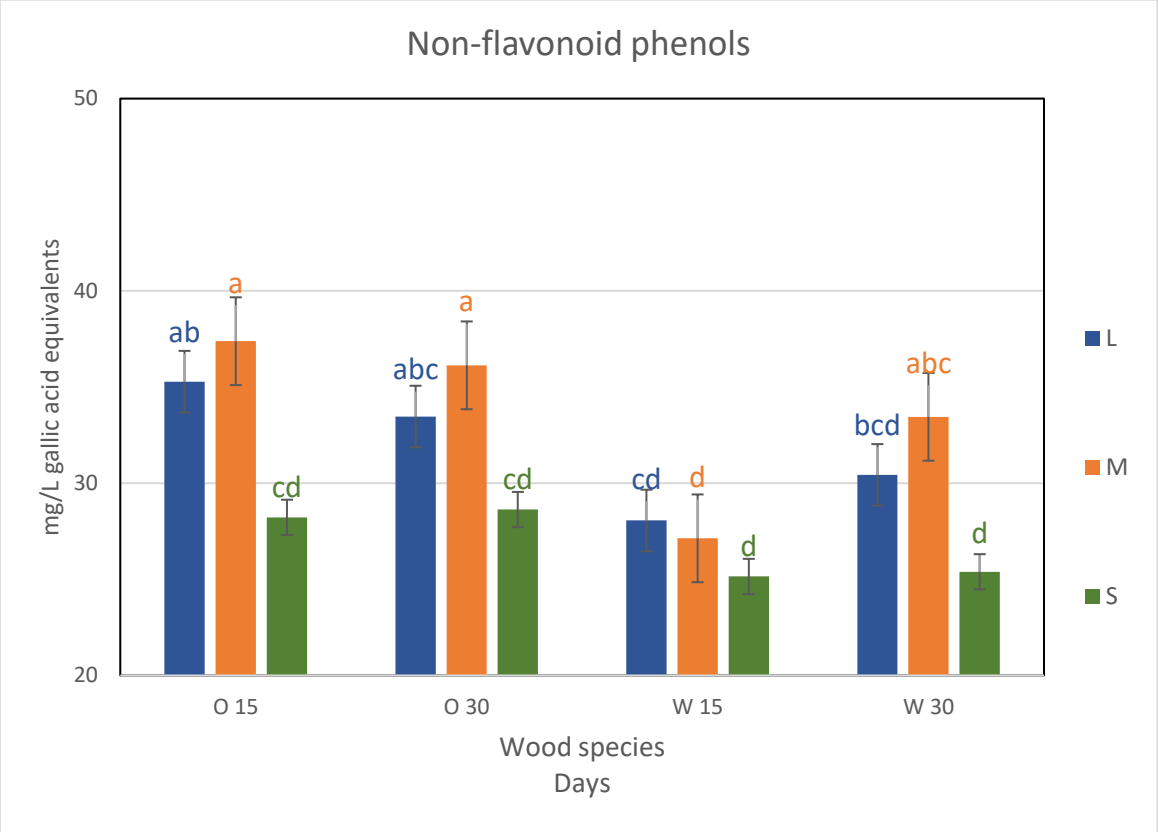

***Figure 23.**. Impact of the interaction between toasting level, maceration time and wood species on the concentration of non-flavonoid phenols extracted. The statistical analysis considers the interaction at three factors between the toasting level, the maceration time and the wood species. Results with the same letter are not significantly different ($p<0.05$). Legend: L) Light toast; M) Medium toast; S) Strong toast; O) Oak wood; W) Walnut wood; 15) 15 days of maceration; 30) 30 days of maceration.*

4.5. Toasting level, wood specie and maceration time effects on the color intensity of the wood extracts

The analysis of the influence on color is useful to understand the effect that these types of wood and the various factors to which they are subjected, have on the color changes of the alcoholic solution, and therefore of a hypothetical wine.

As we can see in Figure 24 a), the level of toasting had no significant effect on the color of the samples. This is because the statistical analysis of the main effects of the single toasting factor did not lead to any significant difference.

It can be said that toasting, in both woods and at both maceration times, did not influence the final color intensity of the model alcohol solution used.

It is instead evident, as we can see in Figure 24 b), that there is a strong influence of the type of wood on the color of the alcoholic solution model.
In fact, the statistical analysis showed an important significant difference between the walnut and oak wood samples. It has been seen that the samples containing walnut wood have a significantly greater absorbency than those with oak wood.
It is therefore clear that it is walnut wood that leads to having an alcoholic solution with the highest color intensity, because it has a statistically greater influence than that of oak wood.

However, it remains to be understood why walnut wood brings more color to the alcoholic solution and what is due to this. The coloring substance extracted from wood is one of the compounds whose analysis results have been shown in the previous paragraphs.
From Figure 24 c) of the statistical analysis of the main effects of maceration time, there are no significant differences between 15 and 30 days.
It can therefore be deduced that the coloring intensity was not given by a longer maceration time, but other factors, such as the level of toasting or the type of wood, have acted in this sense.
However, a slight increase in the color intensity is visible, although not significant, in the samples that have undergone 30 days of maceration, compared to the others.
Given this, it is evident that only the type of wood has a significant influence on the color of the alcoholic solution, but this influence could also be related to the simultaneous action of two or more factors, which together could still have a certain influence.
For this also for intensity of color it is useful to perform the statistical analysis based on the effect of the interacting factors. By doing so, it will be possible to understand if the color change is due to a single factor or to the combination of two or more factors.

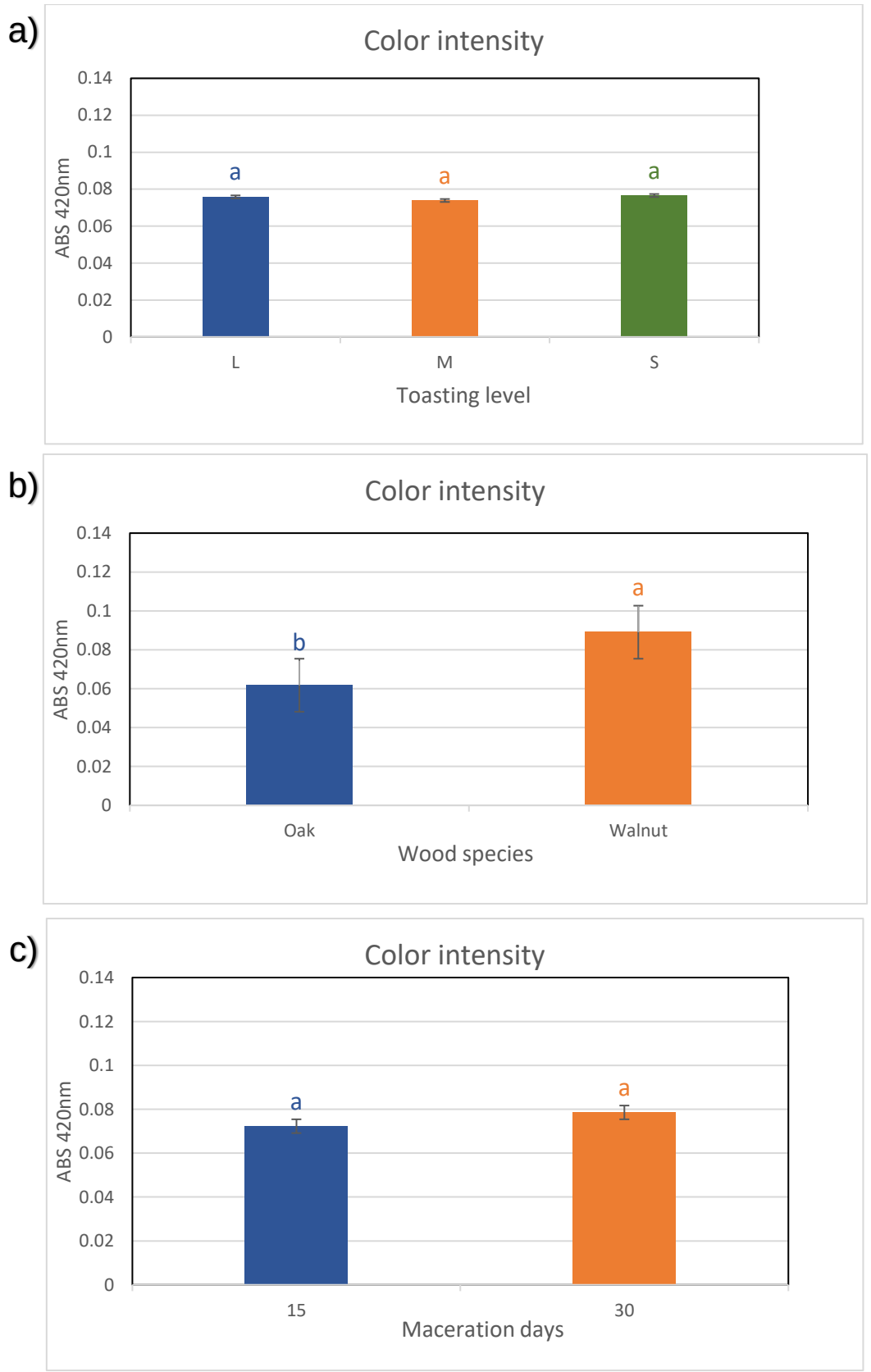

Figure 24. *a) Impact of the level of toasting on the absorbance at 420 nm; b) Impact of the level of wood species on the absorbance at 420 nm; c) Impact of the level of maceration time on the absorbance at 420 nm. Results with the same letter are not significantly different (p<0.05). Legend: L) Light toast; M) Medium toast; S) Strong toast.*

Going on to analyze the interaction between the toasting levels and the type of wood on a statistical level, significance emerged which confirms the relationship between these two factors in influencing the coloring intensity of the samples studied.

It has been seen, in Figure 25 a), that the highest absorbance values belong to the walnut wood samples with light toasting.

The lowest absorbance values, on the other hand, can be found in the oak samples with light and medium toasting.

The only values of the samples with oak wood that approach those with walnut are those of the samples with strong toasting.

The statistical analysis also confirms that walnut has an important influence on the coloring intensity, but here it is highlighted that this influence is also given in relation to the level of toasting, more than to oak wood.

Instead, the analysis of the interaction between the maceration time and the type of wood did not produce any significant results.

In fact, as we can see from Figure 25 b), also observing the height of the columns colored in the same way, the absorbance values detected are not influenced by the interaction ratio between these two factors.

Figure 25 b) shows us how the coloring intensity is not related to the joint action of the maceration time and the type of wood, but, as we have seen before, it is only the second factor that has this type of influence.

The last two-factor interaction statistical analysis was carried out with the toasting level and the maceration time.

This did not detect any significant difference, therefore there was no interaction between the two factors that influenced the coloring intensity.

Looking at Figure 25 c), there are no big differences between the samples, there is simply a trend of higher values in the samples with 30 days of maceration, but without significantly difference.

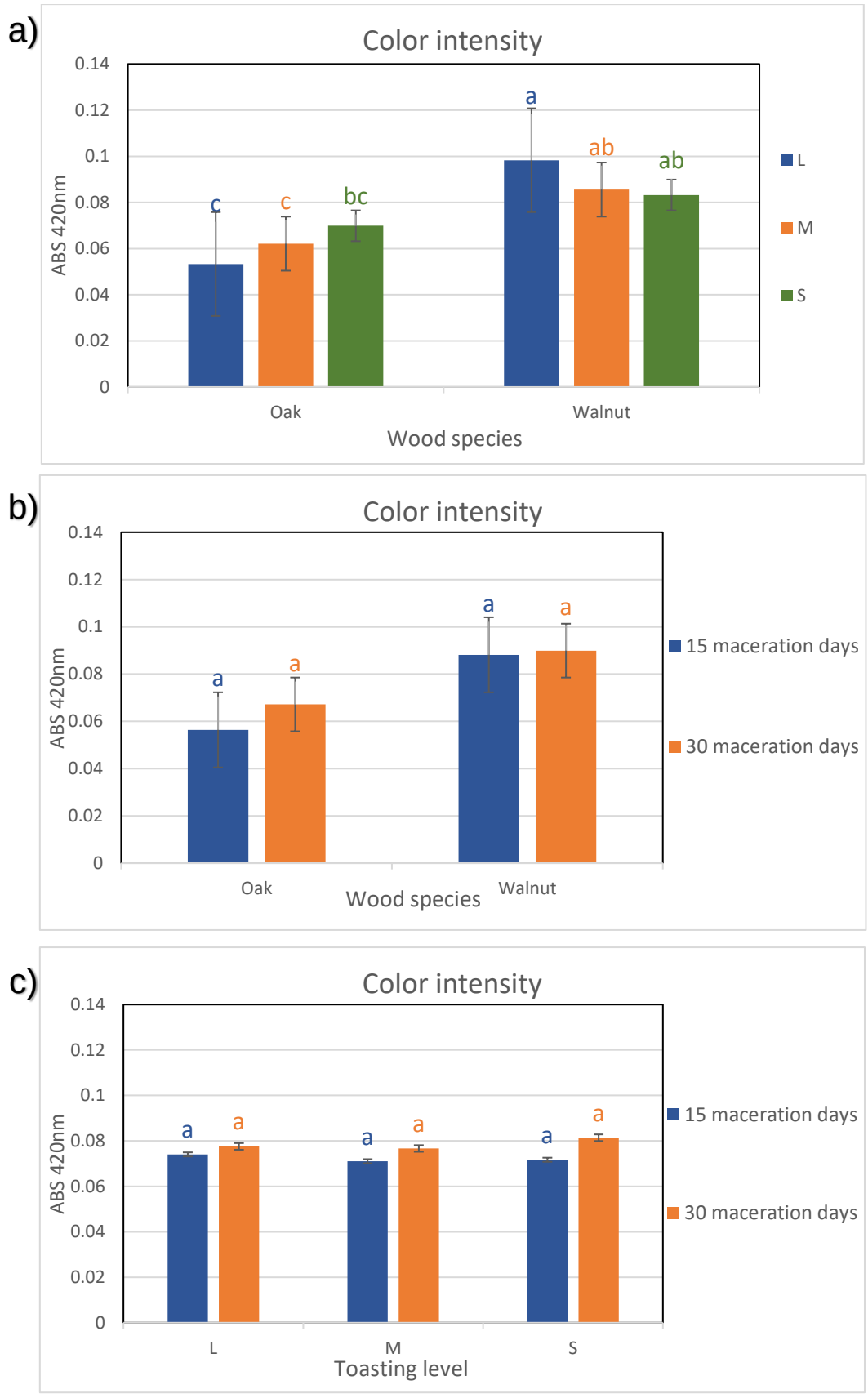

Figure 1. *a) Impact of the interaction between toasting level and wood species on the absorbance at 420 nm; b) Impact of the interaction between maceration time and wood species on the absorbance at 420 nm; c) Impact of the interaction between toasting level and maceration time on the absorbance at 420 nm. Results with the same letter are not significantly different ($p<0.05$). Legend: L) Light toast; M) Medium toast; S) Strong toast.*

Even the statistical analysis of the three-factor interaction did not give significantly relevant results.

In Figure 26 it is in fact possible to see how the columns of the groups, separating them by type of wood, behave in a similar way, with constancy.

Both in the 15 days of maceration and in the 30 days, the values of the oak wood samples tend to have a greater coloring intensity as the toasting level increases. The opposite is true for walnut samples.

It is also clear how walnut has a greater influence on the intensity of the color than oak, as has already been seen in the study of the single factor.

It can therefore be deduced that the three factors did not interact with each other in changing the coloring intensity of the model alcohol solutions used in this experiment.

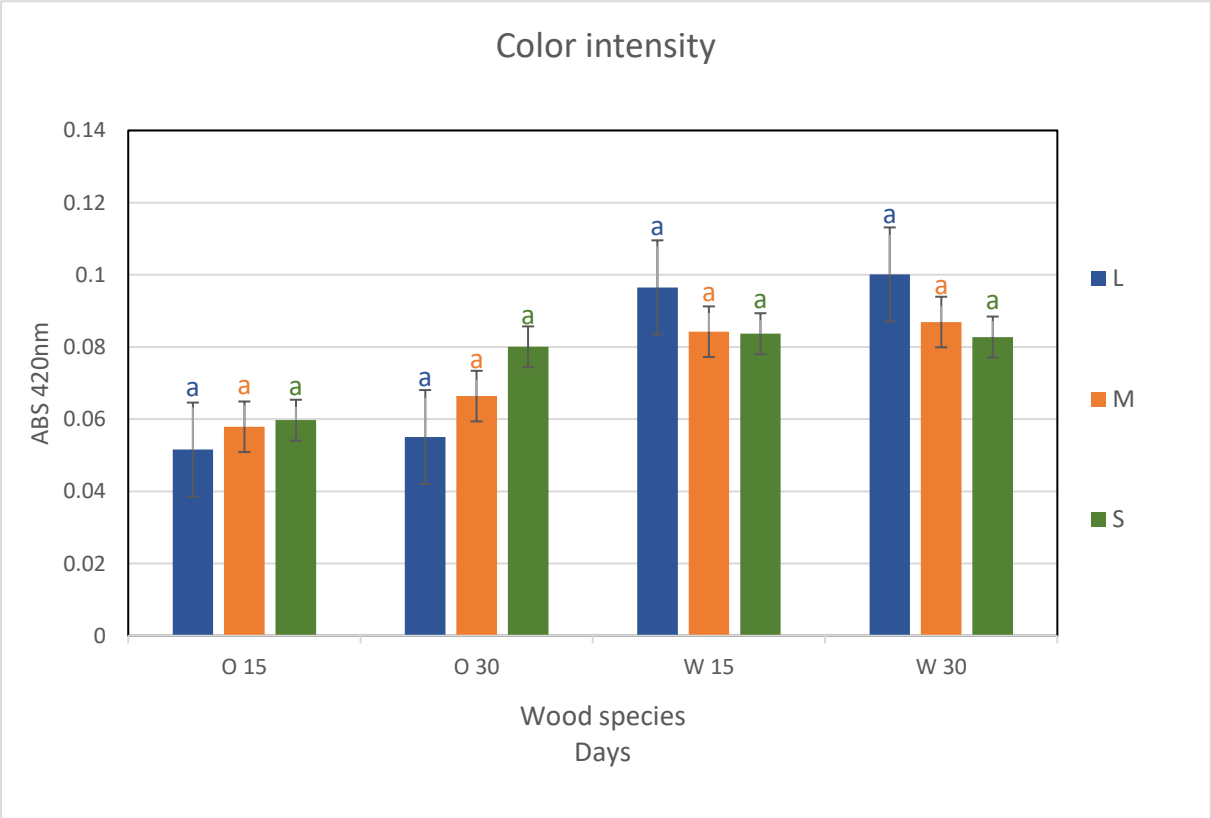

Figure 26. *Impact of the interaction between toasting level, maceration time and wood species on the absorbance at 420 nm. The statistical analysis considers the interaction at three factors between the toasting level, the maceration time and the wood species. Results with the same letter are not significantly different ($p<0.05$). Legend: L) Light toast; M) Medium toast; S) Strong toast; O) Oak wood; W) Walnut wood; 15) 15 days of maceration; 30) 30 days of maceration.*

5. CONCLUSIONS

In this study, the effects on the content of phenolic compounds given by the extraction of walnut and oak chips at two different maceration times and three toasting levels were evaluated.

The different statistical methods used to analyze the results obtained from the analysis of the phenolic content and of the color, carried out on the samples prepared for this study, responded differently but quite clearly.

The total phenol content analyzed with the first statistical method showed that the 30 days of maceration led to a final higher concentration of total phenols extracted in solution. The trend in the concentration of total phenols is higher in samples with medium toasting, followed by those with light and finally strong toasting. The highest content was found in the medium-toasted oak wood sample, while the lowest in a strong-toasted oak sample. Beyond this, it did not significantly show differences, for both maceration times, between the two types of wood. From this it can be deduced that the two types of wood behave similarly during a maceration in an alcoholic solution, as regards the extraction of total phenols.

Also as regards the extracted content of flavonoid phenols, not significantly differences were found between the two types of wood, it can therefore be said that, also for this parameter, the walnut wood chips behaved similarly to that of oak wood.

Instead, analyzing the results of the extracted content of non-flavonoids, the statistical analysis highlighted important significantly differences. In fact, after 15 days of maceration, the oak wood samples, light and medium toasted, produced a significantly higher content than the walnut wood samples, at the same levels of toasting. From this result walnut wood, at light and medium toasting levels, has a lower extraction capacity in alcoholic solution than oak, in the first 15 days of maceration. This difference is attenuated, until it is no longer significant, when you reach 30 days of maceration, i.e. the non-flavonoids contained in walnut wood, need more time to be extracted in an alcoholic solution.

As for the coloring intensity, the statistical analysis of the results showed how, at the level of light toasting, after 15 days of maceration, walnut wood leads to a higher coloring intensity than the corresponding sample of oak wood. This result must be considered in oenology, as there could be rapid and difficult to control changes in color, using walnut wood in winemaking.

The second statistical method gave relatively different results from the first method, but in general they were consistent.

The strong toasting is the one that led to a decrease in the phenolic content in the alcoholic solution, in fact for the total phenols, the flavonoids and the non-flavonoids the difference was significant. This happens regardless of the type of wood used.

Walnut wood, on the other hand, has a negative effect on the extraction of phenolic compounds, in fact it brings to the alcoholic solution a lower content of both total phenols and non-flavonoid phenols.

The extraction of flavonoids, on the other hand, changes between the two woods only in the first 15 days of maceration, which is not enough time for the oak to release the same number of flavonoid phenols as the walnut. Difference that levels out after 30 days of maceration. These capabilities of the walnut are to be taken into consideration for its possible use in oenology, it could therefore be less effective than oak wood, and depending on the suitable oenological objective.

The most effective maceration period, for both types of wood, for the extraction of phenolic compounds, was that of 30 days. In fact, after 30 days of maceration, significantly higher quantities of total phenols and flavonoids were found. This effect was not present on non-flavonoids. The influence of the maceration time on the extraction of phenolic compounds is to be considered during winemaking, longer macerations will lead to higher contents. Walnut wood had an important influence on the coloring intensity of the alcoholic solution, significantly increasing it, especially with a light toasting level. The solution studied, however, is not wine, but a model solution, therefore without other phenolic compounds already present, the final color is indicative, the reaction on the wine could be different, therefore it will be necessary to test the walnut wood directly on the wine, as well as for the extraction of phenolic compounds.

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